Neurovascular Pharmacology of Prospective Antimigraine Drugs

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Neurovascular Pharmacology of Prospective Antimigraine Drugs

Neurovasculaire farmacologie van toekomstige antimigraine geneesmiddelen

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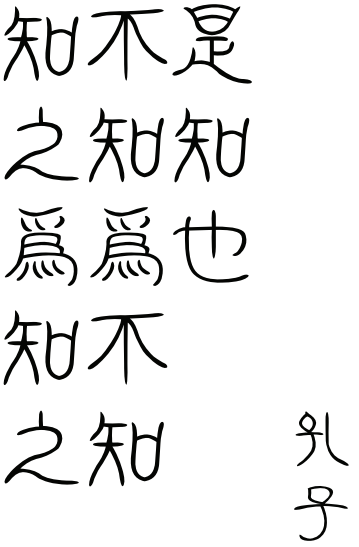

Knowing what you know and knowing what you do not know.
That is true wisdom (Confucius)

Part A: Introduction

Chapter 1
Neurovascular pharmacology of migraine

Based on: A. MaassenVanDenBrink and K.Y. Chan (2008),
European Journal of Pharmacology, 585; 313-319.

Abstract

Migraine is a paroxysmal neurovascular disorder, which affects a significant proportion of the population. Since dilatation of cranial blood vessels is likely to be responsible for the headache experienced in migraine, many experimental models for the study of migraine have focussed on this feature. The current review discusses a model that is based on the constriction of carotid arteriovenous anastomoses in anaesthetized pigs, which has during the last decades proven to be of great value in identifying potential antimigraine drugs acting via a vascular mechanism. Further, the use of human isolated blood vessels in migraine research is discussed. Thirdly, we describe an integrated neurovascular model, where dural vasodilatation in response to trigeminal perivascular nerve stimulation can be studied. Such a model not only allows an in-depth characterization of directly vascularly acting drugs, but also of drugs that are supposed to act via inhibition of vasodilator responses to endogenous neuropeptides, or of drugs that inhibit the release of these neuropeptides. We discuss the use of this model in a study on the influence of female sex hormones on migraine. Finally, the implementation of this model in mice is considered. Such a murine model allows the use of genetically modified animals, which will lead to a better understanding of the ion channel mutations that are found in migraine patients.

Introduction

Migraine is a recurrent incapacitating neurovascular disorder characterized by unilateral and throbbing headaches associated with photophobia, phonophobia, nausea, and vomiting. It affects a significant proportion of the adult population, with a 2-3 fold higher prevalence in women than in men.[1] The pathophysiology of migraine is not yet completely understood, but putatively involves leakage through (genetically) predisposed ion channels in the brainstem, leading to a decrease in cerebral blood flow and subsequent release of neuropeptides such as calcitonin gene-related peptide (CGRP)[2] from trigeminal nerves, which induces a potent dilatation cranial extracerebral blood vessels (Figure 1). This dilatation stimulates the trigeminovascular system, eventually leading to headache and associated symptoms.[3-4]

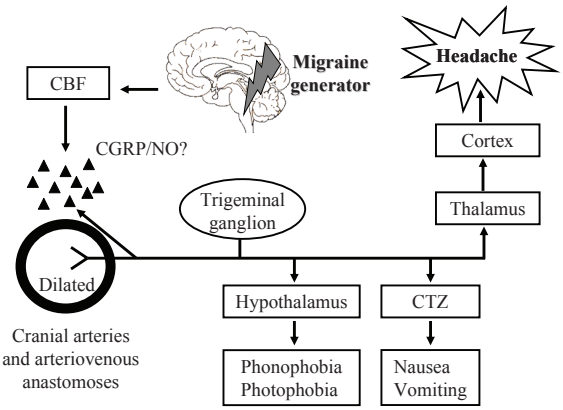

Figure 1: Schematic representation of the pathophysiology of migraine. The pathophysiologic changes in migraine putatively stem from ion leakage through channels in the brain stem, leading to a decreased cerebral blood flow (CBF), possibly owing to cortical spreading depression and, subsequently, neuropeptide release and dilatation of cranial extracerebral blood vessels. The increased pulsation in these blood vessels stimulates the trigeminovascular system, setting in peripheral and central sensitization and leading to headache and associated symptoms (nausea, vomiting, phono- and/or photophobia). CTZ: chemoreceptor trigger zone, NO: nitric oxide.[5]

Current specific drugs used in the acute treatment of migraine interact with vascular receptors, a fact that has raised concerns about their cardiovascular safety. In the past, α-adrenoceptor agonists (e.g. ergotamine, dihydroergotamine, isometheptene) were used. The last two decades have witnessed the advent of 5-$HT_{1B/1D}$ receptor agonists (sumatriptan and the other triptans), which have a well-established efficacy in the acute treatment of migraine. At present, research focuses on drugs that do not induce vasoconstriction, such as CGRP receptor antagonists[6] and glutamate receptor antagonists.[7]

Our group has in the past years investigated the vascular properties of current and prospective antimigraine drugs using several models that are based on the vascular involvement in the pathophysiology of migraine. These models include studies on constriction of carotid

arteriovenous anastomoses in anaesthetized pigs *in vivo* and the study of human arterial contraction *in vitro*. Although these models mainly focus on direct vasomotor responses, they can also be used to study the contribution of perivascular nerves by administration of the neuropeptide-releasing agent capsaicin or by transmural electrical stimulation of isolated arteries. More recently, we have introduced an integrated neurovascular migraine model, where the role of perivascular nerves, which may release neuropeptides that are important in vascular responses, can be studied in further detail. Our research has focused on the pharmacological characterization of the vascular actions of current and future antimigraine drugs, e.g. by assessing their cranioselectivity. Currently, we are also exploring the mechanisms underlying the influence of female sex steroid hormones on the prevalence of migraine, as well as the role of ion channel mutations in the neurovascular pharmacology of migraine.

Constriction of carotid arteriovenous anastomoses in anaesthetized pigs

As described above, a complete understanding of migraine pathogenesis remains elusive, but there seems to be little doubt that dilatation of cranial blood vessels, including carotid arteriovenous anastomoses, is involved in the headache phase of migraine.[8-9] Indeed, migraine patients also experience facial paleness, a reduction in facial temperature, an increase in temporal artery pulsations and swelling of the frontal vein on the side of the headache[10-11] in addition to their headaches. Thus, Heyck[12] measured the oxygen saturation difference between arterial and external jugular venous blood samples (A-V SO_2 difference) during and after the headache phase of migraine and compared it with those of healthy controls. The A-V SO_2 difference was decreased during the headache phase of migraine, likely due to dilatation of carotid arteriovenous anastomoses, which was normalised after spontaneous or drug-induced (ergotamine) alleviation of the headache.[12]

Arteriovenous anastomoses are precapillary communications between the arteries and veins (Figure 2); they are predominantly located in the head skin, ears, nasal mucosa, eyes and dura mater in several species, including humans and pigs.[13] In conscious animals, arteriovenous anastomoses are constricted due to the sympathetic neuronal tone, thus shunting only a small (<3%) fraction of the total carotid blood flow.[14-15] In contrast, under pentobarbital anaesthesia, approximately 80% of the total carotid blood flow in the pig is shunted via arteriovenous anastomoses into the jugular venous circulation.[15] Consequently, opening of the carotid arteriovenous anastomoses during migraine shunts a large quantity of oxygenated blood directly into the veins thereby resulting in facial pallor, a lowering of skin temperature and an increase in vascular pulsations.[13] This increase in vascular pulsations stimulates the so-called ‘stretch receptors’ present in the wall of blood vessels, with ensuing activation of perivascular trigeminal nerves containing peptides such as CGRP.[8-9] The trigeminal cranial

nerve conveys nociceptive information to central trigeminal nuclei that relay the pain signals to higher centers where headache is perceived.[2]

In accordance with the above findings, it is reasonable to assume that counteracting carotid arteriovenous anastomotic vasodilatation may abort migraine.[8-9] Therefore, an animal experimental model in anaesthetized pigs was developed; radioactive microspheres were used to measure the carotid arteriovenous anastomotic blood flow and the effects of antimigraine drugs on this parameter.[13] Over the years, this model has proven predictive of antimigraine activity in the clinic.[8-9] The major advantage of the porcine model is that one can simultaneously study different vascular beds in order to evaluate the cranioselectivity of antimigraine drugs.[8] Based on this notion, all acutely active antimigraine agents, including ergot alkaloids, triptans as well as α-adrenoceptor agonists potently constrict the porcine carotid bed and the corresponding arteriovenous anastomoses;[8,16-20] Figure 3 shows that ergotamine, sumatriptan and phenylephrine dose-dependently decrease arteriovenous anastomotic blood flow. These results, together with Heyck's[12] observations of decreased A-V SO_2 difference during migraine headache, shed light on the mechanisms of action of acutely-acting antimigraine drugs. Hence, the porcine carotid (arteriovenous anastomotic) circulation is an experimental model highly predictive of antimigraine activity, but it should be taken into account that this model will only pick up potential antimigraine drugs acting via vascular mechanisms.

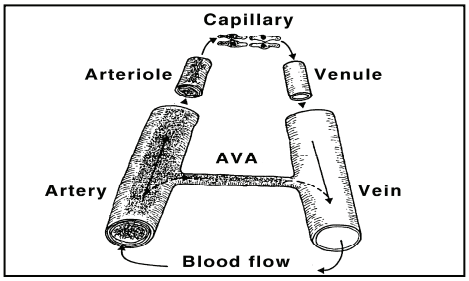

Figure 2: Schematic representation of a vascular bed containing arteriovenous anastomosis (AVA), which is a precapillary communication between artery and veins. When AVAs are dilated, as proposed to be the case during migraine,[12] arterial blood flow will be shunted to the venous side.

Chemical stimulation of sensory nerve fibres with capsaicin

Stimulation of sensory nerve fibres results in the release of neuropeptides in both the central and peripheral nervous system.[21] In the former, these neuropeptides transmit nociceptive signals to the spinal cord and brain, whilst in the latter they promote neurogenic inflammation, such as plasma protein extravasation, neuropeptide release and vasodilatation. This model allows us to study the inhibitory interactions between antimigraine agents and peripheral

trigeminal fibres.

Several studies have shown that sensory nerves innervating the cerebral vasculature contain substance P and CGRP;[2] however, capsaicin-induced relaxation of guinea-pig isolated basilar artery is mainly mediated by CGRP.[22-23] In this context, in an established porcine migraine model, capsaicin was infused into the carotid artery and the carotid haemodynamic responses as well as trigeminal CGRP release were investigated with the help of olcegepant, a selective CGRP receptor antagonist.[24] The results clearly show that capsaicin-induced carotid and arteriovenous anastomotic vasodilatation are mainly mediated by CGRP (Figure 4). Admittedly, as reported earlier,[25] vasodilator responses to capsaicin tend to wear off following subsequent infusions of capsaicin, suggestive of some tachyphylaxis, although this is limited because of a neuronal reuptake of released CGRP into capsaicin-sensitive perivascular nerves.[26]

Human isolated blood vessels

Studies on isolated blood vessels offer several advantages: (i) drug-receptor interactions at equilibrium, (ii) the possibility to carry out a detailed pharmacological analysis, mounting multiple segments of blood vessel in parallel, (iii) no influence by pharmacokinetic factors, (iv) exclusion of central and autonomic mechanisms as well as the effects produced by circulating hormones, distending pressure, etc., (v) the possibility to remove the endothelium from the blood vessel, which provides information whether the receptors are present on the endothelium or in the vascular smooth muscle of arteries, (vi) the possibility to gain insight into downstream signalling, such as measurements of second messengers[28,29] and, most importantly, (v) the possibility to study human preparations. Notwithstanding the benefits provided by the *in vitro* models, these are only complementary to the information obtained with *in vivo* models (see above). The human *in vitro* models that stand out in characterizing potential antimigraine drugs are isolated cranial (meningeal, temporal, basilar) and coronary arteries; the results obtained from these blood vessels allow assessment of cranioselectivity of these drugs.

Isolated cranial arteries

As previously pointed out, the therapeutic efficacy of acutely acting antimigraine drugs is most likely, at least for a major proportion, mediated by constriction of dilated cranial arteries.[8-9,30] Therefore, this model considers the use of three cranial arteries, namely the middle meningeal, the basilar and the temporal.[29,31-35] The human middle meningeal artery is heavily innervated with afferent sensory fibres containing substance P, neurokinin A and CGRP and originating from the trigeminal ganglion.[36] Cumulative concentration response curves are used to determine the vasoconstrictor potency and efficacy of the prospective

antimigraine agent. The rank order of potency of 5-HT receptor agonists in this preparation positively correlates with affinity measurements in cell lines expressing the 5-HT_{1B} receptor.[31] Molecular studies have detected mRNA encoding predominantly 5-HT_{1B} receptors, but also other 5-HT receptors like 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_4 and 5-HT_7.[37-38] In line with the above findings, it has been demonstrated that during a migraine attack the blood flow velocity in the middle cerebral artery significantly decreases, and this is normalised after administration of sumatriptan.[39] Indeed, sumatriptan also constricts cranial vessels *in vitro*.[32-33,40] Therefore, studying human isolated cranial blood vessels gives reliable information regarding the behaviour of these vessels in migraine headache and the effects of novel therapeutic agents.

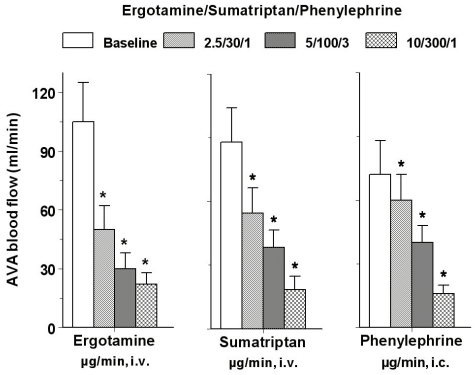

Figure 3: Arteriovenous anastomotic (AVA) blood flow measured at baseline and after infusions of ergotamine (2.5, 5 and 10 µg/kg, i.v.) sumatriptan (30, 100 and 300 µg/kg, i.v.) and phenylephrine (1, 3 and 10 µg/kg/min, intracarotid artery infusion). All values are expressed as mean±SEM *, P<0.05 vs. baseline values. Data are taken from Arulmani *et al* (2006).[27]

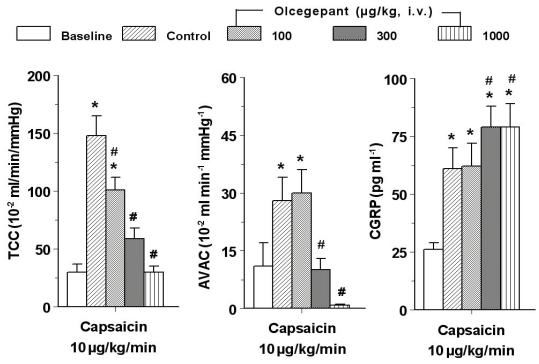

Figure 4: Total carotid conductance (TCC), arteriovenous anastomotic conductance (AVAC) and jugular venous plasma CGRP concentrations measured at baseline and following infusions of capsaicin (10 µg/kg/min, intracarotid artery infusion) given in anaesthetised pigs before (control) and after i.v. administrations of olcegepant (100, 300 and 1,000 µg/kg). *, P<0.05 vs. baseline values; #, P<0.05 vs. response after the corresponding volume of vehicle (data not shown). Data are taken from Arulmani *et al* (2006).[27]

Isolated coronary arteries

Human isolated coronary arteries are useful in analyzing the coronary side-effect potential of antimigraine drugs. Although the chest symptoms (chest pressure, tightness and pain) commonly experienced after the use of antimigraine drugs[43-44] are in most cases not likely to be due to coronary vasoconstriction, cardiac ischemia after antimigraine drugs has indeed been reported.[45] The right epicardial coronary artery has been the most commonly used segment for studies on the contractile effects of antimigraine drugs, which is an appropriate choice for the study of coronary vasospasms, since these tend to occur in epicardial conduit arteries, converting it into a major resistance vessel impeding myocardial blood flow.[46] Moreover, the right epicardial coronary artery is more frequently involved in coronary artery spasm than the left anterior descending artery, the circumflex artery or the main trunk.[47-49] However, for the studies on the role of neuropeptides like CGRP, that induce vasorelaxation, distal portions of the coronary artery seem to be a better model, since the efficacy of CGRP is inversely related to the vessel diameter.[28]

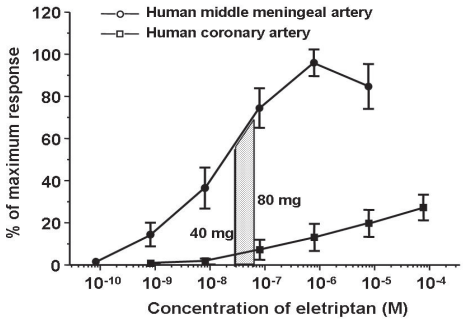

Figure 5: Contractions to eletriptan (expressed as percentage of the maximum response in the respective tissue) in the human isolated middle meningeal artery and the human isolated coronary artery. Dashed bar represents the plasma concentrations occurring at the free (i.e., plasma protein unbound) C_{max} values after 40 mg and 80 mg of eletriptan (for references on the calculations[52]). It is clear that contractions to eletriptan in the middle meningeal artery at clinical plasma concentrations are considerably larger than those in the coronary artery. Data taken from MaassenVanDenBrink *et al* (2000).[52]

It is now well known that triptans constrict coronary arteries, but the effect on cranial vessels, where, contrary to peripheral arteries, the 5-HT_{1B} rather than 5-HT_2 receptor dominates, is more marked.[50-51] Eletriptan (Figure 5),[34,52] sumatriptan,[34,52] rizatriptan,[37] frovatriptan,[53] almotriptan[54] as well as donitriptan[55] have been demonstrated to be several fold more potent in contracting the human middle meningeal artery than the coronary artery. More importantly, the magnitude of contraction elicited by triptans in cranial vessels is much more than that in the coronary arteries. Therefore, at therapeutic plasma concentrations, triptans contract cranial vessels much more than the coronary artery.[34,52-55] The reason for this is not clear, but it may be related to the higher density of 5-HT_{1B} receptors in the cranial compared

to coronary arteries.[37]

The magnitute of coronary artery contraction by triptans at therapeutic maximum plasma concentrations (C_{max}), corrected for the fraction of the drug bound to plasma proteins, has been calculated by interpolating concentration response curves.[56] As shown in Figure 6, the contraction induced by zolmitriptan, eletriptan, and possibly rizatriptan, almotriptan, and frovatriptan may be somewhat smaller than that with sumatriptan. However, both zolmitriptan and rizatriptan have a pharmacologically active N-desmethyl metabolite, which may cause additional contraction. Similarly, a pharmacologically active N-desmethyl metabolite has been described for eletriptan. Although this metabolite is similar to the parent compound with respect to the affinity at 5-HT_{1B} receptors, its plasma concentration remains 7- to 9-fold lower than that of eletriptan, and is thus unlikely to add to the coronary artery contraction induced by eletriptan. Thus, human coronary artery contraction induced by some triptans may be somewhat less than that elicited by sumatriptan, but these subtle differences may be due to the use of relatively low therapeutic doses of newer triptans compared to 100 mg of sumatriptan. Clearly, all triptans are cranioselective and are thus safe medications, although they obviously remain contraindicated in patients with coronary artery disease.

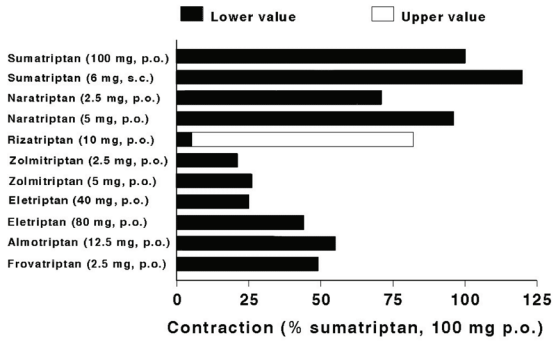

Figure 6: Predicted contraction of the human isolated coronary artery at therapeutic free C_{max}, i.e. at the protein-unbound fraction of the maximum therapeutic plasma concentration, expressed as percentage of contraction elicited after 100 mg sumatriptan p.o. (100%). Closed bars represent the lower value, open bars represent the upper value when data of more than one laboratory were available. Contraction to sumatriptan, obtained as a reference, was always derived from the same study as the respective compound.[56] Because data from literature were used, no statistics were performed.

Studies on the pharmacological characterization of CGRP in the human isolated coronary artery have demonstrated that olcegepant, the only CGRP receptor antagonist evaluated in clinical trials for acute antimigraine therapy thus far, does not constrict coronary arteries.[28,41] Although this seems a clear advantage over the triptans, it should be realized that the antagonism of coronary artery dilatation by olcegepant may have consequences during pathophysiological conditions where endogenous CGRP is important, such as we demonstrated with olcegepant in cardiac preconditioning experiments in rat isolated hearts.[57]

Intravital microscopy on a closed cranial window in rats and mice

Since evidence is evolving that the pathogenesis of migraine is an integrated process involving the trigeminovascular system,[8-9] innovative models of migraine have been developed in which the trigeminal ganglia/nerves are stimulated and the neuroinflammatory processes, such as vasodilatation, trigeminal nociception and trigeminal CGRP release are determined,[8-9] as well as the effect of (prospective) antimigraine medication on these parameters. Triptans attenuate cranial vasodilatation induced by trigeminal stimulation as well as CGRP release in rats. A model has been developed in which trigeminal sensory Aδ-fibres that only release CGRP are electrically stimulated and the dural blood vessel diameter is measured by an intravital microscope through a closed cranial window.[58-60] Electrical stimulation of this cranial window as well as intravenous infusion of substance P and CGRP in rats increase dural blood vessel diameter.[58,61] Interestingly, the NK_1 receptor antagonist, RP 67580, clearly antagonised substance P-induced vasodilatation, but not the neurogenic vasodilatation.[58] In contrast, the CGRP receptor antagonist, $CGRP_{8-37}$, abolished the vasodilatation induced by both CGRP and neurogenic stimulation,[59,62] demonstrating that the neurogenic vasodilatation is mediated by endogenous CGRP released from trigeminal sensory nerves. This observation is consistent with clinical data showing that the levels of CGRP, but not those of substance P, are elevated during the headache phase of migraine.[3] A recent study on this model showed that olcegepant induced a significant inhibition of both CGRP- and electrically-induced increases in the diameter of meningeal arteries, but not of pial and cerebral arteries.[63] Significantly, sumatriptan attenuated dural vasodilatation following trigeminal stimulation, but not the response to CGRP,[60] probably via presynaptic inhibition of CGRP release. The use of selective antagonists suggest that the above inhibitory effect of sumatriptan is mediated via prejunctional 5-HT_{1B} receptors in rats and 5-HT_{1D} receptors in guinea-pigs, cats and humans.[58]

Studies on female sex steroids in migraine

As mentioned in the introduction of this review, migraine has a higher prevalence in women than in men.[1,64] The prevalence of migraine before puberty is higher in men than in women, while after puberty the prevalence increases by two to three fold in females.[65] Migraine attacks in females are aggravated during menstruation,[66] while most migraineurs experience improvement during pregnancy[67] and after menopause.[68] Thus, profound changes occur in female hormone profile during several phases of life, which seem to correlate with changes in migraine attack frequency. Therefore, it is reasonable to assume that there is a hormonal factor involved in the pathogenesis of migraine.[69]

Indeed, the homeostasis of CGRP is strongly influenced by sex steroids. In female rats, for example, it has been shown that ovariectomy decreases the plasma CGRP concentration,

whilst subsequent treatment with 17β-estradiol, progesterone or their combination restores it significantly.[70] Moreover, it is reported that myometrium sensitivity to CGRP increases during human pregnancy,[71] and plasma concentrations of CGRP are elevated in postmenopausal women undergoing hormone replacement therapy.[72] Although these findings suggest a positive correlation between plasma levels of CGRP and 17β-estradiol, admittedly, hormone replacement therapy has shown contradictory results in relation to the occurrence of migraine in postmenopausal women.[73-74] Nevertheless, taken collectively, these findings indicate that changes in the levels of female hormones, like 17β-estradiol and progesterone, may be involved in migraine pathogenesis, but an established neurobiological explanation in this context is lacking.

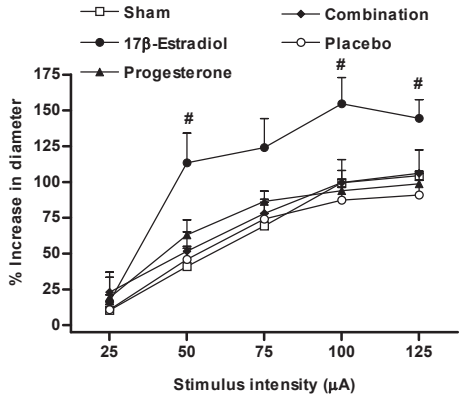

Figure 7: Effect of increasing intensities of periarterial nerve stimulation on middle meningeal artery diameter in sham-operated or ovariectomized rats receiving placebo or hormone treatments. #Significantly higher ($P<0.05$) than the corresponding value in the placebo group. Data taken from Gupta *et al* (2006).[75]

On the above basis, we have investigated the effects of the female sex hormones, 17β-estradiol and progesterone, separately and in combination, on dural vasodilatation induced by CGRP, periarterial electrical stimulation and capsaicin in female rats, using intravital microscopy. Although the responses to CGRP and capsaicin were similar among all groups, the vasodilator response to electrical stimulation was significantly increased in rats treated with 17β-estradiol (Figure 7), suggesting increased CGRP release from perivascular nerves.[75] Although obviously more research in this area is needed to fully explain our findings, our observations may represent one of the mechanisms through which 17β-estradiol exacerbates migraine attacks in women.

Development of a murine intravital microscopy model

The experimental setup of intravital microscopy as discussed above uses wild-type rats, whereas in some cases, animals with a genetic predisposition to migraine should be preferred.

Until now, three migraine genes have been identified in families with familial hemiplegic migraine (FHM), a rare autosomal dominant subtype of migraine with aura.[76-78] FHM genes CACNA1A, ATP1A2 and SCN1A are all involved in ion transport and encode subunits of $Ca_v2.1(\alpha_{1A})$ voltage-dependent P/Q type calcium channels, Na^+/K^+ pumps, or neuronal voltage-gated $Na_v1.1$ sodium channels, respectively. The importance of the FHM genes in common migraine still needs validation.

The identification of migraine gene mutations in patients provided unique opportunities to generate transgenic mice with specific pathogenic mutations (e.g. knock-in mice), and thus a genetic predisposition to the disease. The recently described transgenic knock-in mouse model expressing the human R192Q pure FHM-1 mutation showed pure gain of function effects (e.g. an increased calcium influx through mutant calcium channels, an increased neurotransmitter release, and a reduced threshold for cortical spreading depression, the underlying cause of the migraine aura) and appears a promising model to study migraine pathophysiology.[79]

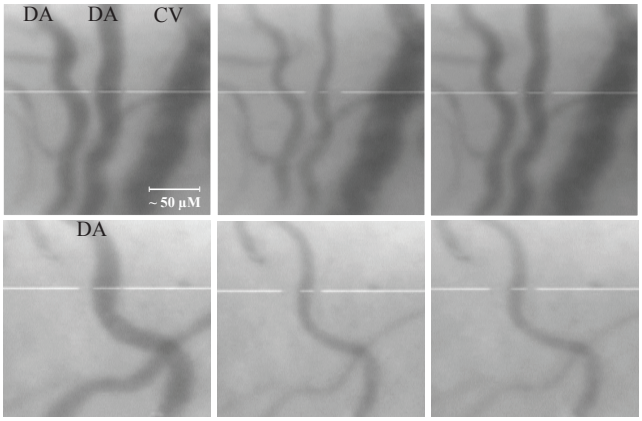

Figure 8: Representative video-microscopic images of dural arteries (DA) in two mice treated with vehicle (top panels) or 100 µg/kg olcegepant (bottom panels). Left panels, 5 min after the administration of vehicle or olcegepant (baseline); middle panels, 2 min after the administration of ET-1 (10 µg/kg); and right panels, 2 min after the administration of CGRP (10 µg/kg). The white line in the middle of each panel tracks vessel diameter. Note that ET-1 constricts dural arteries in both mice, while CGRP induced vasodilatation in the vehicle-treated, but not in olcegepant-treated mouse. The cerebral vessel (CV) to the right of the dural artery in the top panels does not respond to ET-1 or CGRP. Data taken from Gupta *et al* (2006).[80]

Using these mutant mice *in vivo* in an intravital experimental setup clearly seemed a step forward in understanding the role of these mutations in the pathogenesis of migraine, but required modification of the rat intravital microscopy model to the other species. Therefore, we developed a mouse model using the intravital microscopy technique on a closed cranial window, and studied the role of exogenous and endogenous CGRP-induced vasodilatation in wild-type C57BL/6Jico mice.[80] In contrast to the rat model, arteries in mice need to be

preconstricted with endothelin-1, before vasodilatation by CGRP, capsaicin, or transcranial electrical stimulation of perivascular trigeminal nerves can be observed in Figure 8.[80] Similar to earlier observations in the rat model, the CGRP receptor antagonist olcegepant blocked responses evoked by CGRP, capsaicin and electrical stimulation, whereas sumatriptan blocked electrical stimulation-induced vasodilatation only. This model is likely to prove useful in dissecting aspects of the trigeminovascular system and this can be used for exploring various pathophysiological aspects of migraine, especially in future studies using transgenic mice with mutations relevant to those observed in patients with migraine.

玉不琢不成器

三字經

An unpolished gem will never get its true value
(San Zi Ying)

Chapter 2

Potential mechanisms of prospective antimigraine drugs; a focus on vascular (side) effects

Based on: K.Y. Chan, S. Vermeersch, J. de Hoon, C.M. Villalón & A. MaassenVanDenBrink, Pharmacology and Therapeutics (In press).

Abstract

Currently available drugs for the acute treatment of migraine, i.e. ergot alkaloids and triptans, are cranial vasoconstrictors. Although cranial vasoconstriction is likely to mediate -at least a part of- their therapeutic effects, this property also causes vascular side effects. Indeed, the ergot alkaloids and the triptans have been reported to induce myocardial ischemia and stroke, albeit in extremely rare cases, and are contraindicated in patients with known cardiovascular risk factors. In view of these limitations, novel antimigraine drugs devoid of vascular (side) effects, are being explored. Currently, calcitonin gene-related peptide (CGRP) receptor antagonists, which do not have direct vasoconstrictor effects, are under clinical development. Other classes of drugs, such as 5-HT_{1F} receptor agonists, glutamate receptor antagonists, nitric oxide synthase inhibitors, VPAC/PAC receptor antagonists and gap junction modulators, have also been proposed as potential targets for acute antimigraine drugs. Although these prospective drugs do not directly induce vasoconstriction, they may well induce indirect vascular effects by inhibiting or otherwise modulating responses to endogenous vasoactive substances. These indirect vascular effects might contribute to the therapeutic efficacy of these compounds, but may alternatively also lead to vascular side effects. As described in the current review, some of the prospective antimigraine drugs with a proposed non-vascular mechanism of action may still have direct or indirect vascular effects.

Introduction

Migraine is defined as a neurovascular disorder characterized by attacks of a severe, debilitating and throbbing unilateral headache associated with autonomic nervous dysfunction including nausea and vomiting, photophobia and phonophobia as well as neurologic symptoms.[3,81] Based on clinical features, three distinct phases of migraine can be discerned: a trigger, an aura and a headache phase.[3] In Western countries this disorder affects approximately 18% of women and 6% of men[8] Migraine represents an enormous socio-economic burden to the individual as well as to society[83] and profoundly affects the patient's quality of life.[84]

Pathophysiology of migraine

Although elusive for a long time, our understanding of the pathophysiology of migraine progressed significantly, evolving slowly from a malady of supernatural causes[85] to a disorder of vascular,[86-87] neurogenic[88-89] or neurovascular[90-91] origin. Currently, migraine is considered a neurovascular disorder involving activation of the trigemino-vascular system,[81] with the primary dysfunction located in brainstem centers regulating vascular tone and pain sensation.[92] This activation results in cranial vasodilatation mediated by the release of vasoactive neuropeptides including calcitonin gene-related peptide (CGRP), which seems to play a pivotal role in migraine pathophysiology.[91]

Currently available antimigraine drugs

The history of the treatment of headache in general, and migraine in particular, spans millennia, from the Neanderthal era to the Space Age.[93] With this long history, it is surprising that effective antimigraine drugs have been, until recently, limited in number. In the last decades, there have been big steps in the development of antimigraine drugs.[94] Beside analgesics, specific antimigraine drugs can be divided into: (i) agents that abolish an individual migraine attack (acute antimigraine drugs; i.e. ergots and triptans); and (ii) agents aimed at its prevention (prophylactic drugs; such as β-adrenoceptor blockers, anti-epileptics, etc.). Many patients need treatment to abolish attacks (acute treatment), but only patients with frequent attacks additionally need prophylactic treatment by drugs taken daily to reduce the number and/or severity of attacks.[95]

In acute antimigraine treatment triptans represent a considerable advance,[3] but their vasoconstrictor side effects warrant caution in patients with cardiovascular pathologies.[45] Other side effects such as dizziness, nausea, fatigue, chest symptoms and paresthesia prevent some patients from using triptans. Furthermore a number of patients do not respond well to the triptans; indeed, triptan monotherapy is ineffective or poorly tolerated in 1 out of 3 migraineurs and in 2 out of 5 migraine attacks.[96] The advent of CGRP receptor antagonists such as olcegepant (previously referred to as BIBN4096BS)[97] and telcagepant

(MK-0974)[98-100] bodes well for migraineurs who are poor or non-responders to triptan treatment. As will be discussed later in this review, these "gepants", which have an efficacy comparable to triptans, seem to have a better safety and tolerability profile.[91,101]

Ergot alkaloids

The ergot alkaloids, also referred to as "ergots", ergotamine and dihydroergotamine (DHE), were the first specific acute antimigraine drugs for several decades until the advent of the triptans.[102] The ergots were originally developed as sympatholytics, but it was later suggested that their therapeutic efficacy was probably mediated by vasoconstriction of cranial blood vessels.[103] As both ergotamine and DHE display affinity for a wide variety of receptors including 5-HT (5-hydroxytryptamine, serotonin), dopamine and noradrenaline receptors[103] they are considered "dirty drugs". As expected from this pharmacological profile, their most important pharmacological effect is arterial constriction.[103-104] Indeed, at therapeutic concentrations, ergotamine and DHE induce a potent vasoconstriction in the external carotid (extracranial) vascular bed of anaesthetized dogs mainly by activation of α-adrenoceptors and 5-HT (mainly 5-HT_{1B}) receptors.[105-106] Whereas both ergotamine and DHE constrict the cranial vascular bed, there is a difference in their capacity to constrict peripheral blood vessels. Ergotamine induces contraction of peripheral arteries, including the pulmonary,[107] cerebral,[103] temporal[108] and coronary[109] arteries. In contrast, DHE is a more potent constrictor of venous capacitance vessels than of arteries.[110] In humans, blood pressure is transiently increased for about three hours after parenteral therapeutic doses of ergotamine and DHE,[111-112] which is likely caused by an increased peripheral resistance.[113] Moreover, a much longer lasting constrictor effect on peripheral arteries (ergotamine) or veins (DHE) is induced. This is most likely caused by a slow diffusion from the receptor biophase;[114] the effects last much longer than expected from the plasma concentrations.[109,115-116] Thus, overall, based on *in vitro*, *in vivo* animal data and human clinical research, both ergotamine and DHE have the propensity to induce potent and longer lasting clinical effect in some patients, although the side-effect profile of DHE is more favourable compared to that of ergotamine.[117-118]

Besides a vascular mode of action, which was originally believed to be the exclusive mechanism of the antimigraine efficacy of ergot alkaloids, the neuronal properties of these compounds most probably also contribute to their clinical effects. The neuronal activity is probably mediated via the agonist activity at 5-HT_{1B}, 5-HT_{1D} and 5-HT_{1F} receptors on trigeminal nerve terminals resulting in the inhibition of the neuronal release of vasoactive peptides and preventing vasodilatation in migraine.[119]

Triptans

Triptans are 5-HT receptor agonists, displaying affinity mainly at the 5-HT_{1B} and 5-HT_{1D} receptor subtypes.[85] The development of the triptans was prompted by the hypothesis that

5-HT was involved in the pathophysiology of migraine. The factor restricting the clinical use of 5-HT as an antimigraine agent was the prevalence of side effects on the gastrointestinal and cardiovascular systems[120-121] as well as the need for intravenous infusion of 5-HT. The antimigraine efficacy of 5-HT clearly suggested the existence of a specific 5-HT receptor involved in the relief of migraine headache. The identification of the 5-HT receptor type (nowadays called the 5-HT_{1B} receptor) responsible for the beneficial effects of 5-HT provided the possibility to develop antimigraine drugs devoid of the side effects observed with the ergot alkoids.[122] The first triptan developed, sumatriptan, was introduced in the early 1990s,[40] and it did indeed change the lives of numerous migraineurs.[3] Compared to the ergot alkaloids, sumatriptan induces fewer side effects due to the increased selectivity on the 5-HT_{1B} and 5-HT_{1D} receptor,[44] thereby avoiding peripheral vasoconstriction as mediated, e.g., by the 5-HT_{2A} receptor for which ergotamine displays affinity. Further, the vasoconstrictor effects of sumatriptan are not sustained during a long period as is the case for the ergot alkaloids.[109] Limitations of sumatriptan are its low oral bioavailability (14%),[123] and headache recurrence within 24 hours in about one third of patients; nevertheless recurrence can be treated effectively with a subsequent dose of sumatriptan.[124-125] In order to overcome these limitations, over time, additional triptans have been developed with chemical structures similar to sumatriptan, but with a higher lipophilicity.[85] Whereas the pharmacodynamic profile of these so-called 'second generation' triptans resembles that of sumatriptan, there are differences in their pharmacokinetic properties, which may lead to advantages including earlier onset of action.[126] The antimigraine action of triptans is most likely mainly based on their potent vasoconstrictor effect on cranial blood vessels mediated via the 5-HT_{1B} receptor.[5,40] The high intracranial 5-HT_{1B} receptor density compared to extracranial blood vessels probably renders the triptans relatively selective for the intracranial vasoconstriction.[37] Nevertheless, in keeping with their agonist activity at the 5-HT_{1B} receptor, the triptans also have the potential to induce extracranial vasoconstriction. *In vivo*, in humans it was shown that sumatriptan as well as second-generation triptans induce vasoconstriction, increase blood pressure and decrease buffering capacity of conduit arteries after the intake of equipotent therapeutic dosages.[127-129] *In vitro*, constriction of coronary arteries was confirmed,[109,130] which is larger in distal than in proximal sections of the coronary artery.[131] Consequently, the triptans are contra-indicated in individuals with active cardiovascular disease and uncontrolled hypertension.[45] Nevertheless, recently, a retrospective case-control study demonstrated that the use of triptans in patients with cardiovascular risk factors (for whom these drugs are contraindicated) did not increase the incidence of ischemic cardiovascular complications.[132] Moreover, 5-HT_{1B} receptor expression does not differ between normal and atherosclerotic coronary arteries.[133]

It is generally accepted that, besides inducing vasoconstriction in cranial blood vessels, two additional mechanisms of action probably contribute to the therapeutic action of the triptans: (i) inhibition of the release of neuropeptides in perivascular nerve terminals of the

trigeminovascular system[134] and (ii) direct inhibition of neuronal activation, reducing central pain transmission via activation of 5-HT_{1D} and 5-HT_{1F} receptors.[3,135]

Vascular effects of prospective antimigraine drugs

Since the currently available antimigraine drugs have shortcomings and may cause cardiovascular side effects due to their vasoconstrictor properties, research now has focused on the development of antimigraine drugs devoid of vasoconstrictor effects. Several ligands that act centrally and affect neuronal transmission have been described to be potential targets for the prophylactic or acute treatment of migraine.[136] Some of these compounds may, however, also affect the release or action of vasoactive mediators. Examples of potential neuronal targets for future antimigraine drugs, are: the CGRP receptor, glutamate receptor, VPAC/PAC receptor, NOS synthase, 5-HT_{1F} receptor and gap junctions. However, if such antimigraine compounds would indeed inhibit the release of vasoactive agents or block the receptors involved in vasodilatation, these compounds will directly or indirectly induce vascular (side) effects. On this basis, the present review analyzes the preclinical as well as clinical experimental data on the vascular effects of several prospective antimigraine drugs. In the following sections, the (neurogenic) mechanism of action of a number of prospective antimigraine drugs, their (potential) vascular (side) effects and, when possible, their main clinical benefits and limitations are discussed.

CGRP receptor antagonists

CGRP is a potent vasodilator in several species[28,137-138] and is expressed throughout the central and peripheral nervous system.[139] Several lines of evidence support that CGRP plays an important role in the pathogenesis of migraine.[140] Elevated levels of CGRP have been observed in the jugular vein during a migraine attack and these levels were normalized after pain relief with sumatriptan.[141] Moreover, intravenous (i.v.) administration of CGRP can induce a migraine-like headache in migraine patients[142] and, thus, disruption of CGRP signaling represents a valid strategy for the treatment of migraine.[6,99] CGRP is found in the central nervous system (particularly in striatum, amygdalae, colliculi and cerebellum), as well as in the vessel wall of intracranial arteries.[90,92,143] CGRP is located in primary spinal afferent C and Aδ fibers projecting to the trigeminal nuclear complex in the brainstem.[144] Moreover, in the trigeminal nucleus caudalis and at C1/C2 levels, CGRP acts at second order neurons to transmit pain signals centrally through the brainstem and midbrain to the thalamus and higher cortical pain regions.[145] In addition, components of the functional CGRP receptor complex, such as calcitonin-like receptor and receptor activity modifying protein 1 (RAMP1) have recently been localized on trigeminal neurons,[146] and it is suggested that they modulate

the prejunctional CGRP production.[147] In the cranial circulation, CGRP is released by perivascular nerve fibers after trigeminal nerve activation where it induces vasodilatation of cranial arteries by binding to the CGRP receptor.[148-150] CGRP initiates vasodilatation through interaction with these G-protein coupled receptors of the B-type that are primarily coupled to the activation of adenylyl cyclase. *In vitro*, this vasodilatation is independent of endothelium in human cerebral, meningeal[35,148,151] and coronary[28] blood vessels, although in animals an NO-dependent component seems to be present,[152] while other studies provided evidence against an endothelial mechanism, but suggested that the dilatation is associated with activation of adenylyl cyclase.[148] Likewise, *in vivo* in the human peripheral circulation, CGRP-induced vasodilatation is, at least in part, dependent on the release of nitric oxide.[153] Blockade of the functional CGRP receptor complex, which consists of the calcitonin-like receptor component and RAMP1,[154] prevents vasodilatation induced by CGRP. CGRP receptor characterization has in the past relied on the use of the peptide antagonist $CGRP_{8-37}$. Recently the more potent CGRP receptor antagonists olcegepant and telcagepant which are effective in the acute treatment of migraine, became available.[97,99] Although these CGRP receptor antagonists were originally designed to prevent the neurogenic vasodilatation occurring in the pathogenesis of migraine, these antagonists also seem to display central effects that may be clinically important in the treatment of this disorder.[91]

Central effects of CGRP receptor antagonists

It remains unclear whether the antimigraine action of CGRP receptor antagonists is mediated via a central or a peripheral mechanism. Several arguments suggest a central mechanism of action, namely: (i) the lack of presynaptic CGRP receptors in the meninges, which suggests that exogenous CGRP is unlikely to directly modify the innervating sensory nerve fibers;[147] (ii) the fact that exogenous CGRP in the meninges, including meningeal vasodilatation, is not sufficient to activate or sensitize meningeal nociceptors in the rat.[155] This suggests that an action of CGRP on the dura mater cannot account for the activation of peripheral afferents during migraine;[155] (iii) olcegepant inhibits the postsynaptic nociceptive transmission in the trigeminal system by intravenous administration, but not by topical administration on the dura;[156] and, (iv) given the very high clinical doses reported to achieve antimigraine efficacy compared to the *in vitro* receptor binding characteristics of telcagepant, the need for penetration through the blood-brain barrier has been suggested.[97,99,157] However, the discrepancy between the high clinical doses and the binding affinity *in vitro* is probably not only explained by penetration of the blood-brain barrier, but also by other factors, which will be explained below.

Vascular effects of CGRP receptor antagonists

Olcegepant has a high affinity for the primate CGRP receptor (K_i: 0.014 nM), and potently

antagonizes *in vitro* the vasodilator response to CGRP in human cranial arteries.[35,41,158] Besides its direct antagonist effect in cranial arteries, olcegepant is capable of blocking the vasodilatation induced by stimulation of the trigeminal nerve. Moreover, olcegepant: (i) dose-dependently antagonized CGRP-mediated neurogenic vasodilatation caused by trigeminal ganglion stimulation in monkeys and rats;[6] (ii) blocked the changes in facial blood flow induced by brainstem trigeminal nucleus caudalis activation in rats;[159] and (iii) attenuated capsaicin-induced carotid arteriovenous anastomotic vasodilatation.[24] However, due to its physicochemical properties and for reasons of bioavailability, olcegepant needs to be administered intravenously, which reduces its therapeutic value. In contrast with olcegepant, telcagepant (MK-0974) is orally bioavailable,[160] and has also been shown to be effective as an antimigraine drug.[98-99,161] Pharmacological characterization of telcagepant showed that this drug displays equal affinity for native and cloned CGRP receptors as determined by radioligand binding experiments (K_i: 0.77 nM).[162] Moreover, telcagepant antagonized in a concentration-dependent manner: (i) CGRP-induced cAMP accumulation in cells expressing the recombinant human CGRP receptor;[162] (ii) the vasodilator effect of αCGRP in human isolated cranial arteries;[163] and (iii) capsaicin-induced dermal vasodilatation, which is caused by endogenous CGRP release via TRPV1 receptor activation in the rhesus forearm.[162]

Besides its role in the cranial circulation, CGRP is also important in the peripheral cardiovascular system. CGRP is a potent peripheral vasodilator which affects the myocardium. The human coronary circulation is innervated by a dense supply of CGRP-positive fibers[164] and the CGRP receptor components are found in human coronary arteries.[28,165] Indeed, both olcegepant[28] and telcagepant[165] antagonize the vasodilator effect of αCGRP in human isolated coronary arteries. Moreover, increased cAMP levels induced by αCGRP are reduced when coronary arteries are pre-treated with olcegepant[28] or telcagepant,[165] suggesting that the blocking effect is mediated via the CGRP receptor. CGRP increases heart rate[166] and has positive inotropic effects on isolated human trabeculae.[167] Hence, blockade of the CGRP receptors might affect cardiovascular responses induced by CGRP. However, *in vivo* hemodynamic studies in dogs have reported no effect of $CGRP_{8-37}$ on coronary or myocardial regional blood flow.[168] Olcegepant has also been reported to have no effect on myocardial vascular conductance in rat and pig,[24,169] nor does olcegepant alter baseline hemodynamics in animals.[169] These data suggest that endogenous CGRP is not important in cardiovascular regulation under resting conditions in cardiovascularly healthy subjects. On the other hand, CGRP receptor antagonists might display adverse effects in pathophysiological conditions. CGRP is proposed as a pivotal player in ischemia-reperfusion[170-171] and ischemic preconditioning.[172] Indeed, CGRP has a protective effect during coronary[173-174] and cranial ischemia,[175-176] which was demonstrated both in pre-and post- conditioning ischemic reperfusion models. Moreover, $CGRP_{8-37}$[177] and olcegepant[57] blocked the protective effect of CGRP in isolated rat heart model. Based on these observations, it may be suggested that

a CGRP receptor antagonist, especially after chronic use as would be the case when used as a prophylactic drug, might attenuate the cardioprotective effect of CGRP. Nevertheless, the effect of CGRP receptor antagonism on ischemic preconditioning has until now been demonstrated only in isolated rodent hearts, and the pathophysiological significance of this observation is uncertain. In addition, in the setting of pathology, *in vivo* studies of $CGRP_{8-37}$ and olcegepant in rat and pig report no effect on coronary ischemia/reperfusion infarct size.[178-179] Further, $CGRP_{8-37}$ had no effect on myocardial blood flow in dogs with heart failure produced by previous myocardial infarction and rapid ventricular pacing,[180] while another study in dogs reported no effect of topically administered $CGRP_{8-37}$ onto the left ventricular surface on coronary artery microvessel diameter prior to and at 10 min following coronary artery occlusion.[181]

Taken together, although CGRP receptor antagonists under some pathophysiological conditions might negatively affect the body's protective mechanisms, it should be kept in mind that CGRP receptor antagonists do not have vasoconstrictor properties per se in human cranial and coronary arteries,[28,35,165] as the triptans do. This, as will further be discussed below, may be an advantage in view of cardiovascular safety, particularly in migraine patients suffering from cardiovascular pathologies.[91]

Clinical effects of CGRP receptor antagonists

Boehringer-Ingelheim's BIBN4096BS (olcegepant) was the first CGRP receptor antagonist entering the clinical development phase and provided proof-of-concept for the involvement of CGRP in migraine pathophysiology.[97] Being the first of a generation of CGRP receptor antagonists, olcegepant showed promising cerebral and systemic hemodynamics in humans, and thus putted this potential new class of anti-migraine drugs in favor of the triptans.[182] The study by Petersen *et al.* (2005)[182] with olcegepant was inconsistent with the assumption that CGRP receptor antagonists could alter the tone of cerebral and extracerebral arteries. Under resting conditions, no effect of olcegepant was seen on regional and global cerebral perfusion, middle cerebral artery (MCA) blood flow velocity, temporal and radial artery diameter, blood pressure or heart rate. The same group further showed that olcegepant effectively prevents CGRP-induced headache and extracerebral vasodilatation without significant effects on cerebral hemodynamics.[182] After h-αCGRP infusion, olcegepant had no effect on blood flow velocity and regional perfusion of the MCA compared to placebo, however, it prevented dilatation of the superficial temporal and radial arteries as well as reflex tachycardia resulting from systemic vasodilatation.

Although an additional neuronal action cannot be ruled out, in line with its limited ability to penetrate through the blood-brain barrier,[183] it is suggested that olcegepant treats migraine headache predominantly by acting at the level of the large dural blood vessels which are not protected by the blood-brain barrier. Resolving the question whether CGRP

antagonists exhibit their anti-migraine effect in the CNS or through a vascular mechanism of action outside the CNS, is a very relevant issue in the future development of new and safe CGRP-antagonists. To that end, additional research is needed in order to answer this question. Unfortunately, due to its low oral bioavailability, the further development of olcegepant was early terminated. Recently, with BI44370,[101] the company has a new CGRP receptor antagonist ready for phase III clinical development. Given the fact that the efficacy of olcegepant was comparable to that of the triptans in acute migraine, while it had the major advantage of not inducing a direct vasoconstrictor response, validated the CGRP receptor as a valuable target and inspired many companies to develop CGRP receptor antagonists as an effective and safe alternative for the triptans.

The first orally available CGRP-receptor antagonist developed after olcegepant was the Merck compound MK-0974 (telcagepant). It is structurally derived from olcegepant[160,184] and has a promising anti-migraine profile.[99] Indeed, telcagepant showed an efficacy comparable to that of zolmitriptan, but with fewer side-effects. The most commonly frequent adverse effects with telcagepant were dry mouth, somnolence, dizziness, nausea and fatigue.[185] Adverse effects that occur after the use of triptans like asthenia, paraesthesia, chest discomfort, fatigue, myalgia, dizziness and throat tightness, are less frequent after the use of telcagepant.[186] Remarkably, an efficacy and tolerability study in patients with stable coronary artery disease[98] did not demonstrate a significant difference in 2-hours headache freedom between telcagepant (13/52, 25.0%) and placebo (10/53, 18.9%). However, the study design and lower than expected number of patients enrolled may have been the reason for this negative result. The side-effect profile of telcagepant with intermittent dosing, as required for the acute treatment of migraine, looks excellent. Unfortunately however, a small number of patients taking telcagepant twice daily for three months as prophylactic treatment showed marked elevations in liver transaminases.[187] It has been suggested that this is a results of drug accumulation with daily dosing, since this is not seen in acute intermittent dosing. As a consequence of this potential for hepatotoxicity, Merck Research Laboratories announced to delay filing of the U.S. application for telcagepant for the treatment of acute migraine.

During the exploratory phase of the clinical development, studies focused on a biomarker model involving capsaicin-induced changes in dermal blood flow which are the result of local CGRP release. Therefore, inhibition of the increase in dermal vascular blood flow by telcagepant served as a surrogate for the dose at which clinical efficacy could be expected.[188] After topical application of capsaicin on the human forearm, orally administered telcagepant effectively inhibited the CGRP mediated increase in dermal blood flow.[189-190] Comparable results were obtained with a follow-up high potent CGRP-receptor antagonist, MK-3207.[191] As neither MK-0974 nor MK-3207 affected dermal blood flow under resting conditions, this argues against direct vasoconstrictor effects of CGRP receptor antagonists while they are very effective at preventing CGRP-mediated vasodilatation. The blockade by

MK-3207 in the capsaicin biomarker model, guided the dose selection for further clinical trials with the compound. Unfortunately, the clinical development program for MK-3207 was discontinued[192] after delayed, asymptomatic liver test abnormalities in extended Phase I studies where reported.[193]

Limited data about specific vascular effects of telcagepant in a clinical setting are available. No effects on vital signs including blood pressure and ECG were reported in the first published safety data.[99] High doses of telcagepant, i.e. 560 and 600 mg, daily for up to 8 days had no clinically significant effect on 24 hours mean ambulatory blood pressure or heart rate.[194] In a pharmacodynamic drug-interaction study with sumatriptan (100 mg),[195] a supratherapeutic dose of telcagepant (600 mg) did not significantly increase mean arterial blood pressure when administered as monotherapy to migraineurs during the interictal period. Co-administration, however, with sumatriptan resulted in an increased mean arterial blood pressure, comparable to the increase reported after administration of sumatriptan alone.[127]

When looking specifically at cardiac safety, one study investigated the effect of telcagepant on spontaneous ischemia in patients with stable coronary artery disease.[196] No episodes of chest pain were reported on the days of dosing of telcagepant or placebo. No obvious treatment-related changes in vital-signs or ECG safety parameters appeared. Apparently, two 300 mg doses of telcagepant, administered 2 hours apart, did not exacerbate spontaneous ischemia. However, important questions including the long term safety of CGRP antagonists, e.g. in a prophylactic setting, or in patients having an ischemic event, remain unanswered. With the medical need for patients with coronary artery disease in mind, the effect of telcagepant on the hemodynamic response to therapeutic doses of nitroglycerine (NTG) was investigated in healthy volunteers in order to exclude a potential pharmacodynamic interaction.[197] First, telcagepant did not influence brachial artery diameter under resting conditions. Secondly, telcagepant did not affect NTG-induced decrease in arterial stiffness, neither did it affect NTG-induced increase in brachial artery diameter. The study concluded that CGRP receptor antagonists have no influence on the vasodilatory response to an exogenous NO donor.

Although available data show no interference of CGRP receptor antagonists on resting hemodynamics, CGRP blockade under clinical ischemic conditions could have clinical consequences. However, in an acute setting, a single oral dose of telcagepant did not reduce exercise tolerance in patients with exercise-induced myocardial ischemia at T_{max} post telcagepant.[198] Exercise duration, maximum heart rate, chest pain or maximum ST segment depression did not differ between placebo and telcagepant in patients with chronic angina and limiting exercise-induced cardiac ischemia.[198] Although these data show no interference of acute CGRP receptor antagonism with myocardial ischemia, the effects of chronic CGRP blockade under clinical ischemic conditions remain unknown.

In the wake of telcagepant, many companies target the development of CGRP receptor antagonists, now that its clinical efficacy in migraine has been demonstrated. Therefore, more

studies will be performed in the future to test the vascular effects of this new class of drugs, which will provide more information about central and/or peripheral mode of action and additional reassurance about their cardiovascular safety profile.

Discussion of CGRP receptor antagonists

Summarizing data from both clinical and preclinical studies, it seems clear that: (i) CGRP receptor antagonists are clinically effective in the treatment of migraine, probably to a similar extent as the triptans. It is not known yet whether responders and non-responders to triptans will respond to CGRP receptor antagonists in a similar way; and (ii) the site of action of CGRP receptor antagonists is not yet clear, and may be both vascular and neuronal. The discrepancy between the high clinical doses of gepants and the *in vitro* binding affinity (the plasma concentrations used to achieve antimigraine efficacy are considerably higher than their *in vitro* pA_2) is not only explained by the penetration of the blood brain barrier, but also by other factors, namely: (i) high protein binding of these compounds (about 95-96%);[185] (ii) a concentration of drug equal to the pA_2 value may not be sufficient to decrease a functional responses since it only shifts the concentration response curves two-fold to the right; most likely a concentration of a least 10 times pA_2 would functionally inhibit relaxations to CGRP; (iii) as nerve terminals releasing CGRP are located in the adventitia close to the media layer of the blood vessels, the concentration of telcagepant at the receptors may be substantially lower than that at the lumen of the blood vessel, i.e. the plasma concentration. The latter phenomenon is unlikely to occur *in vitro*, where the antagonist can reach the CGRP receptors from both the luminal and abluminal sides. Thus, although both a vascular and neuronal action of CGRP receptor antagonists may currently not be excluded, it seems that the fact that these drugs do not induce direct vasoconstriction is an advantage over the currently available antimigraine drugs. Moreover, in dogs, during acute regional myocardial ischemia induced by atrial pacing in the presence of coronary stenosis, neither CGRP nor $CGRP_{8-37}$ affected coronary flow and severity of ischemia, whereas sumatriptan exacerbated ischemia severity with concomitant reduction in coronary blood flow.[199-200] Likewise, in the dog, $CGRP_{8-37}$ had no effect on myocardial reactive hyperemic response following brief mechanical coronary artery occlusion, whereas sumatriptan reduced peak reactive hyperemic coronary artery blood flow, reactive hyperemic flow and the repayment of coronary blood flow debt.[201] These findings are consistent with the contractile response, unrelated to relaxation to CGRP, observed with triptans in human healthy[109,165] and diseased[202] coronary arteries. Notwithstanding, the above-mentioned studies on the safety of CGRP receptor antagonists, the consequences of CGRP blockade under ischemic conditions remain unknown. As it has not been excluded in human studies that CGRP is involved in ischemia-reperfusion and ischemic preconditioning, CGRP receptor antagonists should be used cautiously in patients with ischemic heart disease. Therefore, specific prudence is called for CGRP antagonists in a prophylactic setting.

5-HT receptor ligands

Serotonin (5-hydroxytryptamine; 5-HT) was one of the first monoamines proposed to be involved in the pathophysiology of migraine on the basis of several lines of evidence, including: (i) some drugs that deplete monoamines (reserpine) can provoke a migraine attack;[121] (ii) high quantities of 5-hydroxyindole acetic acid, a metabolite of 5-HT, are excreted during a migraine attack;[203] and (iii) a slow i.v infusion of 5-HT can abort an attack of migraine.[120-121] Side effects and the need for i.v. infusion precluded the clinical use of 5-HT as an antimigraine agent. Side effects included: gastrointestinal effects, changes in heart rate, vasodilatation in some vascular beds (e.g. cutaneous blood vessels) and vasoconstriction in others (e.g. the external carotid bed).[120-121] The antimigraine efficacy of 5-HT clearly suggested the existence of a specific 5-HT receptor involved in the relief of migraine headache but, admittedly, the association between 5-HT and the mechanisms underlying the pathogenesis of migraine is circumstantial. It is undeniable that the cranial vasoconstrictor activity of sumatriptan and the second-generation triptans, mediated by the 5-HT_{1B} receptor, is associated with their efficacy in the acute treatment of migraine.[85,204] Unfortunately, the 5-HT_{1B} receptor, being not exclusively confined to cranial blood vessels, is most likely also responsible for the moderate hypertension and coronary constriction noticed with these drugs. The development of antimigraine agents without cardiovascular side effects, but capable of inhibiting trigeminal CGRP release would avoid the vasoconstrictor action of the triptans and would represent a major improvement over current treatments.[136] Therefore, in an attempt to avoid coronary vasoconstriction, other avenues have been explored: (i) 5-HT_{1D} and 5-HT_{1F} receptor agonists; and (ii) 5-HT_{7} receptor antagonists.

As described above, the triptans have a high affinity for 5-HT_{1B} and 5-HT_{1D} receptors. However, most triptans show also high pK_i values for the 5-HT_{1F} receptor.[204-205] The 5-HT_{1B} receptor has now clearly been linked to vasoconstriction,[85] whereas stimulation of 5-HT_{1D} or 5-HT_{1F} receptors induces inhibition of the trigeminovascular system without vasoconstriction.[204-205] This led to the synthesis of a series of isochroman-6-carboxamide derivatives, including PNU-109291 and PNU-142633, which have been described as highly selective 5-HT_{1D} receptor agonists.[206-207] In addition, three potent and selective 5-HT_{1F} receptor agonists have been reported (with their corresponding pK_i values at 5-HT_{1B}, 5-HT_{1D} and 5-HT_{1F} receptors, respectively), namely: (i) LY344864 (pK_i values: 6.3, 6.2 and 8.2); (ii) LY334370 (pK_i values: 6.9, 6.9 and 8.8); and lasmiditan (also known as COL-144 and LY573144, pK_i values: 5.9, 5.8 and 8.6).[208-211]

Despite acknowledging that most of the evidence supporting the role of 5-HT in the pathophysiology of migraine is circumstantial, this monoamine has been shown to produce, via activation of 5-HT_{7} receptors: (i) direct vasodilatation of cranial blood vessels;[212-213] (ii) excitation in neuronal systems;[214] (iii) hyperalgesic pain and neurogenic inflammation,[215-216] (iv) neuroinflammatory processes;[217] and (v) central sensitization and

activation of pain pathways.[218] All of these processes have been demonstrated to participate in migraine pathophysiology.

Central effects of 5-HT receptor ligands

The 5-HT_{1F} receptor agonist LY334370 exerts a central mechanism of action by inhibiting the transmission of nociceptive impulses within the trigeminal nucleus caudalis.[219] Likewise, the selective agonists at the 5-HT_{1D}, PNU 142633[207] and the 5-HT_{1F}, LY334370[209] receptors inhibit the trigeminovascular system.[210] This led to the exploration of the effects of selective 5-HT_{1D} and 5-HT_{1F} agonists as antimigraine drugs that would partly act like the triptans, but without vascular effects. There is a high correlation between the potency of various 5-HT_1 receptor agonists in the guinea-pig dural plasma protein extravasation assay and their 5-HT_{1F} receptor binding affinity.[210] However, the relevance of plasma protein extravasation in migraine is no longer tenable.[220] More recently, Nelson *et al.*[211] have shown that lasmiditan: (i) is a more selective agonist for 5-HT_{1F} receptors (selectivity ratio of about 500-fold relative to other 5-HT_1 receptor subtypes) than the first generation 5-HT_{1F} receptor agonist LY334370 (selectivity ratio of about 100-fold relative to other 5-HT_1 receptor subtypes); (ii) potently inhibited, when given orally to rats, markers associated with trigeminal ganglion stimulation, including induction of immediate early gene *c*-Fos in the trigeminal nucleus caudalis; and (iii) displays chemical properties and a pharmacological profile that differ from that of the triptans. Furthermore, 5-HT_{1F} receptors are located on glutamate-containing neurons and their activation might inhibit glutamate release,[221] which may be relevant to its antimigraine action.[222] Indeed, most triptans show high pK_i values for 5-HT_{1F} receptors.[204-205,223]

On the other hand, Agosti[224] has hypothesized that activation of 5-HT_7 receptors may mediate the release of neuropeptides (substance P and CGRP), neurogenic inflammation, and hyperalgesia in the trigeminovascular system during a migraine attack. In agreement with this hypothesis, it has recently been shown in anesthetized rats that the selective 5-HT_7 receptor antagonist SB269970 caused a significant decrease in serum CGRP concentrations following electrical stimulation of the trigeminal ganglion. This effect was reversed by the putative 5-HT_7 receptor agonist AS19.[225]

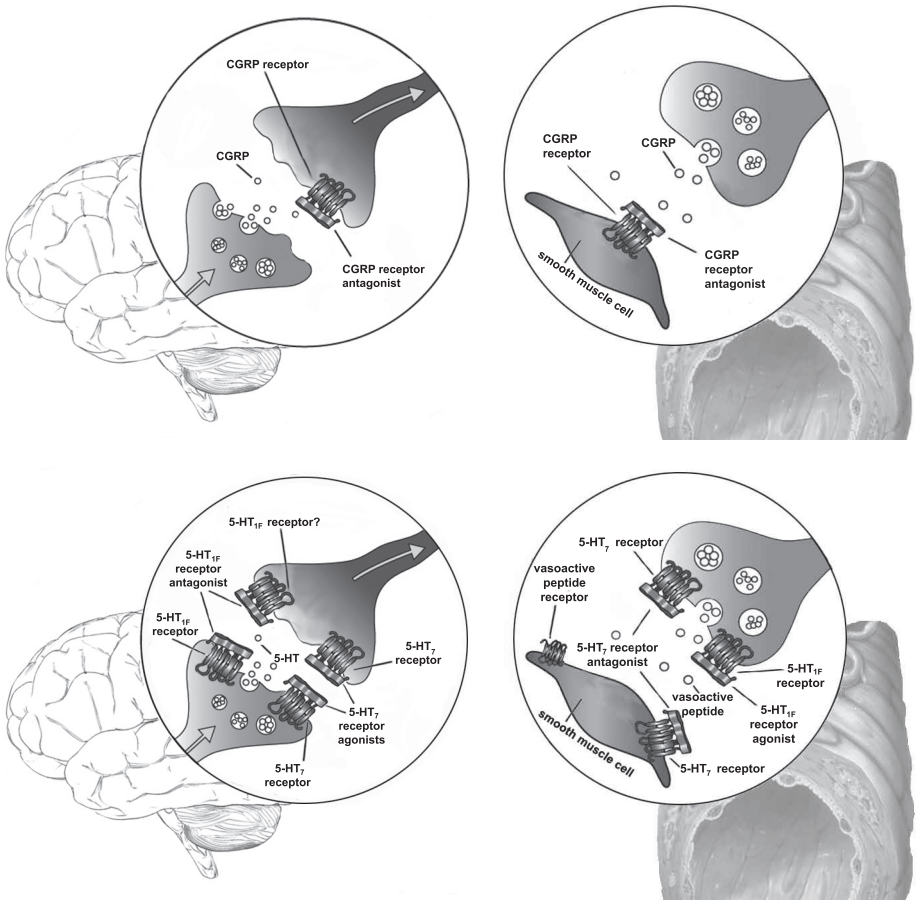

Figure 1: Schematic representation of a synapse in the CNS (left panels) and a neurovascular junction (right panels). **Upper panels:** stimulation of postsynaptic CGRP receptors in the CNS by CGRP released from CGRPergic neurons may produce neuronal activation (left panel), whereas stimulation of vascular CGRP receptors by CGRP released from sensory perivascular nerve terminals may induce vasodilatation (right panel). Thus, CGRP receptor antagonists may affect neurotransmission as well as vasodilatation. **Lower panels:** stimulation of pre- and post-synaptic 5-HT_7 and 5-HT_{1F} receptors in the CNS by 5-HT released from serotonergic neurons may affect neurotransmission. On the other hand, on the neurovascular junction, stimulation of prejunctional 5-HT_7 receptors may increase the release of neuromediators (heteroreceptors; e.g. CGRP) resulting in vasodilatation, whereas stimulation of prejunctional 5-HT_{1F} receptors may inhibit the release of neuromediators (heteroreceptors; e.g. CGRP). Stimulation of vascular 5-HT_7 receptors results in vasodilatation, whereas 5-HT_{1F} receptors are not functional in blood vessels. Thus, both 5-HT_7 receptor antagonists and 5-HT_{1F} receptor agonists may block neurotransmission in the trigeminovascular system.

Vascular effects of 5-HT receptor ligands

Unlike the triptans (5-$HT_{1B/1D/1F}$ receptor agonists), 5-HT_{1D} and 5-HT_{1F} receptor agonists are devoid of contractile effects on coronary and cerebral blood vessels.[207,226] PNU-109291 and PNU-142633 do not produce vasoconstriction in *in vivo* (canine external and internal carotid bed)[227] or *in vitro* (cerebral arteries)[226] preparations. Likewise, LY344864, LY334370 and lasmiditan were devoid of vasoconstrictor activity.[211,226] Together with the fact that the 5-HT_{1B} receptor antagonist SB224289, which displays little affinity at the 5-HT_{1F} receptor,[228] completely blocked sumatriptan-induced external carotid vasoconstriction,[229] it is clear that the 5-HT_{1F} receptor is not involved in the vascular effects of triptans. It therefore implies that if LY334370 and lasmiditan turn out to be effective in migraine at clinical doses devoid of 5-$HT_{1B/1D}$ receptor interaction, the mechanism of action will not be via cranial vasoconstriction.

Interestingly, prophylactic antimigraine drugs such as methysergide[230] and lisuride[231] display high affinity for 5-HT_7 receptors[232] and are capable of blocking 5-HT_7 receptor-mediated vasodilatation in the canine extracranial external carotid circulation,[213] which shows a direct (relaxant) effect of the 5-HT_7 receptor on cranial blood vessels. More recently, it has been shown in anesthetized rats that the selective 5-HT_7 receptor antagonist SB269970 caused a significant decrease in serum CGRP concentrations following electrical stimulation of the trigeminal ganglion and that this effect was reversed by the putative 5-HT_7 receptor agonist AS19.[225] These findings, taken together, suggest that 5-HT_7 receptors may play a role in the pathophysiology of migraine.

Clinical effects of 5-HT receptor ligands

Despite the above trigeminal inhibition, PNU-142633 proved to be ineffective in the acute treatment of migraine,[233] whilst LY334370 did show some efficacy when used in doses which may have interacted with 5-HT_{1B} receptors.[210,234] Though clinical studies demonstrated that LY334370 is effective in treating migraine headaches without coronary side effects,[210] it was recognized that more studies on the role of the 5-HT_{1F} receptor in migraine were warranted.[205] In this respect, the potency, selectivity and pharmacological profile of lasmiditan at 5-HT_{1F} receptors (see above) made it an ideal drug to definitely test the involvement of 5-HT_{1F} receptors in the therapy of migraine headache.[211] Indeed, Ferrari *et al.*[235] have recently reported the results of a randomized, multicenter, placebo-controlled, double-blind, group-sequential, adaptive treatment-assignment, proof-of-concept and dose-finding study using lasmiditan in 130 subjects during a migraine attack. Lasmiditan (at 20 mg i.v. and higher doses) proved effective in the acute treatment of migraine without inducing: (i) serious adverse events or withdrawals due to non-serious adverse events; (ii) triptan-like chest symptoms or chest discomfort; and (iii) significant changes in vital signs or ECG parameters or in hematological or clinical chemistry parameters. Adverse effects were generally mild and included dizziness, paresthesia and sensations of heaviness.[235] Further studies to asses

the optimal oral dose and full efficacy and tolerability profile are expected with great interest. Clearly, lasmiditan's non-vascular neuronal mechanism of action may offer an alternative antimigraine treatment, particularly in patients with cardiovascular pathologies and for whom antimigraine vasoconstrictor agents are contraindicated.

Discussion of 5-HT receptor ligands

The clinical efficacy of 5-HT_{1F} receptor agonists has been demonstrated, some preclinical experiments and clinical observations argue in favour of the potential effectiveness of selective 5-HT_{1F} agonists in migraine. While it remains to be confirmed that the 5-HT_{1F} receptor agonists are devoid of 5-HT_{1B} receptor activity at clinical doses, these antimigraine drugs have potential advantages compared to the triptans.

Furthermore, the preclinical data on 5-HT_7 receptors suggests that this receptor may play a role in the pathophysiology of migraine. The antimigraine efficacy of selective 5-HT_7 receptor antagonists in clinical trials is awaited with great interest. However, the involvement of the 5-HT_7 receptor in vasodilatation and CGRP release suggests potential direct and indirect vascular effects. Therefore, the safety of 5-HT_7 receptor antagonists should be well considered.

Glutamate receptor antagonists

Glutamate is an excitatory neurotransmitter in the mammalian CNS and plays an important role in the mediation of excitatory synaptic transmission. Glutamate exerts its effects by activating ionotropic (ligand-gated ion channels) and metabotropic (G-protein coupled) receptors. Glutamate has been suggested to be involved in the pathophysiology of migraine[236] as it is found in neurons of structures related to migraine pathophysiology, including the trigeminal ganglion, trigeminocervical complex and the thalamus.[237] Indeed, glutamate and CGRP are co-released from trigeminal ganglion neurons by calcium channel-dependent mechanisms,[238] and increased levels of glutamate have been found in the trigeminocervical complex after stimulation of dural structures.[239] Moreover, glutamate levels were found to be elevated in the cerebrospinal fluid of migraine patients compared with controls, suggesting an excess of neuroexcitatory amino acids in the CNS.[240] In addition, cutaneous allodynia, which is a sign for the development of central sensitisation, has been observed in migraine patients during an attack,[241] and glutamate release (and in some extent glutamate receptor activation) is involved in central sensitisation induced by peripheral sensory stimulation.[242]

Since activation of glutamate receptors by glutamate triggers post-synaptic excitatory potentials,[243] and experimentally produced pain increased the extracellular levels of glutamate in rat ventroposteromedial thalamic nucleus (VMP),[243] it has been suggested that glutamate also plays a role in the transmission of nociceptive information in the sensory thalamus. Moreover, the *N*-methyl-*D*-aspartate (NMDA) glutamate receptors are activated

during cortical spreading depression (CSD), which is considered to be involved in migraine aura.[243-244] In view of the fact that glutamate seems to play a significant role in migraine processes, pharmacological management of glutamate receptors may provide further insight into potential therapy for the treatment of migraine. Indeed, several studies have suggested that ionotropic glutamate receptor antagonists affect processes involved in the pathophysiology of migraine.

The ionotropic glutamate receptors are ligand-gated ion channels and are divided into NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors.[245] They primarily mediate fast synaptic transmission and have been identified in the superficial laminae of the trigeminal nucleus caudalis[246] and the sensory thalamus among other pain-related areas of the rat brain.[247] Moreover, messenger RNA of NMDA and kainate receptors has been found in the trigeminal ganglion.[248]

Central effects of glutamate receptor antagonists

Different NMDA and non-NMDA glutamate receptor antagonists have demonstrated to attenuate mechanisms that are putatively involved in the pathophysiology of migraine, including inhibition of trigeminovascular nociception in the trigeminocervical nucleus.[249-251] Further, the NMDA receptor has been implied to mediate antinociceptive effects in the descending brainstem nuclei.[252] The NMDA receptor antagonist MK801 reduces c-fos protein expression in the trigeminal nucleus caudalis after intracisternal capsaicin injection or other painful stimuli,[253-255] while it increases c-fos-like immunoreactivity in the periaquaductal grey, dorsal raphe nucleus and nucleus raphe magnus.[253] Moreover, MK801 blocks cell firing in the trigeminal cervical complex induced by electrical stimulation of the superior sagittal sinus.[250-251] Interestingly, the NMDA receptor antagonists MK801 and memantine prevent CSD, while the AMPA/kainate receptor antagonist, NBQX (2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(F)quinoxaline) has no effect.[256-257] Since systemic administration of the glycine site selective NMDA receptor antagonist, L701324, prevents the induction of CSD,[258] a role for the NMDA receptor subunit 1 that carries the glycine binding site of the NMDA receptor has been suggested.[259] Taken together, the NMDA receptor mediates diverse mechanisms that may be of clinical relevance in the treatment of migraine.

The AMPA/kainate receptor antagonists CNQX and NBQX are also capable of reducing c-fos protein expression in the trigeminal nucleus caudalis after intracisternal capsaicin injection.[254-255] Moreover: (i) trigeminal ganglion stimulation induced c-fos expression in the trigeminal cervical complex, and this response was attenuated after intravenous administration of the AMPA/kainate receptor antagonist, tezampanel (LY293558 or NGX424);[260] (ii) cell firing in response to electrical stimulation of dural structures in the trigeminocervical complex is blocked by CNQX; and (iii) trigeminovascular-evoked responses in the cat trigeminal cervical complex is dose-dependently inhibited by the AMPA receptor antagonist, GYKI52466.[251] Since the AMPA/kainate receptor antagonist, tezampanel, blocked the dural

plasma protein extravasation after electrical stimulation of the trigeminal ganglion, while the specific AMPA receptor antagonist, LY300168, had no effect,[261] suggesting that the effect of tezampanel might be mediated via the kainate receptor rather than the AMPA receptor.[261]

The clinically active kainate receptor antagonist, LY466195, inhibits c-fos expression in the trigeminal cervical complex induced by trigeminal ganglion stimulation.[260] Moreover, the kainate receptor antagonist UBP302 blocked the cell firing induced by electrical stimulation of dural structures in the ventroposteromedial (VPM) nucleus and post-synaptic firing in response to kainate receptor activation.[262] It is noteworthy that the VPM nucleus may be involved in the transmission of painful sensory information to the cortex when the trigeminovascular system is active;[263] hence, the pharmacological effect of LY466195 in the treatment of migraine might be partly explained by blocking the glutamatergic neurotransmission through kainate receptors in the VPM nucleus.[263]

Vascular effects of glutamate receptor antagonists

Although glutamate plays an important role in the mediation of excitatory synaptic transmission, it may also induce vascular effects since activation of ionotropic glutamate receptors on neurons may lead to the release of vasoactive substances and production of nitric oxide (NO), which is mediated by calcium influx, and therefore activation of intracellular signalling pathways.[264] A direct effect on vascular tone may be less likely, since it has been demonstrated that increased glutamate levels did not affect vascular tone in pial arteries of rat, cat and humans.[265]

As described in the previous section, it is suggested that treatment with ionotropic glutamate receptor antagonists would be active at central sites involved in the pathophysiology of migraine, without affecting vascular mechanisms. However, the finding that the NMDA receptor antagonist, MK801, reduced capsaicin-evoked CGRP release[266] points to potential indirect vascular effects of glutamate receptor antagonists. This is supported by the finding that the NMDA receptor antagonists ketamine and MK801 are capable of inhibiting neurovascular CGRP release.[267] Moreover, activation of neuronal NMDA receptors results in the release of NO, which causes vasodilatation.[268] However, the AMPA receptor antagonist, GYKI52466, did not affect CGRP release nor the vasodilatation induced by endogenous CGRP;[267] these findings are in accordance with the finding that AMPA receptors are absent in the peripheral trigeminovascular system.[248]

The kainate receptor antagonist, LY466195, does not induce vasoconstriction *per se,* nor does it affect the vasoconstriction to sumatriptan in the rabbit saphenous vein.[260] Moreover, LY446195 and the kainate receptor agonist UBP302 did not affect the vasodilatation of dural blood vessels induced by electrical stimulation or exogenous CGRP in a neurogenic dural vasodilatation model.[262,267] In contrast with LY446195 and UBP302, the antiepileptic drug topiramate, which is effective in migraine prophylaxis, probably at least partly

through blockade of kainate receptors, attenuated the vasodilatation induced by electrical stimulation and infusion of an NO donor, but not the CGRP-induced vasodilatation in the same model.[269] The fact that activation of kainate receptors effectively blocked neurogenic dural vasodilatation suggests that kainate receptor antagonists may be capable of indirectly preventing the vasodilatation induced by activation of the kainate receptor during a migraine attack. Although the vascular effects of kainate receptor antagonists are not known, based on the agonist effect it is suggested that kainate receptor antagonists might indirectly affect vascular tone.

Clinical effects of glutamate receptor antagonists

In patients taking the NO-donor nitroglycerin for reducing the risk of cardiac ischemia, infusion of ketamine, an NMDA receptor antagonist, was proposed to be effective against NO-induced headache.[270] Moreover, in a small open-label study, intranasal ketamine reduced the severity and duration of the neurologic deficits due to the aura in 5 out of 11 patients with familial hemiplegic migraine.[271]

Several kainate receptor antagonists are effective in the acute treatment of migraine, including the mixed AMPA/kainate receptor antagonist tezampanel[7] and the kainate receptor antagonist LY466195.[272] Tezampanel is well tolerated and showed no vasoconstrictor liability in clinical trials.[273] However, due to the mixed AMPA and kainate receptor action of tezampanel, it is not clear which receptor is responsible for its anti-migraine effect.[273] Tezampanel has been FDA approved to enter phase 3 for acute migraine treatment, its oral prodrug NGX426 is awaiting outlicencing for the migraine and pain program.[274] Although LY466195 is effective in the treatment of migraine, its therapeutic potential may be limited because of mild reversible visual distortions.[272]

In the wake of successful proof of concept of glutamate receptor antagonists for migraine treatment, other compounds are in clinical development. The AMPA receptor antagonist, BGG492 from Novartis, is currently under investigation, but no results have been reported so far.

Discussion of glutamate receptor antagonists

The finding that the NMDA receptor antagonist MK801 reduced capsaicin-evoked CGRP release[266] points to potential indirect vascular effects of glutamate receptor antagonists. This is supported by the fact that ketamine and MK801 are capable of inhibiting neurovascular CGRP release.[267] Moreover, activation of neuronal NMDA receptors results in NO release, which causes vasodilatation.[268] This property may represent a therapeutic mechanism of action of glutamate receptor antagonists in the treatment of migraine, but might also result in cardiovascular side effects.[267] However, before conclusions may be drawn about the relevance of such effects, more clinical data on the use of NMDA receptor antagonists

should be available. Moreover, obviously, it has to be kept in mind that ionotropic glutamate receptors are involved in several mechanisms in the brain and spinal cord; thus, blockade of these receptors may induce neurological side effects. Based on the study of glutamate receptor antagonists in stroke, it is known that antagonism of the NMDA receptor causes adverse psychotomimetic effects, including hallucinations, agitation, peripheral sensory disturbance, catatonia, nausea and vomiting. Moreover, except for neurological symptoms, NMDA receptor antagonists also induce effects associated with stimulation of the sympathetic nervous system, like hypertension.[275] In addition, recently glutamate has been described to uncouple blood flow and glucose metabolism, however, this is not mediated via the ionotropic glutamate receptor.[276]

In contrast to NMDA receptor antagonists, as described above, AMPA receptor antagonists do not affect CGRP release and vascular tone,[248,267] although they block responses in the trigeminocervical complex.[251] This suggests that AMPA receptor antagonists might display antimigraine efficacy, which would most likely be unrelated to a vascular mode of action. However, blockade of the AMPA receptor might have toxic effects on glial cells, at least in patients with brain ischemia.[277] These observations warrant further research on the potential central side effects of these ligands.

Finally, the kainate receptor antagonist LY466195, which is effective in the treatment of migraine,[272] seems to be devoid of vascular effects.[260,267] Given its effects on the trigeminocervical complex and the VPM nucleus, it seems that the antimigraine efficacy of LY466195 could involve a purely central effect, unrelated to vascular CGRPergic pathways and/or its receptors. However, kainate receptor antagonists have been described to induce anxiolytic-like effects in an animal model, and thus further human studies are needed to predict their safety in humans.

VPAC/PAC receptor antagonists

The parasympathetic nervous system has for a long time been implicated in the pathophysiology of migraine and, indeed, the parasympathetic outflow to cephalic vasculature may trigger the activation and sensitization of perivascular sensory afferents and thereby migraine pain.[278] Pituitary adenylate cyclase activating polypeptides (PACAPs) and vasoactive intestinal peptide (VIP), which are released by the parasympathetic efferent nerves to regulate cerebrovascular tone and hemodynamics of the brain,[279] have been suggested to play a role in the pathophysiology of migraine. In fact, these peptides also activate or sensitize intracranial sensory nerve fibers leading to the perception of pain.[280-282] PACAPs and VIP are structurally closely related peptides and belong to the secretin/glucagons/VIP peptide family. They are widely distributed in the central and peripheral nervous systems and are associated with various physiological functions.[283] The action of PACAP is mediated via the $VPAC_1$, $VPAC_2$ and PAC_1 receptors, while VIP induces its effects only via the $VPAC_1$ and $VPAC_2$ receptors.

These three receptors are G_s-protein coupled receptors and activate adenylate cyclase to induce their effects.[283-284] Elevated VIP levels have been reported in the plasma of the cranial circulation in a subgroup of migraine patients with pronounced autonomic symptoms.[141]

Central effects of VPAC/PAC receptor antagonists

VIP and PACAP are widely distributed throughout the brain and periphery.[283] In relation with migraine, immunostainings reported the presence of PACAP and VIP in different regions of the brainstem nuclei[285] as well as in perivascular nerves.[282] However, only PACAP was detected in the trigeminal cervical complex and in the C1 and C2 levels.[286] Moreover, mRNA expression of VPAC and PAC receptors is found in the trigeminal, otic and superior cervical ganglia.[287]

Migraine-like headache induced by PACAP38 has been suggested to be caused by activation of peripheral sensory trigeminal fibers mediated via direct sensitization.[281] Moreover, mast cell degranulation caused neuronal activation of C-fibers innervating the dura;[288] significantly, mast cells surrounding cerebral and dural blood vessels are in close proximity to parasympathetic and sensory nerve fibers.[289-291] Hence, it has been suggested that PACAP38 may activate peripheral sensory trigeminal fibers via mast cell degranulation. In addition, a facilitatory effect of PACAP38 on second order trigeminal neurons has been suggested as a possible mechanism for the migraine-like headache induced by PACAP38.[281] In agreement with these findings: (i) the PAC receptor antagonist, PACAP6-38, attenuates nociception in animal models of chronic inflammatory as well as persistent pain;[292-293] and (ii) inflammatory pain disappears in PACAP gene knockout mice.[294]

Vascular effects of VPAC/PAC receptor antagonists

In vitro and *in vivo* studies have demonstrated that VIP and PACAP act as potent vasodilators on cranial blood vessels in various species, including humans.[283-284] VPAC/PAC receptor agonists induced vasorelaxation with different efficacy and potency in the human cranial and coronary arteries.[151,167,295-296] Different vasodilator responses to PACAP and VIP between several blood vessels were described in: (i) the rabbit posterior cerebral artery and coronary artery;[297] (ii) the rat basilar artery and middle cerebral artery;[298] (iii) the guinea pig aorta and pulmonary artery;[299] and (iv) the human proximal and distal coronary arteries.[167,296]

It can be suggested that vasodilatation induced by PACAP and VIP vary not only in different species, but also in the region of the arteries from the same species. Possible explanations for these differences in the vascular responses are selective activation of the three types of the VPAC/PAC receptors on different tissues, tissue-dependent factors such as the levels of receptor protein expression and coupling efficiency of the receptors.[283] Moreover, several splice variants of the receptors, which are to a certain extent tissue specific, have been described to affect cellular function by altering receptor pharmacology and signaling.[283]

VIP is more potent to induce vasodilatation than PACAP38 in human cranial and coronary arteries.[151,295-296] In accordance with these findings in human arteries, a lower vasodilator potency of PACAP was also found in the rabbit posterior cerebral artery[297] and the rat basilar artery,[298] but this difference was not seen in the rat middle meningeal artery.[298] Taken together, since several lines of evidence indicate a lower vasodilator potency of PACAP38 compared to that of VIP, it seems that PAC_1 receptors, which are activated by PACAP38, but not by VIP, are of minor importance in mediating vasodilatation.

It is suggested that the PACAP- and VIP-induced vasodilatation of the temporal artery seen in healthy volunteers and migraine patients[281,300-301] is probably mediated by perivascular nerve activation,[296] since: (i) these peptides are present in perivascular nerves;[282,286] and (ii) direct stimulation with these peptides in human isolated meningeal arteries induced only minor relaxations.[296] The PAC_1 receptor is most likely involved in this mechanism since only the PAC_1 receptor antagonist PACAP6-38, but not $VPAC_1$ receptor antagonists, blocked the vasodilatation induced by neurogenic dural stimulation[302] as well as the neuronal firing in the trigeminal cervical complex after salivatory nucleus stimulation.[302]

Clinical effects of VPAC/PAC receptor antagonists

PACAP38 and VIP have been shown to (i) decrease mean blood flow velocity in the middle cerebral artery; and (ii) induce vasodilatation in the superficial temporal artery.[281,300-301] Although VIP induces mild headache in healthy volunteers and migraine patients, it does not induce a migraine-like headache.[300-301,303] In contrast, PACAP38 induces migraine-like headaches in healthy volunteers and migraine patients.[281] Since PACAP38 displays a higher affinity for the PAC_1 receptor, activation of this receptor may result in migraine-like headaches. Accordingly, antagonism of the PAC_1 receptor may be a putative target for migraine treatment. However, to date, no selective PAC_1 receptor antagonists have been investigated in migraine.

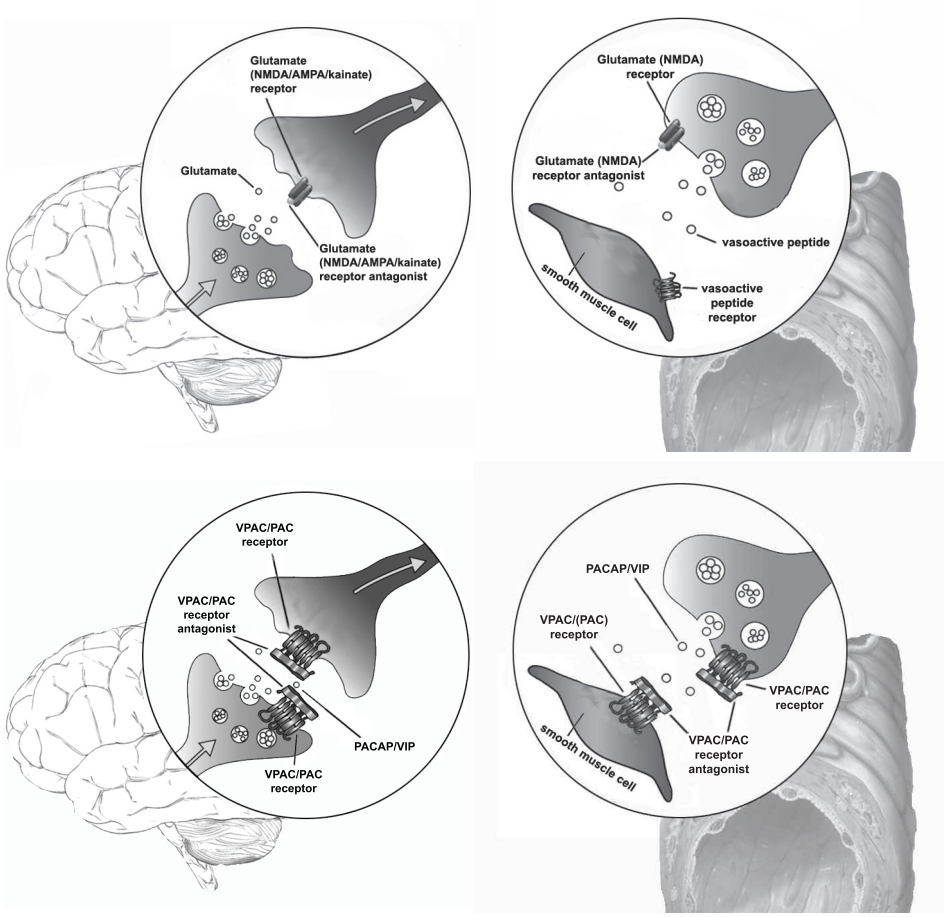

Figure 2: Schematic representation of a synapsis in the CNS (left panels, except for the lower panel) and a neurovascular junction (right panels). **Upper panels:** stimulation of postsynaptic ionotropic glutamate receptors in the CNS by glutamate released from glutamatergic neurons may produce neuronal activation (left panel), whereas stimulation of neurovascular glutamate NMDA receptors by glutamate released from nerve terminals increases the release of vasoactive peptides from sensory perivascular nerves (right panel). Therefore, glutamate receptor antagonists may affect neurotransmission, while NMDA receptor antagonists additionally will affect vasodilatation. **Lower panels:** stimulation of pre- and post-synaptic VPAC/PAC receptors in the CNS by PACAP or VIP released from peptidergic neurons may produce neuronal activation (left panel), whereas stimulation of neurovascular VPAC/PAC receptors by PACAP or VIP released from peptidergic neurovascular terminals may produce release of vasoactive peptides and vasodilatation. Taken together, VPAC/PAC receptor antagonists might affect neurotransmission as well as vasodilatation.

Discussion of VPAC/PAC receptor antagonists

Since PACAP38, but not VIP, induces migraine-like headaches in migraine patients,[281,301] and only PACAPs interact with the PAC_1 receptor, it could be suggested that the PAC_1 receptor is involved in migraine pathophysiology. This mechanism is unlikely to be related to cranial vasodilatation, since PACAP induces only a limited cranial vasodilatation. In contrast, VIP, which does not induce migraine-like headaches, induced a more pronounced vasodilatation than PACAP in cranial arteries.[296] Therefore, the PAC_1 receptor may play a role in activating the central mechanisms involved in migraine, and antagonists for the PAC_1 receptor may be considered as potential antimigraine drugs with a limited vascular side effect. However, since PACAP and VIP display a high degree of homology, antagonists for the PAC_1 receptor may also have affinity for the VPAC receptor, which could lead to an increased vascular tone via inhibition of vasodilator responses. Further, we cannot categorically exclude the possibility that PAC_1 receptor inhibition will affect cerebrovascular tone and hemodynamics of the brain. Obviously, clinical data are needed to confirm or exclude the therapeutic potential of this target.

Nitric oxide synthase inhibitors

Nitric oxide (NO) is a signaling molecule that is present in most tissues throughout the body. The formation of NO from L-arginine is catalyzed by three different enzyme isoforms of nitric oxide synthase (NOS) and involves several cofactors. Endothelial NOS (eNOS; which is expressed in vascular endothelium) and the neuronal NOS (nNOS; which is found in both central and peripheral neurons) are activated by an increase in intracellular calcium, whereas inducible NOS (iNOS; which is normally not detectable), can be activated in many cells by a variety of stimuli unrelated to intracellular calcium.[304] Moreover, iNOS can produce 1,000 times more NO than eNOS and nNOS.[305] One of the mechanisms in which NO is involved is the decrease in intracellular calcium by phosphorylation of ion channels mediated via activation of a cascade of second messengers and kinase.[305] This mechanism induces relaxation of smooth muscle cells, whereas in neurons it has a variety of functions such as involvement of nociceptive processes.[305] Since the NO donor glyceryl trinitrate (GTN) (i) triggered headache, and (ii) migraine patients are more sensitive to this trigger,[305] the role of NO in migraine has extensively been investigated. Although dilatation of cranial blood vessels induced by NO was considered as the cause of migraine headache, more recently primarily neuronal effects have also been forwarded as a potential mechanism.[306]

Central effects of nitric oxide synthase inhibitors

NO is suggested to be a key molecule in the cascade of nociceptive processes in the CNS that lead to migraine pain and other vascular headaches. Sensitization of the spinal cord may be associated with the generation of NO,[307] which is primarily caused by nNOS activation

since nNOS inhibition reduces central sensitization.[308] However, eNOS may also play a role as suggested in nNOS deficient mice.[179,309] Certainly, pain responses are increased by NO donors in neuropathic and inflammatory pain models.[308,310] In addition, responses to facial and dural stimulation in rats,[311] as well as to noxious stimulation of the first synapse in the trigeminal spinal nucleus,[312] are potentiated by NO. GTN infusion leads to fos protein expression in the trigeminocervical complex, which indicates that NO donors can activate the trigeminal system.[313] Moreover, neurons within the trigeminocervical complex that express NOS are activated by GTN.[314] The systemic administration of GTN increases levels of NOS-immunoreactive neurons in the rat dura mater with a delay of hours.[315] This is in line with the biphasic response in trigeminal neurons induced by NO,[305] which is parallel to the biphasic headache response reported in migraine patients after exposure to GTN, as will be discussed below.[316]

Inhibition of NOS decreased the activity of neurons with meningeal input in the rat spinal trigeminal nucleus.[317] The non selective NOS inhibitor, Nω-nitro-l-arginine methyl ester (L-NAME) inhibits c-fos expression in the trigeminocervical complex after stimulation of the superior sagittal sinus in the cat,[318] while the iNOS inhibitor GW274150 has an analgesic effect in rat models.[319]

Vascular effects of nitric oxide synthase inhibitors

Since NO is a potent endogenous vasodilator, NO may induce migraine by inducing cranial vasodilatation.[305] In animal models, i.v. administration of NO-donors causes meningeal vasodilatation.[152] Further, the NOS inhibitor L-NAME reduces resting dural arterial blood flow as well as electrically-evoked dural increases in flow.[320] Interestingly, L-NAME inhibited dural vasodilatation induced by endogenous and exogenous CGRP, while nNOS inhibitors, but not inhibitors of eNOS, blocked only the dural vasodilatation induced by endogenous CGRP.[152] This suggests an indirect action of NO produced by nNOS,[152] which seems to be in line with the fact that NO has been described to activate sensory nerve fibers to release CGRP.[321] Alternatively, both NO and CGRP may display direct vascular effects and can be released with trigeminal fibre activation. Indeed, the presence of nNOS in neurons in the trigeminal ganglion as well as those colocalized with CGRP has been demonstrated.[280] Moreover, eNOS might be responsible for vasodilatation induced by *exogenous* CGRP since this was partially blocked by L-NAME and eNOS inhibitors in rat dural arteries.[152] In contrast, in human blood vessels, the dilatation to CGRP seems to be mediated mainly via an increase in cAMP levels in smooth muscle cells, without involvement of the endothelium.[28,42,165] Nevertheless, CGRP-induced vasodilatation in the human forearm, was shown at least in part, to be mediated by NO.[153]

Since NO synthesized by eNOS is known to have a variety of antiatherosclerotic actions, it was suggested that inhibition of eNOS activity might result in an increased risk

for myocardial infarction.[322] However, the absence of eNOS in transgenic mice did not cause spontaneous myocardial infarction, which was possibly the results of compensatory mechanism by other NOS isoforms, since nNOS was upregulated in eNOS deficient mice.[322-324] Since disruption of all the three NOS isoforms causes myocardial infarction,[325] the design of NOS inhibitors as a treatment target for migraine has to be selective.

Clinical effects of nitric oxide synthase inhibitors

NO causes an immediate headache in migraine sufferers and less often in control subjects.[305] GTN dilates cerebral and extracerebral arteries in humans;[223,326] these pronounced vascular effects might suggest that this vasodilatation is the trigger to mediate NO-induced migraine. However, several arguments have been forwarded against such a vascular mechanism, namely: (i) in a 3T magnetic resonance angiography study, migraine induced by GTN was not associated with cerebral and meningeal vasodilatation,[326] and (ii) the phophosdiesterase inhibitor sildenafil, inhibiting the breakdown of cGMP, the second messenger of NO, did induce migraine-like headache in migraine patients, while in that study cerebral arteries were not dilated, and cerebral blood flow was not increased.[327] Although these observations are important and interesting, it should be kept in mind that: (i) Schoonman *et al.* could only study the large, extracranial parts of the middle meningeal artery due to methodological limitations,[328] and (ii) although Kruuse and colleagues did not observe dilatation of cerebral arteries, sildenafil has been demonstrated by others to affect the cerebrovascular reactivity.[329]

It is interesting that migraineurs experience a delayed headache several hours after a NO-donor infusion, which might be partly mediated by the increase nNOS activity in the trigeminal system that induces CGRP release and dural vasodilatation, since nNOS inhibitors inhibit the vasodilatation induced by perivascular electrical stimulation. Moreover, it has been shown that the premonitory symptoms reported in spontaneous migraine[330] are also seen in nitroglycerin-induced migraine[331] and these symptoms occur well after any vascular change would have occurred.[153] Although, NO does not contribute to a basal tone in human cerebral arteries, it has a mild dilator tone in cerebral arterioles.[332] Moreover, inhibition of NOS with L-NAME produced increase in systemic blood flow without changes in the velocity of blood in middle cerebral artery or on the diameter of the radial artery.[333] However, L-NAME has been shown to increase blood pressure and decrease heart rate.[332-333]

Since Lassen *et al*[334] first described efficacy of the non-selective NOS inhibitor L-NMMA in the treatment of migraine, clinical research on the role of NO in migraine was accelerated as reviewed by Olesen.[305] Although L-NMMA showed encouraging results, its clinical potential is rather limited in view of its pharmacokinetic profile and its vasoconstriction.[332] As L-NMMA inhibits nNOS, iNOS and most importantly also eNOS, NOS inhibitors should be selective when used as anti-migraine drugs. Since the role of

eNOS in migraine is debatable,[335-336] and, most significantly, blocking eNOS could disturb systemic blood pressure and heart rate,[337] selective eNOS inhibitors do not seem a logical pharmacological target for prospective antimigraine drugs. Two selective iNOS inhibitors, GW274150 and GW273629, were developed for inflammatory conditions.[338-341] In preclinical studies, GW274150 seemed to reduce organ injury in hemorrhagic shock[342] and reduced experimental renal ischemia/reperfusion injury,[339] which increased the hope for a beneficial vascular profile of iNOS inhibitors. Although GW274150 seemed to display analgesic effects in rat models of inflammatory and neuropathic pain,[319] clinical studies could not establish its efficacy in acute migraine treatment.[343] This lack of efficacy might be assigned to the unfavorable pharmacokinetic profile of GW273629.[343] However, other randomized controlled trials with GW274150, another potent and selective iNOS inhibitor with a more favorable pharmacokinetic profile than GW273629, also failed in the prevention[344] and acute treatment[345] of migraine. This suggests that iNOS is not a promising target for migraine treatment.[346] Only one selective nNOS inhibitor is currently in clinical development, namely NXN-188.[347] However, this molecule also shows affinity for 5-$HT_{1B/D}$ receptors and, therefore, its clinical effects cannot exclusively be attributed to nNOS inhibition. At the moment, NXN-188 appears to be well tolerated in healthy volunteers and exhibits linear pharmacokinetics over the dose range studied in five phase I, randomized, double-blind parallel studies with single and multiple doses.[347] Further clinical investigation will be performed to overview the pharmacodynamic profile of NXN-188, to assess its efficacy in acute migraine treatment and to obtain more data about its vascular mechanism of action.

Discussion of nitric oxide synthase inhibitors

In view of the pharmacological lines of evidence described above, it seems clear that NO is involved in the pathophysiology of migraine, probably via both vascular and neuronal mechanisms. Although NOS inhibitors may represent an interesting therapeutic option in migraine, the use of non-selective NOS inhibitors or selective eNOS inhibitors seems impeded by their cardiovascular side effects such as increased blood pressure and decreased heart rate. While the results of clinical trials with iNOS inhibitors in migraine were disappointing, the result of trials investigating the effects of nNOS inhibition is awaited with great interest. In addition to the NOS inhibitors, other pharmacological targets that inhibit the formation of NO should be explored, as for example tetrahydrobiopterin, which is the most important cofactor in the conversion of L-arginine to NO and L-citrulline.[348] The role of NOS inhibition in the regulation of gap junction coupling is also under investigation,[349-350] based on the facts that NO: (i) enhances *de novo* formation of endothelial gap junctions by increasing incorporation of Cx40 into the plasma membrane due to protein kinase A activation;[351] (ii) regulates coupling in cells expressing Cx35, a connexin expressed in neurons throughout the central nervous system;[352] (iii) is involved in the control of gap junction intercellular communication and

Cx43 expression;[353] and (iv) inhibits the intercellular transfer of small molecules by a specific influence on Cx37.[354] Taken together, these studies suggest interesting parallels between the NOS system and gap junction modulation (see below) in anti-migraine drug development.

Gap junction modulators

As discussed above, the pathophysiology of migraine is not yet fully understood. However, CSD is thought to provide the basis for migraine aura, and may serve as a trigger for migraine pain.[355-356] Prophylactic drugs, such as topiramate and valproate, suppress CSD in a dose dependent manner.[357] Further, the neuronal changes in CSD have been demonstrated to be preceded by vasomotor changes in the cortex,[358] raising the question whether CSD should be considered as a primary neuronal or primary vascular event.[359] Both CGRP and NO are likely to be involved in the vasodilatation induced after CSD.[360]

Central effects of gap junction modulators

Tonabersat (SB-220453) is a benzopyran compound, which has been demonstrated to inhibit CSD in animal models,[361] CSD-induced release of NO,[362] as well as trigeminal nerve ganglion stimulation-induced carotid vasodilatation[363] and plasma protein extravasation.[364] While it was not originally known which mechanism mediated these effects of tonabersat, it was later demonstrated that this drug may act, at least partly, via inhibition of increased neuron satellite glia signaling via gap junctions.[365] In general, gap junctions: (i) are formed between the cell membranes of two adjacent cells and serve as intercellular conduits that allow for direct transfer of small molecular weight molecules, such as ions, that regulate cellular excitability, metabolic precursors, and second messengers; (ii) consist of two hemichannels (each from one cell), each consisting of a hexamer of connexins, arranged around a central pore; (iii) are found in most neurons and glial cells and function to facilitate neuron-neuron, glia-glia, and neuron-glia communication; and (iv) are abundant in the CNS and allow for extensive intercellular coupling between cells that form a communication network.

Vascular effects of gap junction modulators

Besides the neuronal functions mentioned above, gap junctions have also been postulated to be responsible for the endothelium-derived hyperpolarizing factor (EDHF) phenomenon.[366-367] The gap junctions may allow passive spread of agonist-induced endothelial hyperpolarization through the blood vessel wall via direct intercellular communication. Although we have previously demonstrated that tonabersat does not display direct vascular effects[368] and the compound produced no cardiovascular effects in experimental animals,[369] it is not clear whether tonabersat affects endothelium-dependent relaxations. As mentioned below, only few cardiovascular adverse events have been reported in clinical trials with tonabersat, while a causal relationship with administration of the drug was not always evident. In view of the

knowledge now available about the supposed mechanism of action of tonabersat, it would, however be prudent to perform experimental studies specifically devoted to the potential effects of tonabersat on endothelium-dependent relaxation. In this context, it is interesting to mention that the role of EDHF is dependent on estrogen plasma levels[370-371] while this dependency seems to differ between cranial and peripheral blood vessels.[371-373] These facts, together with the higher prevalence of migraine in women also seems to be related to changes in estrogen levels. Thus, it seems feasible that tonabersat would display a hormone-dependent effect in migraine. Obviously it would first have to be determined whether such indirect vascular effects of tonabersat, mediated via EDHF, have any clinical relevance.

Clinical effects of gap junction modulators

Currently, only one compound specifically targeting CSD, tonabersat (SB-220453), was clinically tested. In a phase II proof-of-concept study, tonabersat failed to meet the primary endpoints,[374] i.e. reduction in migraine days between tonabersat and placebo. The reduction in mean monthly headache days after 3 months, 4.4 days in the tonabersat group and 3.7 days in the placebo group, was not significantly different. However, secondary endpoints were more positive: responder rate, defined as a 50% reduction in migraine attacks, was 62% for tonabersat and 45% for placebo, and rescue medication was reduced by 1.8 days in the tonabersat group compared with placebo. As tonabersat was also well tolerated, further clinical research was initiated. Indeed, a randomized double-blind, placebo-controlled crossover study showed promising effects of tonabersat in aura prophylaxis.[375] In this clinical trial, patients with at least one attack of migraine aura per month were included. The number of attacks of aura significantly decreased from 3.2 during placebo treatment to 1.0 during tonabersat treatment. The number of migraine headache days, however, did not significantly differ between placebo and tonabersat treatment. Thus, tonabersat was effective in preventing attacks of migraine aura, but had no effect on non-aura attacks. These results are in line with the hypothesis that auras are caused by CSD and that this phenomenon is not involved in attacks without aura.

Besides the use of tonabersat in a prophylactic setting, several trials were also conducted to test its efficacy in the acute treatment of migraine.[369,376] Unfortunately, tonabersat was not superior to placebo. However, it should be pointed out that these trials were conducted in a heterogeneous group of migraine patients (with and without aura), limiting the power of the study. Overall, tonabersat seems to be well tolerated with no indications of serious cardiovascular side effects. The most common adverse events after treatment with tonabersat were dizziness and nausea.[377]

The fact that tonabersat seems to act specifically on CSD and aura could explain why this drug was not effective in the GTN-induced migraine headache model.[378] As this model only induces migraine-like headache without aura,[379] and tonabersat is only effective in migraine

with aura,[375] negative results could have been expected a priori with the current knowledge. The apparent synergism between GTN and tonabersat resulted in serious hypotension in two subjects.

We conclude that suppression of CSD seems to be most useful in a prophylactic setting to increase threshold of the aura. In view of safety results obtained so far, it is tempting to speculate that this new class of anti-migraine drugs will show a beneficial cardiovascular profile. However, the potential for cerebral hypotension[378] should be kept in mind. Moreover, gap junctions not only have functions in the brain,[380] but also play an important role in the electrical coupling of cardiomyocytes and as such are determinants of the speed and direction of cardiac conduction.[381-384] Consequences of tonabersat's effects on glial cell communication via gap junction (e.g. connexin26)[365] inhibition, could also negatively affect connexins in the myocardium. Indeed, connexin alterations may cause arrhythmias in heart disease.[381-385] Furthermore, gap junction communication is also a key player in the mechanisms leading to ischemic preconditioning-induced tolerance against infarction and arrhythmias during ischemia-reperfusion of the heart.[386] However, as not all connexins are distributed in all tissues,[382,387] connexin-specific drugs may solve this issue in the future.

Listing all the points, namely: (i) the gap junction modulator tonabersat inhibits aura; (ii) gap junctions and connexin alterations play an important role in cardiac disease; and (iii) migraine with aura is associated with an increased risk for cardiovascular disease,[388-389] there is at least a rationale for the hypothesis that connexins form the link between migraine with aura and the increased cardiovascular risk. This is translated into the hypothesis that inhibition of the neuronal gap junction system for migraine aura treatment could possibly be undesirable with respect to cardiovascular safety, although no excessive cardiovascular side effects have been reported so far.

Discussion of gap junction modulators

It remains to be established whether migraine with and without aura are driven by different pathophysiological mechanisms. As inhibition of CSD (which is only observed in migraine with aura[356,390-391] does not prevent the non-aura headache, it seems that aura is a symptom in parallel with the non-aura headache. In conclusion, the therapeutic potential of tonabersat may be limited as it only prevents the aura, but not the non-aura headache.[392] These findings seem to confirm some hypotheses about aura, but, as with all interesting science, more knowledge also means more questions.

Implications, future directions and conclusions

Many prospective antimigraine drugs with a putatively selective neuronal mechanism of action may display indirect vascular effects. In contrast to the currently available antimigraine drugs, these drugs do not directly induce vasoconstriction, but they may inhibit either vasodilatation induced by neuropeptides (e.g., CGRP receptor antagonists) or the release of such peptides (e.g., some glutamate receptor antagonists).

While the vasoconstrictor potential of the ergots does seem to involve a realistic risk,[393] similar concerns existed about the use of the triptans in view of case reports on coronary ischemia in relation to triptan use.[394] The triptans are in clinical use for about two decades, and it is now clear that the incidence of serious cardiovascular events with triptans in both clinical trials and clinical practice appears to be extremely low, and thus the cardiovascular risk-benefit profile of triptans favors their use in the absence of contraindications.[45] Moreover, even in patients with cardiovascular risk factors (for whom these drugs are contraindicated) the use of triptans did not seem to increase the risk for ischemic cardiovascular complications.[132] However, the fact that triptans have the propensity to constrict the coronary artery[109] warrants these drugs to remain contraindicated in patients with cardiovascular disease.

It remains to be elucidated whether the indirect vascular effects of the prospective antimigraine drugs discussed in this review contribute to the therapeutic efficacy of these compounds. Alternatively, the vascular effects could be relevant in view of the cardiovascular side-effect potential of the drugs. For some of the prospective antimigraine drugs, such as telcagepant, data obtained from clinical trials[185] are already available and suggest that telcagepant is cardiovascularly safe, even in patients with increased cardiovascular risk, since telcagepant did not exacerbate spontaneous ischemia in a small cohort of patients with stable coronary artery disease,[196] which confirms its favourable cardiovascular side-effect profile when used as an acute antimigraine treatment. For some of the other prospective drugs no or only few clinical data are available, on theoretical grounds we expect the cardiovascular side-effect profile to be mild as well. Thus, the probably improved cardiovascular safety profile of the new generation antimigraine drugs may be of clinical relevance for patients in which the triptans are contraindicated. However, it should be kept in mind that, when the prospective drugs would be used chronically as prophylactic instead of acute antimigraine drugs, the cardiovascular side effect profile may be less favorable than expected for acute use. Obviously, further research is warranted before any definite statements can be made on this topic.

Finally, some drugs that are discussed in this review, such as kainate receptor antagonists and possibly 5-HT_{1F} receptor antagonists, seem to be completely devoid of vascular effects. The demonstrated efficacy of these compounds confirms that a vascular action is not an essential feature for antimigraine efficacy. Whether a completely non-vascular mode of action will clinically be an advantage over compounds with mild (in)direct vascular effects is awaited with great interest.

飲水思源

庾信

When drinking water remember where it came from
(Yu Xin)

Chapter 3
Aims of the thesis

Aims of the thesis

1. CGRP plays a role in the pathogenesis of migraine. Characterization of the CGRP receptor led to the development of the CGRP receptor antagonist, telcagepant, which is effective in the treatment of migraine. We characterized the effect of telcagepant in human cranial **(chapter 4)** and coronary **(chapter 5)** arteries to investigate the possible mechanism of its therapeutic effect, as well as its potential coronary side effects.

2. Estrogen fluctuations have been associated with migraine and estrogen may regulate the synthesis and receptor expression of CGRP. Therefore, we investigated the dural vasodilatation to exogenous CGRP in female rats after 17β-estradiol withdrawal, using the method of intravital microscopy on a closed cranial window **(chapter 6)**.

3. Compounds targeting central mechanisms that are involved in the pathogenesis of migraine have been described to be potential antimigraine drugs. In this context, it has been suggested that these targets may not cause vasoconstriction like the triptans. We investigated the effects of ionotropic glutamate receptor antagonists on neurovascular vasodilatation **(chapter 7)** and we also characterized the vasodilator effects of PACAP and VIP receptors in human meningeal as well as in coronary arteries **(chapter 8)**.

4. Triptans are 5-HT_1 receptor agonists, which have been shown to be cranioselective, although they are contraindicated in patients with coronary artery disease. However, the effects of the triptans have only been investigated in large diameter human coronary arteries. For this reason, we investigated whether the contractions to sumatriptan are different in proximal and distal parts of the human coronary artery **(chapter 9)**.

5. 5-HT_4 receptor agonists, which are used for the treatment of gastrointestinal disorders, have been associated with cardiac adverse events. This might be caused by activating 5-HT receptors in the heart. We have investigated the vasoconstrictor effect of the 5-HT_4 receptor agonist, tegaserod, in human coronary artery **(chapter 10)** and the inotropic responses of different 5-HT_4 receptor agonists in human myocardial trabeculae **(chapter 11)**.

世上無難事 只怕有心人

三國演義

Nothing is impossible to a willing heart
(Romance of the Three Kingdoms)

Part B: Calcitonin gene-related peptide

Chapter 4

Effect of the CGRP receptor antagonist telcagepant in human cranial arteries

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Abstract

Calcitonin gene-related peptide (CGRP) is a neuronal messenger in intracranial sensory nerves and considered to play a significant role in migraine pathophysiology. We investigated the effect of the CGRP receptor antagonist, telcagepant, on CGRP-induced cranial vasodilatation in human isolated cerebral and middle meningeal arteries. We also studied the expression of the CGRP receptor components in cranial arteries with immunocytochemistry.

Concentration response curves to αCGRP were performed in human isolated cerebral and middle meningeal arteries in the absence or presence of telcagepant. Arterial slices were stained for RAMP1, CLR and actin in a double immunofluorescence staining.

In both arteries we found that: (i) telcagepant was devoid of any contractile or relaxant effects *per se*, (ii) pre-treatment with telcagepant antagonized the αCGRP-induced relaxation in a competitive manner and (iii) immunohistochemistry revealed expression and co-localization of CLR and RAMP1 in the smooth muscle cells in the media layer of both arteries.

Our findings provide morphological and functional data on the presence of CGRP receptors in cerebral and meningeal arteries, which illustrates a possible site of action of telcagepant in the treatment of migraine.

Introduction

The intracranial circulation is supplied by calcitonin gene-related peptide (CGRP)-containing nerve fibers that originate in the trigeminal ganglion.[148,150] Activation of the intracranial trigeminovascular system results in release of CGRP;[149] this occurs in primary headaches and following subarachnoid hemorrhage.[134,141,395-396] CGRP is a potent vasodilator in several species,[28,137-138] increases heart rate,[166] and has positive inotropic effects on isolated human trabeculae.[167] CGRP initiates vascular responses through interaction with G-protein coupled receptors of the B-type that are primarily coupled to the activation of adenylyl cyclase and is independent of endothelium in human cerebral, meningeal[35,148,151] and coronary[28] vessels. CGRP receptor characterizations have relied on the use of the antagonist $CGRP_{8-37}$, but more recently several small molecule di-peptide CGRP receptor antagonists have been reported such as olcegepant (BIBN4096BS).[397] Although this di-peptide derivative is effective in migraine,[97] due to its molecular structure it has until now only used parenterally. Recently, an orally bio available CGRP receptor antagonist, telcagepant (MK-0974), was described[160] and shown to be effective as an antimigraine drug.[98-99]

The aim of the present study was (a) to compare the functional responses to αCGRP and the antagonistic effects of telcagepant in human cerebral and meningeal arteries and (b) to examine the expression of the receptor elements calcitonin like receptor (CLR) and receptor activity modifying protein 1 (RAMP1) with immunohistochemistry in human cerebral and meningeal arteries.

Materials and Methods

Human isolated arteries

Human cerebral (cortex) arteries (5 male, 4 female, age 45-76 years, internal diameter 300-500 μm) were removed at neurosurgical tumor operations in Lund, Sweden. Human meningeal arteries (2 male, 2 female, age 42-62 years, internal diameter 500-750 μm) were obtained perioperatively from patients undergoing neurosurgical procedures at Erasmus MC, Rotterdam, The Netherlands. All vessels were placed in buffer solution, for cerebral arteries, composition in mM: NaCl, 119; KCl, 4.7; $CaCl_2$, 1.5; $MgSO_4$, 1.17; $NaHCO_3$, 25; KH_2PO_4, 1.18; EDTA, 0.027; glucose, 5.5, pH 7.4; for meningeal arteries, composition in mM: NaCl 119, KCl 4.7, $CaCl_2$ 1.25, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11.1; pH 7.4 aerated with 5% CO_2 in O_2 (carbogen) and transported to the laboratory for investigation. The Swedish part of the study was approved by Lund University Ethics Committee (LU99) and had the individual patients' approval, while the human experimentations ethics committee at Erasmus MC, Rotterdam, approved the Dutch part of the study.

Functional experiments

The arteries were cut into cylindrical segments of one or two mm in length for *in vitro* pharmacological experiments. Each segment was mounted on two metal prongs, one of which was connected to a force displacement transducer and attached to a computer, and the other to a displacement device. The position of the holder could be changed by means of a movable unit allowing fine adjustments of vascular tension by varying the distance between the metal prongs.[398-399] The mounted specimens were immersed in temperature-controlled tissue baths (37°C) containing the buffer solution continuously gassed with carbogen, and the artery segments were allowed to equilibrate for approximately 30 min. The vessel tension was continuously recorded and the distance between the pins or wires was adjusted to maintain a resting tone of 4 mN for cerebral arteries or stretched to a tension normalized to 90% of *l*100 (the diameter when transmural pressure equals 100 mmHg)[399] for the meningeal segments. Following the 30 min equilibration period, the contractile capacity of each vessel segment was examined by exposure to a potassium-rich (60 mM) buffer solution, which had the same composition as the standard solution except that the NaCl was exchanged for an equimolar concentration of KCl for the experiments performed in the cerebral artery. In the experiments performed in the meningeal artery, vessel segments were exposed to 30 mM KCl once. Subsequently, the tissue was exposed to 100 mM KCl to determine the maximum contractile response to KCl.

The relaxant effect of human αCGRP was examined by cumulative application of increasing concentrations of the peptide in the absence or presence of various concentrations of the antagonist telcagepant. Segments were precontracted with 1 μM U46619 (cerebral arteries) or 30 mM KCl (meningeal arteries) before αCGRP was added. In the meningeal artery, each segment was exposed to a single cumulative concentration-effect curve and a matched pair's protocol was used, where one segment acted as control (no antagonist present) while in another segment from the same artery, the agonist response was assessed following equilibration (20-30 min) with 1 μM of the antagonist. After wash out, the functional integrity of the endothelium was verified by observing relaxation to substance P (1-10 nM) after precontraction with the thromboxane A_2 analogue U46619 (10 nM). In the cerebral artery, due to the scarcity of the tissue, cumulative concentration-effect curves in the absence or presence of the antagonist were performed in the same segments. The first curve acted as control (no antagonist present). After washout, the next curve was then performed in the presence of the antagonist (10 nM, 100 nM or 1 μM).

Compounds

The following materials were used in the *in vitro* experiments: human αCGRP (NeoMPS S.A., Strasbourg, France, and Sigma, U.S.A., for the Dutch and Swedish experiments, respectively) and U46619 (Sigma, U.S.A.). Telcagepant (MK-0974) was synthesized by the

Medicinal Chemistry Department, Merck Sharp and Dohme Research Laboratories, U.S.A.. The αCGRP and U46619 were dissolved in water and stored as aliquots at -20°C. Telcagepant was dissolved in dimethylsulphoxide (DMSO) and stored as aliquots at -20°C. When the compounds were to be used they were diluted in saline.

Analysis of data

The vasodilator response to CGRP was expressed relative to the contraction evoked by U46619 or KCl, respectively (=100%). For each segment the maximum vasodilator effect (E_{max}) was calculated. The concentration-response curves for all agonists were analysed using nonlinear regression analysis and the potency of agonists was expressed as pEC_{50} (i.e., negative logarithm of the molar concentration of agonist inducing half maximum response) using Graph Pad Prism 4.0 (Graph Pad Software Inc., San Diego, CA, U.S.A.). The blocking potency of the antagonists was estimated by calculating EC_{50} ratios and plotting a Schild[400] using linear regression to get the slope value. The pA_2 represents the negative logarithm of the concentration of antagonist that induces a two-fold shift of the concentration response curve to the right. This parameter can be calculated in the case of competitive antagonism, i.e., when the slope of the Schild plot is equal to unity. In meningeal arteries, only one concentration of telcagepant was studied, in these cases "apparent pK_B" (a parameter similar to the pA_2, which is used in case that antagonism has not been demonstrated to be competitive in nature) values were calculated, constraining the Schild slope to unity. Since it was not feasible to use agonist concentrations higher than 3 μM, concentration response curves in the presence of higher concentrations of antagonist did not always reach a plateau. In these cases, the concentration response curves were extrapolated, considering the maximal response in the absence of antagonist as E_{max}. Data are expressed as mean values±SEM and 'n' refers to the number of patients from whom the vessels were collected. Statistically significant differences in pEC_{50} values were examined by Mann Whitney U-test.

Immunohistochemistry

For immunofluorescence the cerebral and meningeal artery segments obtained perioperatively from patients were immediately snap frozen after arrival in the laboratories and were then embedded in Tissue TEK (Gibo, Invitrogen A/S, Taastrup, Denmark), frozen at -80°C and subsequently sectioned into 10 μm thick slices. Cryostat sections were fixed for 10 min in ice-cold acetone (-20°C) and thereafter rehydrated in phosphate buffered-saline (PBS, pH 7.2) containing 0.25% Triton X-100 (PBST), for 3x5 min. The sections were then permeabilized with PBST and blocked for 1 h in blocking solution containing PBS and 5% normal donkey serum and then incubated overnight at 4°C with either of the following primary antibodies: rabbit anti-RAMP1 (Santa Cruz Biotechnology, CA, U.S.A, sc-11379) diluted at 1:50, rabbit anti-CLR (Alpha Diagnostic International, SA, U.S.A, CRLR-11A) diluted at 1:100. The

dilutions of the primary antibodies were done in PBST, 1% bovine serum albumin (BSA) and 3% normal donkey serum. On the second day sections were room-tempered and rinsed in PBST for 3x15 min. Sections were subsequently incubated with the secondary antibody (1h, room-temperature). The secondary antibody used was Cy™² conjugated donkey anti rabbit (Jackson ImmunoResearch, West Grove, PA, U.S.A., 711-165-152) diluted 1:200 in PBST and 1% BSA. The sections were washed subsequently with PBST and mounted with Crystal mounting medium (Sigma, St.Louis, MO, U.S.A.). To determine the cellular localization of RAMP1 and CLR, double immunofluorescence was performed by addition of a mouse anti-smooth muscle actin antibody (Santa Cruz, sc-53015) diluted 1:200 in PBST, 1% BSA and 3% normal donkey serum. As secondary antibody Texas Red-conjugated donkey anti-mouse was used (Jackson ImmunoResearch, 715-076-150), diluted 1:200 in PBST and 1% BSA. In order to co-localize RAMP1 and CLR, a new CLR antibody was used (anti-goat, 1:50, Santa Cruz, sc-18007). Vectashield medium containing 4',6-diamidino-2-phenylindole (DAPI) staining nucleuses was used on some sections (Vectashield, Vector Laboratories Inc., Burlingame CA, U.S.A.).

Immunoreactivity was visualized and photographed with an Olympus Microscope (BX 60, Japan) at the appropriate wavelength (FITC Filterblock N B-2EC, EX465-495, EM515-555; TRITC Filterblock N G-2EC, EX540/25, EM605/55; DAPI Filterblock N UV-2EC, EX340-380, EM435-485). Adobe Photoshop CS3 was used to visualise co-labelling by superimposing the digital images. Negative controls for all antibodies were made by omitting primary antibodies. In all cases, no specific staining was found; only auto-fluorescence in lamina elastica interna was seen (not shown). To evaluate the auto-fluorescence in lamina elastica interna, controls were made with primary antibodies only.

Results

Functional studies to αCGRP in human isolated arteries

The cumulative administration of αCGRP caused a concentration-dependent relaxation of cerebral arteries precontracted with U46619, yielding a pEC_{50} value of 9.0±0.2 and an E_{max} of 56±6% (Figure 1). Also in the meningeal artery, αCGRP induced a concentration-dependent relaxation, the E_{max} amounting to 50±11% of precontraction with 30 mM KCl and pEC_{50} value of 8.7±0.1 (Figure 2).

Effects of telcagepant in human isolated arteries

The CGRP receptor antagonist telcagepant, tested in concentrations up to 10 μM, did not show any marked vasomotor responses in any of the isolated vessel segments at basal tone (contraction response with E_{max} of 2.0±2.9%, n=9). Pre-treatment with telcagepant at increasing concentrations (10 nM to 1 μM) shifted the concentration response curves to αCGRP to the right without changing the maximal effect (Figure 1). In the meningeal arteries, CGRP induced an E_{max} of 55±7% in the presence of 1 μM telcagepant (Figure 2). The pA_2 value amounted to 9.37±0.12 in cerebral artery; the slope of the Schild plot did not differ from unity (0.97±0.17). In the meningeal arteries, the apparent pK_B value was 8.03±0.16 (pA_2 could not be calculated since only one concentration of telcagepant was studied, the slope of the plot was constrained to unity to calculate the apparent pK_B).

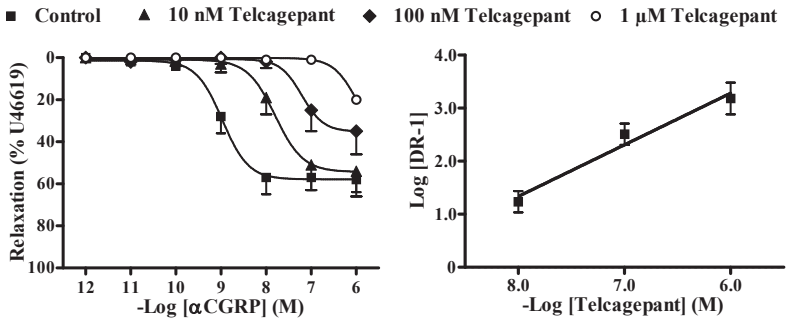


Figure 1: Relaxant effect of αCGRP on human cerebral arteries that were precontracted with U46619. Concentration response curves to αCGRP in the absence or presence of increasing concentrations of telcagepant (left). There was a clear shift to the right in the concentration effect curve. The average Schild plot of the concentration response curves in the cerebral arteries (right). Values given represent mean±SEM, n=9.

Immunohistochemistry of human arteries

The distributions of RAMP1 and CLR in human cerebral (Figure 3A) and meningeal (Figure 3B) arteries were studied by immunohistochemistry. We observed positive immunoreactions for RAMP1 and CLR in the smooth muscle cell layer (media layer) of cerebral and meningeal artery segments. Localization of the CGRP receptor components in the smooth muscle layer was confirmed by double staining with an antibody specific for actin. In separate experiments using another CLR antibody we verified that the two receptor components RAMP1 and CLR co-localized in the smooth muscle cells (Figure 4). There were no obvious positive immunoreactions in the endothelium or in the lamina elastica interna; the latter is strongly autofluorescent, especially in the green filter.

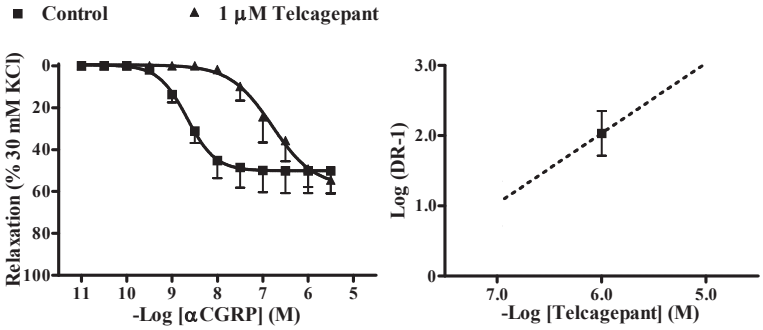


Figure 2: Relaxant effect of αCGRP on human meningeal arteries that were precontracted with 30 mM KCl. Concentration response curves to αCGRP in the absence or presence of 1 µM of telcagepant (left). There was a clear shift to the right in the concentration effect curve. The apparent pK_B value was calculated with a slope (dotted line) constrained to the unity (right). Values given represent mean±SEM, n=4.

Discussion

CGRP receptors have long been regarded as a useful target for the development of novel antimigraine therapies.[140] In this study, we have shown that CGRP induced vasodilatation and that telcagepant had no direct vasoconstrictor or vasodilator effect *per se* on isolated human cerebral and meningeal arteries. However, telcagepant was able to block, in a competitive manner, the vasodilator effect of αCGRP on these blood vessels. We also showed that the CGRP receptor complex, consisting of CLR and RAMP1, is co-expressed in the smooth muscle cells of human cerebral and meningeal arteries and not present in the endothelium or in the adventitia, which is in line with a previous study of human intracranial arteries.[401]

As stated above, telcagepant did not show any evidence of direct vasoconstrictor or vasodilator effect when given alone. If the same will apply to observations made *in vivo*, these findings suggest that CGRP receptors normally do not have a tonus role.[168] This profile of telcagepant agrees well with previous studies of CGRP receptor antagonists such as $CGRP_{8-37}$, Compound 1,[402] and olcegepant.[28,35,41] The relaxant responses to αCGRP were not different in cerebral and meningeal arteries and were in accordance with previous studies.[35,158,402] Telcagepant antagonized relaxations induced by αCGRP with a potency that seemed somewhat lower in the middle meningeal than in the cerebral artery. Because of the small methodological differences between the protocols used for the both tissues no statistical comparison was made. However, since the potency of telcagepant was determined using the control curves in each respectively tissue, the results obtained in the two laboratories should be comparable. Further, in pilot experiments we demonstrated that the different precontractions used in the two laboratories do not affect the response to CGRP (pilot experiments, data not shown).

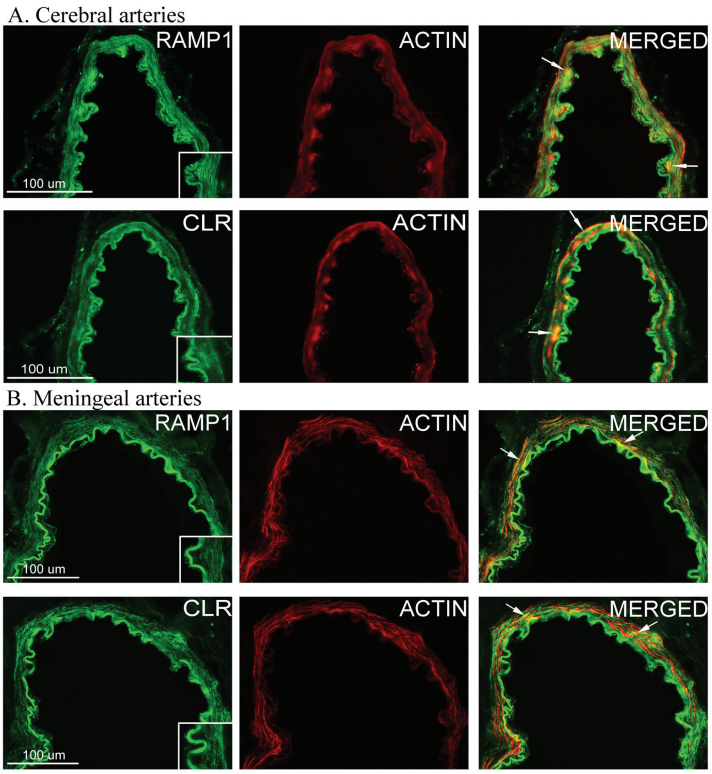


Figure 3: Immunohistochemistry of human cerebral (A) and meningeal (B) artery segments. Antibodies specific for RAMP1 and CLR showed positive staining in the walls of the artery segments (insert, a higher magnification). Co-staining with actin specific antibody revealed the localization of the immunoreactions in the smooth muscle cells. We observed no staining in endothelium or in the adventitial layers. Marker, 100 µm.

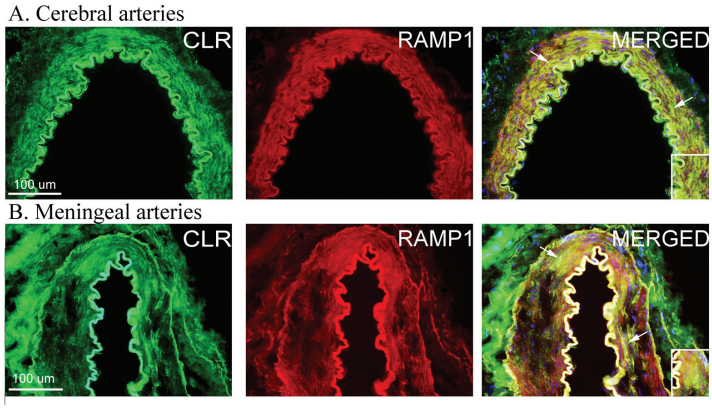


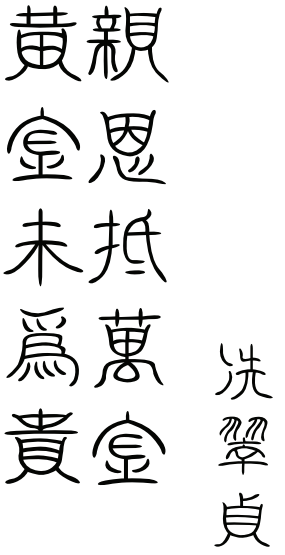
Figure 4: Immunohistochemistry of human cerebral (A) and meningeal (B) artery segments. Antibodies specific for RAMP1 and CLR showed positive staining in the cytoplasm of the smooth muscle cells in walls of the artery. The receptor components co-localized (merged, arrows). DAPI (blue), staining of the nuclei, Marker, 100 µm.

When comparing the effects of telcagepant with those of the CGRP receptor antagonist olcegepant, telcagepant shows activity in higher concentrations (about one to two log units)[35,402] thus, being less potent than olcegepant, which agrees with the K_i of respective compounds (telcagepant: 0.77 nM, olcegepant: 0.014 nM).[6,162] However, telcagepant has the advantage that it can be given as an oral medication,[99] whereas olcegepant is not orally available.[97]

In comparing the clinical effects, it is interesting that both compounds required substantially higher plasma concentrations relative to their *in vitro* pA_2 to achieve clinical efficacy for the acute treatment of migraine.[97,99] For example, plasma concentrations of telcagepant associated with clinical efficacy are in the micro molar range, which is substantially higher than the pA_2 that we have seen for the cranial vascular effect (in the nano molar range). Several factors may be involved in explaining this discrepancy, namely (i) the difference may be due to in part to the high protein binding of these compounds; (ii) a concentration of drug equal to the pA_2 value may not be sufficient to decrease functional responses since it only shifts the concentration response curves two-fold to the right; most likely a concentration of a least 10 times pA_2 would functionally inhibit relaxations to CGRP; (iii) as nerve terminals releasing CGRP are located in the adventitia close to the medial layer of the blood vessels, the concentration of telcagepant at the receptors may be substantially smaller than that at the lumen of the blood vessel, i.e. the plasma concentration. This phenomenon is unlikely to occur *in vitro*, where the antagonist can reach the CGRP receptors from both the luminal and abluminal sides; (iv) finally, the therapeutic effect of CGRP receptor antagonists could obviously also be mediated via other pathways than only inhibition of blood vessel dilatation induced by CGRP. Penetration of telcagepant through the blood-brain barrier may be necessary in addition to the peripheral blockade to achieve anti-migraine efficacy.[157] Arguments in favor of a neuronal mechanism are the lack of presynaptic CGRP receptors in the meninges, which suggests that exogenous CGRP is unlikely to directly modify the innervating sensory nerve fibers.[147] This finding is also in agreement with *in vivo* data obtained in rat, suggesting that an action of CGRP on the dura mater cannot account for the activation of peripheral afferents during migraine.[155] In this study the effects of CGRP in the meninges, including meningeal vasodilatation, were not sufficient to activate or sensitize meningeal nociceptors. Thus, clearly further studies are needed to resolve the therapeutic mechanisms involved of CGRP receptor antagonists.

In conclusion, telcagepant antagonizes relaxations induced by αCGRP with a potency that is consistent between human cerebral and meningeal arteries, without affecting the vascular tone *per se*. To predict potential vascular side effects, the effects of telcagepant obviously have to be investigated in more arteries, including the human coronary artery, as well as an *in vivo* model. Also, it remains to be demonstrated whether inhibition of vasodilatation by CGRP mediates the therapeutic action of telcagepant or that central penetration is also

required. Our findings on the vascular properties of telcagepant provide insight in a possible site of action in the treatment of migraine.



Gold is not as valuable as love of family
(Xian Cui Zhen)

Chapter 5

Characterization of the CGRP receptor antagonist telcagepant in human isolated coronary arteries

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*both authors contributed equally to this work.

Abstract

The sensory neuropeptide calcitonin gene-related peptide (CGRP) plays a role in primary headaches and CGRP receptor antagonists are effective in migraine treatment. CGRP is a potent vasodilator raising the possibility that antagonism of its receptor could have cardiovascular effects. We therefore investigated the effects of the anti-migraine CGRP receptor antagonist telcagepant (MK-0974) on human isolated coronary arteries. Arteries with different internal diameters (ID) were studied to assess the potential for differential effects across the coronary vascular bed. The concentration-dependent relaxation responses to human αCGRP were greater in distal (ID: 600-1,000 μm, E_{max}=83±7%) than proximal coronary arteries (ID: 2-3 mm, E_{max}=23±9%), coronary arteries from explanted hearts (ID: 3-5 mm, E_{max}=11±3%) and coronary arterioles (ID: 200-300 μm, E_{max}=15±7%). Telcagepant alone did not induce contraction or relaxation of these coronary blood vessels. Pretreatment with telcagepant (10 nM to 1 μM) antagonized αCGRP-induced relaxation competitively in distal coronary arteries (pA_2=8.43±0.24), proximal coronary arteries and coronary arterioles (telcagepant 1 μM giving pK_B=7.89±0.13 and 7.78±0.16 respectively). αCGRP significantly increased cAMP levels in distal, but not proximal coronary arteries and this was abolished by pretreatment with telcagepant. Immunohistochemistry revealed the expression and co-localization of the CGRP1 receptor elements calcitonin like receptor (CLR) and receptor activity modifying protein 1 (RAMP1) in the smooth muscle cells in the media layer of human coronary arteries. These findings *in vitro* support the cardiovascular safety of CGRP receptor antagonists and suggest that telcagepant is unlikely to induce coronary side effects under normal cardiovascular conditions.

Introduction

Migraine is thought to be a neurovascular disorder, although its pathophysiology remains elusive. In contrast, the physiology and pharmacology of migraine pain is undoubtedly associated with activation of the trigeminovascular sensory nervous system.[27,403] The trigeminovascular system contains calcitonin gene-related peptide (CGRP)-positive trigeminal sensory nerves that innervate cerebral and meningeal blood vessels and with their central synapses mediate pain signal transmission to central second order sensory neurons within the brainstem trigeminal nucleus caudalis. Activation of the trigeminal nerves during migraine pain has been shown to be associated with the release of CGRP (reviewed in Edvinsson and Linde, 2010)[404] and its importance in the pathophysiology of migraine pain has been confirmed pharmacologically by the clinical anti-migraine efficacy of the CGRP receptor antagonist olcegepant (BIBN4096BS) given intravenously.[97]

The human coronary circulation is densely innervated with CGRP-positive fibers[164] raising the possibility that CGRP receptor antagonists could affect coronary vascular tone. In the current experiments, we have investigated *in vitro* the potential coronary vascular safety profile of telcagepant (MK-0974) a novel orally bioavailable anti-migraine CGRP receptor antagonist[98,160-161] that is currently in late phase clinical trials. The studies investigated the anatomical localization of CGRP1 receptors in coronary vessels using immunohistochemistry and compared the coronary vascular pharmacology of telcagepant with the vasoconstrictor anti-migraine 5-$HT_{1B/1D}$ receptor agonist zolmitriptan in human coronary arteries of different diameter to assess its potential cardiovascular safety profile.

Materials and Methods

Human isolated arteries

The right proximal (internal diameter 2-3 mm) and distal (internal diameter 600-1,000 μm) coronary arteries were obtained within 24 hours after death from heart beating organ donors (8 male, 11 female; age 19-64 years). The donor hearts were provided by the Rotterdam Heart Valve Bank through the Bio Implant Services Foundation / Eurotransplant Foundation (Leiden, The Netherlands) after removal of the aortic and pulmonary valves for homograft valve transplantation. In Sweden, (i) the left internal mammary artery (LIMA, internal diameter 3-5 mm) was removed in conjunction with coronary artery by-pass surgery (5 male, 1 female; age 62-78 years). (ii) Epicardial arteries of somewhat larger diameter (internal diameter 3-5 mm) were removed from 2 explanted hearts in conjunction with heart transplantation (1 male, 1 female, age 56 and 67 years), and (iii) coronary arterioles of small diameter (about 300 μm) were removed during valvular surgery (5 male, 4 female; age 67-84 years).

All vessels were placed in buffer solution aerated with 5% CO_2 in O_2 (carbogen) for transfer to the participating laboratories. The buffer composition (mM) in the Netherlands was NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 8.3; pH 7.4 and in Sweden NaCl 119, KCl 4.7, $CaCl_2$ 1.5, $MgSO_4$ 1.17, $NaHCO_3$ 25, KH_2PO_4 1.18, EDTA 0.027, glucose 5.5; pH 7.4. The Swedish study had the individual patients' approval and was sanctioned by the Lund University Ethics Committee (LU99) and the Human Ethics committee at Erasmus Medical Center, Rotterdam, approved the Dutch experiments.

Functional experiments

For *in vitro* pharmacological experiments the arteries were each cut into cylindrical segments of one to four mm length. Each segment was mounted on two metal prongs, one of which was connected to a force displacement transducer and attached to a computer, and the other to a displacement device. The mounted specimens were immersed in temperature-controlled tissue baths (37°C) containing the buffer solution continuously gassed with carbogen, and the artery segments were allowed to equilibrate for approximately 30 min. Resting tone was 4 mN for LIMA, coronary arteries obtained from explanted hearts and coronary arterioles, and about 15 mN for the proximal coronary artery segments obtained from heart beating organ donors. Distal segments obtained from the heart beating organ donors were stretched to a tension normalized to 90% of the diameter when transmural pressure equals 100 mmHg.[399]

For the experiments in Sweden, after a 30-min equilibration period, the contractile capacity of each vessel segment was examined by exposure to a potassium-rich (60 mM) buffer solution that had the same composition as the standard buffer solution, except that the NaCl was exchanged for an equimolar concentration of KCl. For the experiments in the Netherlands, vessel segments were exposed to 30 mM KCl once (distal segments) or twice (proximal segments). The functional integrity of the endothelium was verified by observing relaxation to substance P (1-10 nM) after pre-contraction with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 1 μM, proximal coronary artery segments)[109] or the thromboxane A_2 analogue U46619 (10 nM, distal segments.[405] After washout, the maximum contractile response of the tissue to 60 mM KCl was determined. The relaxant (vasodilator) effect of human αCGRP (h-αCGRP) was examined by cumulative application of increasing concentrations of the peptide in the absence or presence of various concentrations of the antagonist telcagepant (MK-0974); preliminary data have been presented on autopsy large coronary arteries.[406] The Dutch experiments used both human and rat αCGRP and the data was pooled for analysis as they were equipotent. Each segment was pre-contracted with 1 μM U46619 (Swedish experiments) or 30 mM KCl (Dutch experiments) before αCGRP was added. Pilot experiments (data not shown) showed that these different pre-contraction protocols did not affect the vasorelaxant (vasodilator) response to CGRP. In the Dutch experiments, a matched pair protocol was used where two vessel segments from the same artery were exposed to a single cumulative

concentration-effect curve with one segment acting as control (no antagonist present), and the other to assess the agonist response following equilibration (30 min) with various concentrations of the antagonist. In the Swedish experiments, in view of the scarcity of tissue, cumulative concentration-effect curves were performed in the absence or presence of one or two concentrations of the antagonist in the same segments. The first curve acted as control (no antagonist present). After proper washout and return to baseline, the next curve was then performed in the presence of the antagonist (10 nM, 100 nM or 1 μM).

In a separate set of experiments, contractions to the 5-hydroxytryptamine$_{1B/1D}$ (5-HT$_{1B/1D}$) receptor agonist, zolmitriptan were compared to contractile effects of telcagepant and the relaxations to αCGRP.

cAMP measurements

Human proximal and distal coronary artery segments obtained from heart beating organ donors in The Netherlands were incubated in a medium containing isobutylmethylxanthine (IBMX, 0.5 mM) for 30 min in the absence or presence of telcagepant (1 μM, separate segments as those used for myograph studies). The arterial segments were exposed to h-αCGRP (10 μM) or forskolin (10 μM) for 5 min and then snap frozen. Forskolin, which increases intracellular cAMP concentrations by activating adenylyl cyclase, was used to assess the specificity of telcagepant towards CGRP-mediated increases in cAMP concentrations. The samples were stored at -80°C until cAMP assay using the ELISA kit and manual (R&D Systems Europe Ltd., Abingdon, U.K.).

Compounds

The following materials were used in the *in vitro* experiments: human and rat αCGRP (NeoMPS S.A., Strasbourg, France, and Sigma, U.S.A., for the Dutch and Swedish experiments, respectively), Prostaglandin $F_{2\alpha}$ (tris salt) and U46619 (9, 11-dideoxy-11α, 9α-epoxy-methano-prostagladin $F_{2\alpha}$, Sigma, U.S.A.). Zolmitriptan (from nasal spray, AstraZeneca, U.K. or AK Scientific, U.S.A., for the Swedish and Dutch experiments, respectively) was dissolved in saline. Telcagepant (MK-0974) was synthesized and supplied by the Medicinal Chemistry department, Merck, West Point PA, U.S.A.. αCGRP and U46619 were dissolved in water and stored as aliquots at -20°C. Telcagepant was dissolved in dimethylsulphoxide (DMSO) and stored as aliquots at -20°C. The compounds were diluted in saline for use in the experiments.

Analysis of data

The vasodilator response was expressed relative to the contraction evoked by U46619 or KCl, respectively (=100%). For each segment the maximum vasodilator effect (E_{max}) was calculated. The concentration response curves for all agonists were analysed using nonlinear regression

analysis and the potency of agonists was expressed as pEC_{50} (i.e., negative logarithm of the molar concentration of agonist inducing half maximum response) using Graph Pad Prism 4.0 (Graph Pad Software Inc., San Diego, CA, U.S.A.). The blocking potency of the antagonist was estimated by calculating EC_{50} ratios and plotting a Schild plot[400] using linear regression to get the slope value. In proximal coronary arteries and coronary arterioles, only one concentration of telcagepant was studied, in these cases "apparent pK_B" values were calculated, constraining the slope to unity. Since it was not feasible to use agonist concentrations higher than 3 μM, concentration response curves in the presence of higher concentrations of antagonist did not always reach a plateau. In these cases, the concentration response curves were extrapolated, considering the maximal response in the absence of antagonist as E_{max}. Data are expressed as mean values±SEM (standard error of mean) and 'n' refers to the number of patients from whom the vessels were collected. Statistically significant differences in pEC_{50} values were examined by Mann Whitney U-test, correlations were studied using the SPSS 15.0 statistical program (SPSS, Chicago, IL, U.S.A.). The potency of telcagepant can be compared across the various arterial preparations since each potency value is calculated in relation to its own control.

Immunohistochemistry

For immunofluorescence studies, the distal artery and arteriole segments were embedded in Tissue TEK (Gibco, Invitrogen A/S, Taastrup, Denmark), frozen at -80°C and subsequently sectioned into 10 μm thick slices. Cryostat sections were fixed for 10 min in ice-cold acetone (20°C) and thereafter rehydrated in phosphate-buffered saline (PBS, pH 7.2) containing 0.25% Triton X-100 (PBST), for 3x5 min The sections were then blocked for 1 h in blocking solution containing PBS and 5% normal donkey serum and then incubated overnight at 4°C with either of the following primary antibodies: rabbit anti RAMP1 (1:50, Santa Cruz Biotechnology, CA, U.S.A., sc-11379), rabbit anti CLR (1:100, Alpha Diagnostic International, SA, U.S.A., CRLR-11A). The primary antibodies were diluted in PBST, 1% bovine serum albumin (BSA) and 3% normal donkey serum. On the second day sections were room-tempered and rinsed in PBST for 3x15 min. Sections were subsequently incubated with secondary antibody (1 h, room temperature). The secondary antibody used was Cy™² conjugated donkey anti-rabbit (1:200, Jackson ImmunoResearch, West Grove, PA, U.S.A, 711-165-152) diluted in PBST and 1% BSA. The sections were washed subsequently with PBST and mounted with Crystal mounting medium (Sigma, St.Louis, MO, U.S.A.). To determine cellular localization of RAMP1 and CLR, double immunofluorescence was performed by addition of a mouse anti smooth muscle actin antibody (1:200, Santa Cruz, sc-53015). As secondary antibody Texas Red-conjugated donkey anti-mouse was used (1:200, Jackson ImmunoResearch, 715-076-150). To co-localize RAMP1 and CLR, an additional anti-goat CLR antibody, was used (1:50, Santa Cruz, sc-18007). As secondary antibody, Alexa Flour 488 donkey

anti-goat was used for the double staining (1:400, Invitrogen, La Jolla, CA, U.S.A). Vectashield medium containing 4', 6-diamidino-2-phenylindole (DAPI) staining nuclei was used on some sections (Vectashield, Vector Laboratories Inc., Burlingame CA, U.S.A.).

Immunoreactivity was visualized with an Olympus Microscope (BX 60, Japan) at the appropriate wavelength. Adobe Photoshop CS3 was used to visualise co-labelling by superimposing the digital images. Negative controls for all antibodies were made by excluding primary antibodies and in all cases resulted in no specific staining; only autofluorescence in lamina elastica interna was seen (not shown). As controls, to evaluate the autofluorescence in lamina elastica interna, preparations were made using only the primary antibodies.

Results

Functional studies to αCGRP in human isolated arteries

Pre-contracted proximal coronary arteries did not consistently relax to αCGRP in four out of eight experiments. The mean E_{max} to CGRP, including both responding and non-responding arteries, amounted to 23±9% (n=8, Figure 1; upper left panel) of pre-contraction induced by 30 mM KCl. The mean pEC_{50} of the responding proximal coronary arteries was 7.2±0.2. In coronary arteries obtained from two explanted hearts, αCGRP induced a small relaxation after pre-contraction with U46619 (E_{max} of 11±3% and pEC_{50} of 8.1±0.1). In distal coronary arteries αCGRP induced consistent relaxant responses with an E_{max} of 83±7% of pre-contraction induced by 30 mM KCl and a pEC_{50} of 9.1±0.1 (n=6, Figure 1; middle left panel). The small human coronary arterioles obtained from patients undergoing valvular surgery relaxed upon administration of αCGRP in a consistent concentration-dependent manner with a smaller E_{max} of 15±7% and a pEC_{50} of 8.0±0.1 (Figure 1; lower left panel). There were no noticeable relaxant responses to αCGRP in segments of the LIMA (data not shown).

Effects of telcagepant in human isolated arteries

The CGRP receptor antagonist telcagepant, tested in concentrations up to 100 μM, did not show any vasomotor (contraction or relaxation) responses in any of the isolated vessel segments at basal tone (Figure 2). Pre-treatment with telcagepant at increasing concentrations (10 nM to 1 μM) caused concentration-dependent parallel shifts to the right of the concentration-effect curve to αCGRP without changing the maximum relaxant response in proximal (Figure 1; upper left panel) or distal coronary arteries (Figure 1, middle left panel) and small coronary arterioles from valvular surgery patients (Figure 1; lower left panel). The pA_2 value was 8.43±0.24 in distal coronary arteries; the slope of the Schild plot did not differ from unity (slope=0.8±0.1, p=0.07) (Figure 1, middle right panel).

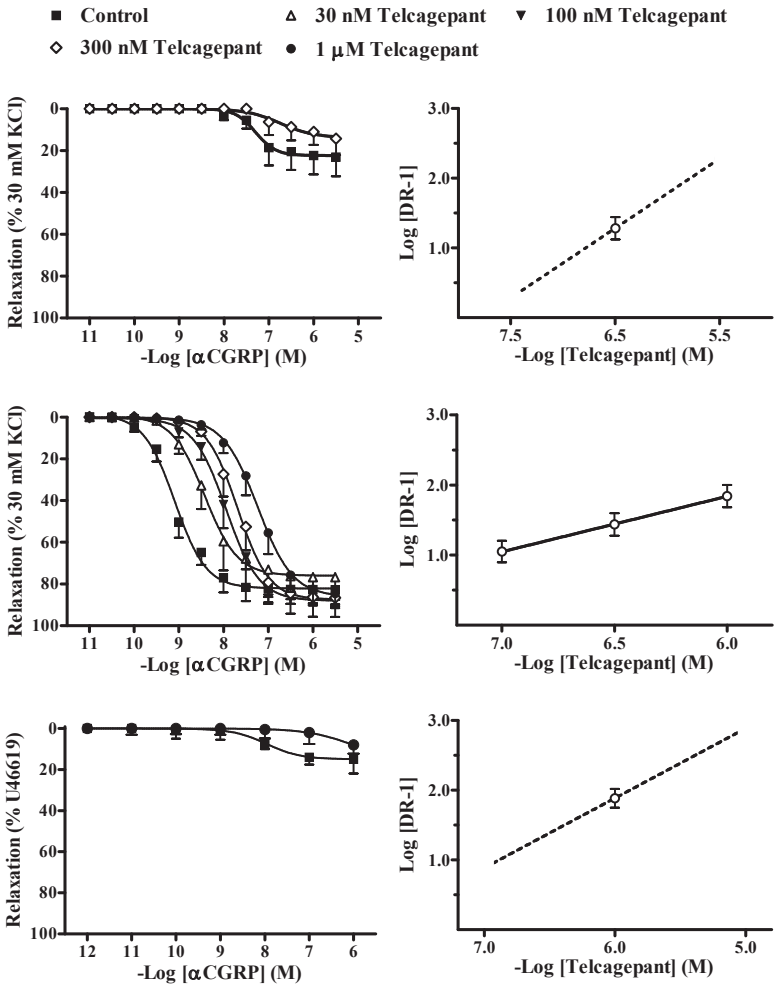


Figure 1: Relaxant effect of αCGRP on human proximal (upper left panel) and distal (middle left panel) coronary arteries, as well as on human coronary arterioles (lower left panel). Concentration-response curves to αCGRP were constructed in the absence or presence of 300 nM (proximal), 30 nM-1 μM (distal) and 1 μM (arterioles) of telcagepant (left panels). Right panels show the average Schild plot of the concentration response curves with the apparent pK_B value, calculated with a slope constrained to unity for the proximal coronary arteries (upper right panel) and coronary arterioles (lower right panel). The middle right panel shows the average Schild plot of the concentration response curves with the corresponding pA_2 in the distal coronary arteries. Values given represent mean±SEM, n=6-8.

Although in the proximal coronary artery and the small coronary arterioles, αCGRP was less potent and less efficacious as a relaxant agent compared to that seen in distal coronary arteries, telcagepant was still an effective antagonist with a apparent pK_B of 7.78±0.16 for the proximal coronary artery and 7.89±0.13 for the coronary arterioles (pA_2 could not be calculated since only one concentration of telcagepant was studied) (Figure 1; upper and lower right panels).

Effect of zolmitriptan in human isolated arteries

In proximal and distal coronary arteries, zolmitriptan induced a concentration-dependent contraction, which was larger in distal (99±44%, n=4) than in proximal (13±3%, n=7) segments (Figure 2; upper panels). In the coronary arterioles, zolmitriptan induced a strong concentration-dependent contraction (155±47%, n=4, Figure 2; lower left panel). All these vessels also responded to αCGRP with relaxation; the E_{max} of zolmitriptan was unrelated to that of αCGRP (Pearson's correlation<0.4 and p>0.05, Figure 2; lower right panel).

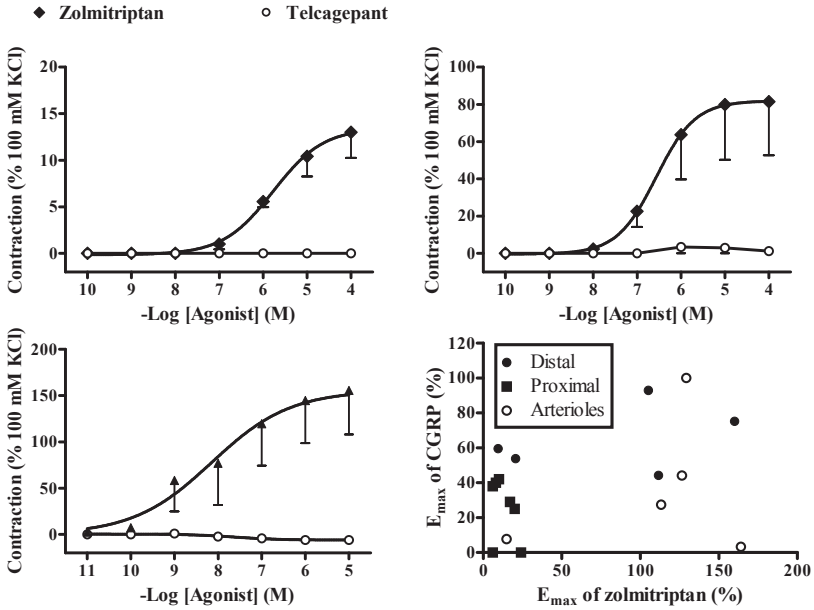


Figure 2: Contractile response to zolmitriptan and telcagepant in proximal coronary arteries (upper left panel), distal coronary arteries (upper right panel) and coronary arterioles (lower left panel) segments. Comparison of the maximum relaxant response to αCGRP with the maximum contractile response to zolmitriptan in proximal coronary artery, distal coronary artery and coronary arteriole segments (lower right panel). Values given represent mean±SEM, n=5-7.

Effect of αCGRP and telcagepant on cAMP levels

In proximal coronary arteries, αCGRP, either in the absence or presence of telcagepant, did not increase cAMP levels (Figure 3; upper left panel). In contrast, αCGRP increased cAMP levels in distal coronary arteries, which was abolished by pretreatment with telcagepant (Figure 3; upper right panel). Forskolin increased the cAMP levels in both proximal and distal coronary arteries; this increase was not inhibited by pretreatment with telcagepant (Figure 3; lower panels). Telcagepant, in concentrations up to 1 μM, did not affect cAMP levels at baseline (data not shown).

Immunohistochemistry of human arteries

The distributions of RAMP1 and CLR in distal coronary arteries (Figure 4; upper panel) and coronary arterioles (Figure 4; lower panel) were studied by immunohistochemistry. Positive immunoreactivity for RAMP1 and CLR was observed in the smooth muscle layer (media layer) of coronary artery segments. The localization of CGRP receptor components in the smooth muscle layer was confirmed by double staining with an antibody specific for actin, which showed a clear co-localization. Using another CLR antibody, we could verify that the two receptor components RAMP1 and CLR co-localized, supporting the presence of functional CGRP1 receptor in these arteries (Figure 5). There were no obvious positive immunoreactions in the endothelium or in the lamina elastica interna; the latter is strongly auto-fluorescent, especially in the green wavelength.

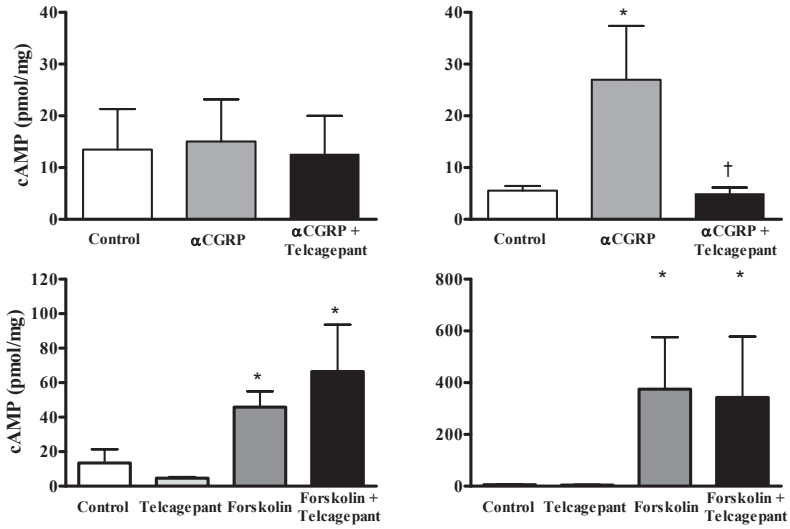


Figure 3: Effect of telcagepant (1 μM) on the increase in cAMP levels induced by αCGRP (1 μM, upper panels) or by forskolin (10 μM, lower panels) in human proximal (left panels) and distal (right panels) coronary arteries. Values given represent mean±SEM, n=4-10. $p<0.05$, * control vs. αCGRP or forskolin -/+ telcagepant and † αCGRP vs. αCGRP + telcagepant.

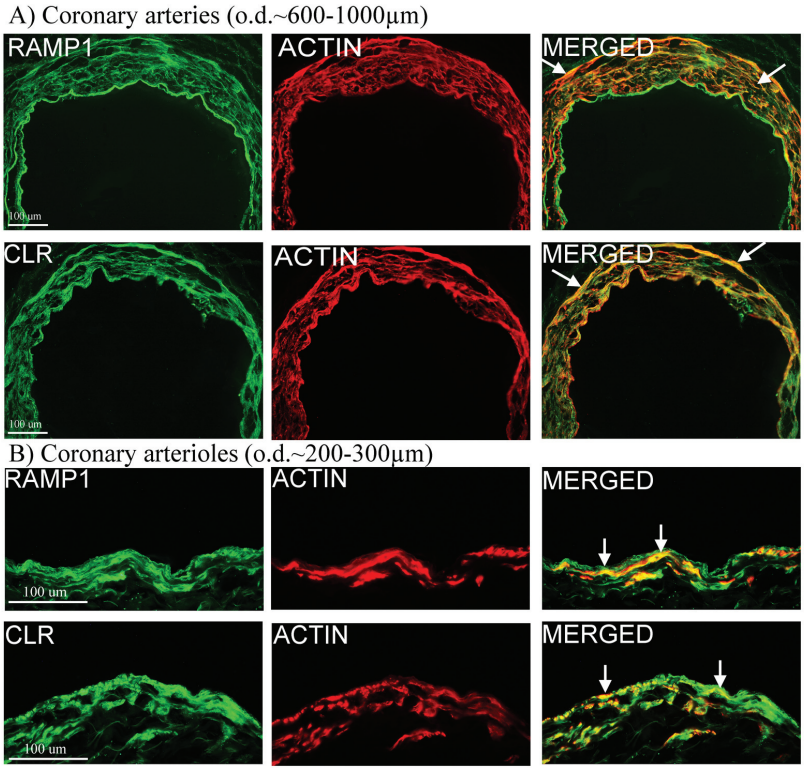


Figure 4: Immunohistochemistry of human distal coronary artery (upper, i.d.=600-1,000 µm) and coronary arteriole (lower, i.d.= about 300 µm) segments. Antibodies for RAMP1 and CLR showed positive staining in the walls of the artery segments. Co-staining with actin antibody revealed the localization of the immunoreactions in the smooth muscle cells (merged, arrows). No staining in endothelium or in adventitial layers. Marker, 100 µm.

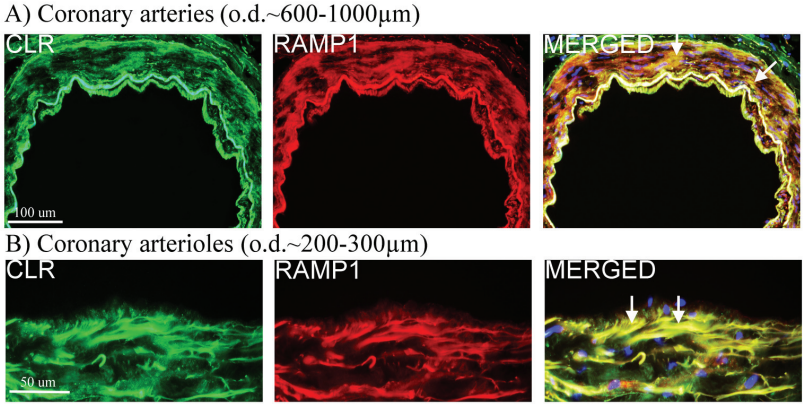


Figure 5: Immunohistochemistry of human distal coronary artery (upper, i.d.=600-1,000 µm) and coronary arteriole (lower, i.d.= about 300 µm) segments. Antibodies for RAMP1 and CLR showed positive staining in the cytoplasm of the smooth muscle cells of the artery. The receptor component co-localized (merged, arrows). DAPI, staining nuclei, is used in the merged pictures (blue). Marker, 100 µm (upper panel) and 50 µm (lower panel).

Discussion

In the current studies, CGRP-induced concentration-dependent relaxations varied in magnitude in coronary arteries of different caliber and were independent of the endothelial quality of the vessel segments and the presence of coronary artery disease. Indeed, similar responses were observed in coronary arteries of similar diameter taken from explanted hearts, and healthy heart-beating donors.[28,164,407] CGRP-induced relaxation was most pronounced in distal coronary arteries than in proximal coronary arteries, coronary arteries from explanted hearts and small coronary arterioles. Our immunohistochemical studies showed no profound difference in the immunoreactivity of CLR and RAMP1, the CGRP receptor components, between proximal and distal coronary arteries, suggesting that the differential dilator effects of CGRP are not due to differences in CGRP receptor density between arteries but may reflect differences in efficiency of intracellular receptor coupling. Interestingly, the EC_{50} for CGRP observed in our isolated coronary artery studies is well below the plasma levels of CGRP (80 pM) observed in external jugular venous blood during a migraine attack,[134] making it unlikely that cranially-derived CGRP released by trigeminal activation during a migraine attack might affect coronary vascular tone.

The antagonist activity of telcagepant in coronary artery tissues was proven by our biochemical assays in which telcagepant reduced the increase in cAMP levels elicited by αCGRP, but not the cAMP increase elicited by forskolin, indicating that the effects of telcagepant were due to specific blockade of CGRP receptors rather than a non-specific mechanism affecting second messenger levels. This response was observed only in distal but not proximal segments, probably reflecting the relatively high number of proximal tissues that were unresponsive to CGRP. In our functional assays in coronary arteries, telcagepant antagonized the vasodilatation induced by αCGRP in a competitive manner as previously shown for its effects in cerebral and meningeal arteries.[163] The potency of telcagepant was almost the same in distal coronary arteries and coronary arterioles (pA_2 of 8.43±0.24, pK_b of 7.89±0.13, respectively), despite the fact that the vasorelaxant effect of CGRP was larger in distal coronary vessels. The potency of telcagepant in coronary arteries seemed somewhat lower than in cerebral arteries (pA_2 of 9.37±0.17), but was comparable to that in meningeal arteries (pK_b of 8.03±0.16).[163] When the potency of telcagepant is compared with that of other CGRP receptor antagonists in human coronary arteries, telcagepant results to be more potent than $CGRP_{8-37}$ and an early-generation small molecule CGRP receptor antagonist Compound 1.[402,407] Telcagepant displays a slightly lower potency than the i.v. anti-migraine CGRP receptor antagonist olcegepant in coronary (pA_2 of 9.37),[28,402] cerebral[41] and middle meningeal[35] (dura mater) arteries.

Studies with the prototypic peptide CGRP receptor antagonist $CGRP_{8-37}$[408] and the small molecule CGRP receptor antagonist olcegepant suggested that endogenous CGRP does not

play a significant role in cardiovascular regulation under resting conditions, since these agents do not induce vasoconstriction *in vitro*[28,35] nor alter baseline hemodynamics in animals.[169] It has however been hypothesized that CGRP is released as part of a protective mechanism under pathophysiological conditions such as ischemia.[57,173-174] Since telcagepant is equipotent in cranial and coronary arteries this raised the question as to whether CGRP receptor antagonism by telcagepant during the treatment of migraine could impair such a response. A complicating factor in this assessment is that telcagepant is highly species-dependent with regard to binding affinity to the CGRP receptor, with significantly lower affinities in rat and dog than in non-human primate and human.[162] This precludes assessment of its hemodynamic effects in common preclinical species. There is nonetheless a significant literature from studies with CGRP receptor antagonists lacking species specificity that can affect this debate. Thus, *in vitro* studies in isolated mouse and rat hearts have reported no effect of the prototype CGRP receptor peptide antagonist $CGRP_{8-37}$ and the small molecule CGRP receptor antagonist olcegepant on ischemic injury or function.[57,177,409-410] Some *in vitro* studies have reported that CGRP receptor antagonism attenuates ischemic preconditioning cardioprotection in isolated mouse and rat hearts, elicited by a set program of ischemia-reperfusion cycles preceding longer periods of myocardial ischemia,[57,177,410] but the pathophysiological significance of these observations is uncertain.

In contrast to studies *in vitro*, preclinical *in vivo* studies in multiple species with $CGRP_{8-37}$ and olcegepant have reported no intrinsic hemodynamic effects. Specifically regarding coronary function, *in vivo* hemodynamic studies in normal dogs have reported no effect of $CGRP_{8-37}$ on coronary or myocardial regional blood flow in dogs[168] and no effect of the small molecule CGRP receptor antagonist olcegepant on myocardial vascular conductance in normal rat and pig.[169,411] Also, *in vivo* ischemia/reperfusion studies in rat and pig reported that $CGRP_{8-37}$ and olcegepant had no effect on infarct size.[178,412] Moreover, $CGRP_{8-37}$ had no effect on myocardial blood flow in dogs with heart failure produced by previous myocardial infarction and rapid ventricular pacing.[180]

In the current studies a strong contractile response was observed with the $5\text{-}HT_{1B/1D}$ receptor agonist zolmitriptan in coronary artery segments consistent with earlier findings with all members of the serotonin agonist class of anti-migraine drugs in healthy[109] and diseased coronary arteries.[133] A recent series of *in vivo* preclinical studies has compared the effects of the $5\text{-}HT_{1B/1D}$ receptor agonist anti-migraine drug sumatriptan to the effects of CGRP or CGRP receptor antagonists on coronary vascular function in dogs in the settings of acute regional myocardial as well as coronary reactive hyperemia. During acute regional myocardial ischemia induced by atrial pacing in the presence of coronary stenosis, neither CGRP nor $CGRP_{8-37}$ affected coronary flow and severity of ischemia, whereas sumatriptan exacerbated ischemia severity with concomitant reduction in coronary blood flow.[200-201] Likewise, $CGRP_{8-37}$ had no effect on myocardial reactive hyperemic response following brief

mechanical coronary artery occlusion, whereas sumatriptan reduced peak reactive hyperemic coronary artery blood flow, reactive hyperemic flow and the repayment of coronary blood flow debt.[199] These preclinical findings indicate that caution should be exercised in the use of triptans in migraine patients with cardiovascular disease; further, they are in agreement with the outcome of recent studies showing that telcagepant did not exacerbate spontaneous ischemia in a small cohort of patients with stable coronary artery disease.[196]

In conclusion, our findings *in vitro* suggest that telcagepant is unlikely to cause coronary side effects under normal physiological conditions in cardiovascular healthy patients. Telcagepant is currently being tested in migraine patients with stable vascular disease (NCT00662818 at www.clinicaltrials.gov).[185] The absence of vasoconstriction with telcagepant suggests a potential cardiovascular safety advantage of the CGRP antagonist class of anti-migraine agents as compared to the triptans.

The early generation plant the tree; the later generation enjoy its shadow (Lu Shi E)

Chapter 6

The effect of 17β-estradiol withdrawal on the dural vasodilatation to exogenous αCGRP in female rats

K.Y. Chan, R. de Vries, I. M. Garrelds, A.H.J. Danser,
C.M. Villalón & A. MaassenVanDenBrink (In progress).

Abstract

The prevalence of migraine is 2-3 fold higher in females than in males, which could relate to changes in the levels of female sex hormones. Such changing hormone levels may regulate the synthesis and receptor expression of calcitonin gene-related peptide (CGRP), which mediates neurogenic dural vasodilatation. Moreover, 17β-estradiol increases dural vasodilatation to endogenous CGRP (caused by electrical stimulation), but not to exogenous αCGRP, suggesting that 17β-estradiol may increase CGRP release from sensory nerves.
This study investigated the dural vasodilatation to exogenous αCGRP after 17β-estradiol withdrawal. Rats were ovariectomized and, 7 days later, subcutaneously implanted with 21-day release pellets of 17β-estradiol or placebo. On day 21, pellets were withdrawn and, on day 22, rats were prepared for intravital microscopy experiments on a closed cranial window. Then, the vasodilator responses to intravenous bolus injections of exogenous αCGRP (10-3,000 ng/kg) were investigated. An extra group without pellet removal was used as control. Exogenous αCGRP induced a dose-dependent dural vasodilatation. There were no differences in vasodilatation between the groups treated with 17β-estradiol or placebo pellet without removing the pellet, which is in line with previous findings. Furthermore, the vasodilatation to exogenous αCGRP in the group where the 17β-estradiol pellet was removed was similar to that in the placebo group or the group where the 17β-estradiol pellet was not removed. Our results suggest that one day withdrawal of 17β-estradiol does not affect the vasodilatation to exogenous αCGRP. The effects of 17β-estradiol withdrawal on the vasodilatation to *endogenous* CGRP (released by dural electrical stimulation) are currently being investigated.

Introduction

Migraine is considered a neurovascular disorder characterized by a severe, debilitating and throbbing unilateral headache associated with anorexia, nausea, vomiting, photophobia, phonophobia and/or diarrhoea,[1,64] In Western countries this disorder affects approximately 18% of women during the reproductive years and 6% of men.[413] This sex difference appears to be related to fluctuations in the levels of female sex hormones since around 50% of the women report an association between migraine and menstruation.[66,414-415] Moreover, most women suffering from migraine without aura and menstrual migraine experience improvement during pregnancy.[416] Migraine prevalence is increased during the perimenopause and decreased after the menopause.[68,417] Evidence supports estradiol withdrawal in the late luteal phase of the menstrual cycle as one of the triggers of menstrual migraine in view that: (i) maintaining high plasma estradiol levels could postpone migraine attacks until plasma estradiol levels decreased;[418] and (ii) stable estradiol levels induced by estrogen supplements are associated with a decrease in the number of migraine attacks, and attacks that do occur are milder and shorter.[419] The change in estradiol levels may regulate the synthesis and receptor expression of calcitonin gene-related peptide (CGRP), which mediates the neurogenic vasodilatation that has been shown to be involved in the pathogenesis of migraine.[220] Indeed, 17β-estradiol increases dural vasodilatation to endogenous CGRP, but not to exogenous αCGRP, suggesting that 17β-estradiol may increase CGRP release from sensory nerves.[75] Moreover, CGRP levels in the root dorsal ganglion are decreased after ovariectomy and are increased after estrogen treatment.[420-421] On this basis, the present study set out to investigate the dural vasodilatation to exogenous αCGRP in female rats after 17β-estradiol withdrawal, using the method of intravital microscopy on a closed cranial window.

Materials and methods

Animals

A total of 26 female Sprague-Dawley rats (200 to 300 g), purchased from Harlan Netherlands (Horst, The Netherlands), were maintained at a 12/12-h light-dark cycle (with light beginning at 7 a.m.) and housed in a special room at constant temperature (22±2°C) and humidity (50%), with food and water freely available in their home cages.

General methods

The rats were divided into 5 groups (3-8) each): (i) sham operation or (ii to v) ovariectomy performed under isoflurane anesthesia followed on day 7 by subcutaneous implantation of pellets releasing over a 21-day period: (ii and iv) a placebo and (iii and v) 17β-estradiol

(0.25 mg). After 14 days incubation, intravital experiments (ii and iii) or pellet removal of (iv) the placebo and (v) 17β-estradiol were performed. In the latter two groups (iv and v), intravital experiments were performed on day 22, thus creating a 24-hours period of 'hormone withdrawal'. Sham-operated rats were only treated with placebo and intravital experiments were done on day 21. Blood samples (300 to 500 µl) and vaginal smears were collected 3-4 times: just before ovariectomy, 7 days after ovariectomy before pellet implantation, 14 days after ovariectomy before pellet removal and 24h after pellet removal. Blood samples were collected from the tail vein under isoflurane anesthesia. The samples were mixed with EDTA, centrifuged at 10,000 rpm for 10 min and the resulting plasma was stored at -80°C until the samples were analyzed for 17β-estradiol. Vaginal smears were made on a glass slide with the help of a cotton bud. After drying the smear, the slides were kept in slideboxes at room temperature until the slides were analysed for different phases of the estrous cycle according to proportions of epithelial cells, cornified cells and leukocytes present in the smear.

During the experiments on day 21 or day 22, the rats were anaesthetized with an intraperitoneal (i.p.) injection of sodium pentobarbital (Nembutal, 60 mg/kg, followed by 18 mg/kg per hour when necessary). The trachea was cannulated and connected to a pressure ventilator (small animal ventilator SAR-830 series, CWE Inc., Ardmore, PA, U.S.A.). End-tidal pCO_2 was monitored (Capstar-100 CWE Inc., PA, U.S.A.) and kept between 35 to 48 mmHg. The left femoral vein and artery were cannulated for intravenous (i.v.) administration of drugs and continuous monitoring of blood pressure, respectively. Two or three samples (100 µl each) of blood (at the beginning and at the end of the experiment) were withdrawn via the femoral artery to monitor blood gases, which were kept between normal values (pH: 7.35 to 7.48; pCO_2: 35 to 48 mmHg; pO_2: 100 to 120 mmHg). These values and therefore normal physiological conditions were kept stable throughout the experiment. The body temperature of animals was monitored via a rectal thermometer and maintained throughout the experiment between 36.5°C and 37.5°C by a homeothermic blanket system for rodents (Harvard Instruments, Edenbridge, Kent, U.K.). The rats were placed in a stereotaxic frame and the bone overlying a segment of the dural meningeal artery was carefully drilled thin, applying cold saline (4°C) during the drilling until the artery was clearly visible. Since drilling of the skull induces vasodilatation, we allowed the animal to recover for 1 hour before proceeding with the experimental protocol. The drilled area was covered with mineral oil to prevent drying and to facilitate visualization of the meningeal artery. The artery was captured with an intravital microscope (model MZ 16; Leica microsystem Ltd., Heerbrugg, Switzerland) using a cyan blue filter on a cold source of light. A zoom lens (80 to 450 × magnification) and a camera were used to display images on a standard television monitor. The blood vessel diameter (30 to 40 µm at baseline) was continuously monitored and measured with a video dimension analyzer (Living Systems Instrumentation Inc., Burlington, VT, U.S.A.) and the effects of αCGRP were studied.

Experimental protocols

First, an i.v. bolus injection of saline was given to analyze the effect of vehicle. After 10 min, increasing doses of r-αCGRP (rat-αCGRP) were administered in quarter log dose steps (10 to 3,000 ng/kg). The first 4 doses were given every 5 min, while for the last 5 doses an interval of 10 min was included to allow the values to return to baseline. The peak increase in arterial diameter and peak decrease in mean arterial blood pressure were measured after every dose. The resulting data were displayed and recorded using a WINDAQ data acquisition system. It is important to note that repeated i.v. infusions (3 to 7 times) of αCGRP have been reported to induce each time similar changes with the first infusion in dural vessel diameter and blood pressure in this model.[182] In our hands also, the responses to αCGRP (560 to 1,000 ng/kg) were highly reproducible after finishing the whole protocol (data not shown).

Data analysis

The effects of αCGRP on the dural vessel diameter were calculated as a percentage increase from the baseline diameter just before i.v. bolus injections of αCGRP. The dose response curves were analyzed to establish the maximum response (E_{max}) and dose required to increase dural vessel diameter by 50% of E_{max} (ED_{50}). The changes in mean arterial blood pressure were expressed as absolute values (mmHg). All data are presented as mean±SEM. At every dose, ANOVA was conducted between all groups, followed by Bonferroni multiple comparisons test, with $P<0.05$ considered statistically significant. Significant differences in peptide concentrations, E_{max} and ED_{50} were calculated using the student T-test.

Compounds

r-αCGRP (NeoMPS S.A., Strasbourg, France) was dissolved in isotonic saline and was stored in aliquots at -80°C until required. Just before use, the stock solutions were further diluted to the appropriate concentration in isotonic saline for injection. The hormone pellets were purchased from Innovative Research of America (Sarasota, FL, U.S.A.).

Results

Changes in body weight

The body weight and its changes in the different groups of rats are listed in Table 1. After 7 days of ovariectomy, there was no difference in the body weight in the sham-operated group and the ovariectomized group. Body weight subsequently increased in all groups, and on day 21 after pellet implantation the body weight increase in the placebo-treated group was larger than the increases in body weight in the 17β-estradiol-treated groups and the sham-operated group ($P<0.05$; see Table 1).

Effect of αCGRP on dural artery diameter

Vehicle (saline) did not induce any significant effect on blood vessel diameter (not shown). αCGRP (10 to 3,000 ng/kg) induced dose-dependent dural artery dilatations (Figure 1). There were no significant differences in the potency (ED_{50}) or maximum responses (E_{max}) between the different groups (Table 2).

	Sham-operated rats	Ovariectomized rats treated with:	
		Placebo	17β-estradiol
Day 1 (before sham or ovariectomy)	243±2	246±8	243±4
Day 7 (after sham or ovariectomy)	242±4	245±4	240±5
Δ Body weight at Day 7	-2±3	0±6	-3±3
Day 21 (after pellet implantation)	261±2	294±4	249±5
Δ Body weight at Day 21	19±6*	49±4	9±3*
Day 22 (after pellet removal)		298±4	250±7
Δ Body weight at Day 22		0 ±1	-1±1

Table 1: Body weight (g) and body weight changes (Δ, g) in rats after different interventions (n=3-8), * $p<0.05$ vs. placebo.

	E_{max} (%)	ED_{50} (ng/kg)
Sham-operated rats	133±31	543±181
Placebo	108±25	590±164
17β-estradiol	82±11	386±72
Placebo withdrawal	100±22	207±33
17β-estradiol withdrawal	99±19	220±34

Table 2: Vasodilator responses to r-αCGRP in rats of the different treated groups (n=3-8).

Effect of αCGRP on mean arterial blood pressure

The baseline values of mean arterial blood pressure (mmHg) in the different groups of animals were: sham ovariectomized rats (72±8) and ovariectomized rats treated with placebo (78±5), 17β-estradiol (72±7), withdrawn placebo (81±4) or withdrawn 17β-estradiol (90±7). Vehicle (saline) did not induce any significant effect on mean arterial blood pressure. Administration of αCGRP (10 to 3,000 ng/kg) induced similar dose-dependent decreases in mean arterial blood pressure in all groups (maximum decrease: 30 mmHg, Figure 1).

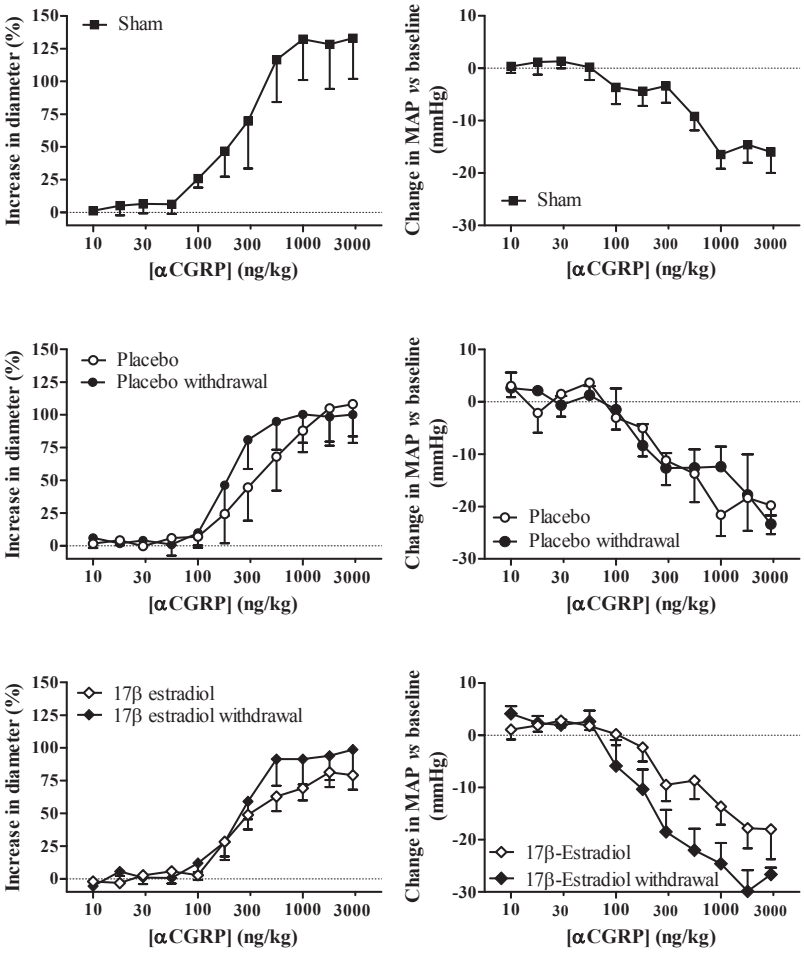


Figure 1: Effect of increasing doses of r-αCGRP (i.v. bolus injections) on dural artery diameter (left panels) and on mean arterial blood pressure (mmHg) (right panels) in sham-operated (upper panels) or ovariectomized rats receiving placebo (middle panels) or 17β-estradiol treatment (lower panels) and with withdrawal of these pellets (n=3-8).

Discussion

The present study investigated the effect of 17β-estradiol withdrawal on dural vasodilatation elicited by exogenous αCGRP. Physiological conditions were kept in the normal range throughout the experiment. Apart from the implications discussed below, our study shows that treatment with 17β-estradiol significantly reduced the weight increase as compared with placebo, as previously shown.[75] The difference between the sham-operated placebo group and the ovariectomized placebo group on day 21 might be caused by the estrogen cycle of the sham-operated group, which is also in accordance with previous data. In contrast with the previous study, no increase in weight was seen on day 7 in the ovariectomized groups. A detailed analysis of the vaginal smears and determination of the plasma estrogen levels might help to explain this discrepancy. These investigations are currently in progress.

Exogenous αCGRP induced a dose-dependent dural vasodilatation that was not affected by ovariectomy. Treatment with 17β-estradiol did not affect this vasodilatation, which is in line with previous findings that suggest the function of CGRP receptors is not affected in the presence of 17β-estradiol.[75] In addition, 17β-estradiol withdrawal did not affect the vasodilatation induced by exogenous αCGRP. Since 17β-estradiol has been suggested to also regulate the expression of the CGRP receptor,[220] which might affect vasodilatation, our data suggest that, at least, a rapid decrease in plasma 17β-estradiol concentration does not affect the vascular function of CGRP receptors. Future studies should now address the protein expression and localization of the CGRP receptors under the various conditions. Based on the results of our data no difference in functional receptor will be expected. However, there might be discrepancies in the receptor protein translation level under the various conditions.

Previous studies have shown that administration of 17β-estradiol increased the dural vasodilatation to endogenous CGRP induced by periarterial electrical stimulation in this intravital microscopy model.[75] Clearly, the effect of 17β-estradiol withdrawal on the dural vasodilatation induced by periarterial electrical stimulation should now be investigated as well. For these experiments we will use the same groups of animals as described in the methods of this chapter; neurogenic dural vasodilatation will be induced by increasing current intensities of periarterial electrical stimulation. In addition, protein expression, receptor localization, functional *in vitro* experiments with the blood vessels and assessment of coronary function are items for further investigations on the effect of 17β-estradiol withdrawal in rats.

經一事長一智

五代史平話

Wisdom is experiences
(History of the Five Chinese Dynasties)

Part C: Other neuronal targets

Chapter 7

Effects of ionotropic glutamate receptor antagonists on rat dural artery diameter in intravital microscopy model

K.Y. Chan, S. Gupta, R. de Vries, A.H.J. Danser, C.M. Villalón, E. Muñoz-Islas & A. MaassenVanDenBrink (2010), British Journal of Pharmacology, 160; 1316–1325.

Abstract

During migraine, trigeminal nerves may release calcitonin gene-related peptide (CGRP), inducing cranial vasodilatation and central nociception; hence, trigeminal inhibition or blockade of craniovascular CGRP receptors may prevent this vasodilatation and abort migraine headache. Several preclinical studies have shown that glutamate receptor antagonists affect the pathophysiology of migraine. This study investigated whether antagonists of NMDA (ketamine and MK801), AMPA (GYKI52466) and kainate (LY466195) glutamate receptors affect dural vasodilatation induced by αCGRP, capsaicin and periarterial electrical stimulation in rats, using intravital microscopy.

Male Sprague-Dawley rats were anaesthetized with i.p. nembutal and the overlying bone was thinned to visualize the dural artery. Then, vasodilator responses to exogenous (i.v. αCGRP) and endogenous (released by i.v. capsaicin and periarterial electrical stimulation) CGRP were elicited in the absence or presence of the above antagonists.

αCGRP, capsaicin and periarterial electrical stimulation increased dural artery diameter. Ketamine and MK801 inhibited the vasodilator responses to capsaicin and electrical stimulation, while only ketamine attenuated those to αCGRP. In contrast, GYKI52466 only attenuated the vasodilatation to exogenous αCGRP, whilst LY466195 did not affect the vasodilator responses to endogenous or exogenous CGRP.

Although GYKI52466 has not been tested clinically, our data suggest that it would not inhibit migraine via vascular mechanisms. Similarly, the antimigraine efficacy of LY466195 seems unrelated to vascular CGRPergic pathways and/or receptors. In contrast, the cranial vascular effects of ketamine and MK801 may represent a therapeutic mechanism, although the same mechanism might peripherally contribute to cardiovascular side effects.

Introduction

Migraine is a neurovascular disorder that has been associated with cranial vasodilatation and release of calcitonin gene-related peptide (CGRP) resulting from activation of perivascular trigeminal sensory nerves that originate in the trigeminal ganglion and the trigeminocervical complex.[91,134] Interestingly, the excitatory neurotransmitter, glutamate, has also been involved in migraine[236] as it is found in neurons of structures related to migraine pathophysiology, including the trigeminal ganglion, trigeminocervical complex and the thalamus.[237] Indeed, glutamate and CGRP are released from trigeminal ganglion neurons by calcium channel-dependent mechanisms,[238] and increased levels of glutamate have been found in: (i) the trigeminocervical complex after stimulation of dural structures;[239] and (ii) the cerebrospinal fluid of migraine patients.[240]

Glutamate exerts its effects by activating ionotropic (ligand-gated ion channels) and metabotropic (G-protein coupled) receptors. Ionotropic glutamate receptors are divided into N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors.[245] Activation of ionotropic glutamate receptors on neurons leads to calcium influx, activation of intracellular signalling pathways and production and release of vasoactive agents like nitric oxide (NO).[264] Moreover, the ionotropic glutamate receptors are activated during cortical spreading depression, which is considered to be involved in migraine aura.[244]

Several preclinical studies have suggested that ionotropic glutamate receptor antagonists affect the pathophysiology of migraine. The NMDA receptor antagonist MK801 and the AMPA receptor antagonist GYKI52466 blocked trigeminovascular nociception in the trigeminocervical nucleus,[249-251] whilst MK801 reduced capsaicin-evoked CGRP release;[266] the latter finding points to potential vascular effects of glutamate receptor antagonists. In addition, blockade of both NMDA and non-NMDA ionotropic receptors reduces c-fos protein expression in the trigeminal nucleus caudalis after intracisternal capsaicin injection.[254-255] Most notably, several glutamate receptor antagonists are effective in the acute treatment of migraine, including the mixed AMPA/kainate receptor antagonist LY293558[7] and the kainate receptor antagonist LY466195.[272] Likewise, the anticonvulsant topiramate is effective in migraine prophylaxis,[269] probably, at least partly, due to blockade of AMPA and kainate receptors. In patients taking nitroglycerin for reducing the risk of cardiac ischemia, infusion of ketamine, an NMDA receptor antagonist, was proposed to be effective against this NO-induced headache.[270] Moreover, in a small open-label study, intranasal ketamine reduced the severity and duration of the neurologic deficits due to the aura in 5 out of 11 patients with familial hemiplegic migraine.[271]

On this basis, the present study set out to analyse the effects of several ionotropic glutamate receptor antagonists on an experimental neurovascular model of migraine, using

intravital microscopy. Accordingly, the NMDA receptor antagonists ketamine and MK801, the AMPA receptor antagonist GYKI52466, and the kainate receptor antagonist LY466195 were tested on the rat dural vasodilatation induced by exogenous and endogenous (released by i.v. capsaicin or periarterial electrical stimulation) CGRP.

Materials and methods

Animals

Male Sprague-Dawley normotensive rats (300 to 400 g), purchased from Harlan Netherlands (Horst, The Netherlands), were maintained at a 12/12-h light-dark cycle (with light beginning at 7 a.m.) and housed in a special room at constant temperature (22±2°C) and humidity (50%), with food and water freely available in their home cages.

General methods

Experiments were carried out in a total of 69 rats. During the experiments the rats were anaesthetized with an intraperitoneal (i.p.) injection of sodium pentobarbital (Nembutal, 60 mg/kg, followed by 18 mg/kg per hour when necessary). The trachea was cannulated and connected to a pressure ventilator (small animal ventilator SAR-830 series, CWE Inc., Ardmore, PA, U.S.A.). End-tidal pCO_2 was monitored (Capstar-100 CWE Inc., PA, U.S.A.) and kept between 35 to 48 mmHg. The left femoral vein and artery were cannulated for intravenous (i.v.) administration of drugs and continuous monitoring of blood pressure, respectively. Two or three samples of blood (at the beginning and at the end of the experiment) were withdrawn via the femoral artery to monitor blood gases, which were kept between normal values (pH: 7.35 to 7.48; pCO_2: 35 to 48 mmHg; pO_2: 100 to 120 mmHg). These values and therefore normal physiological conditions were kept stable throughout the experiment. The body temperature of animals was monitored via a rectal thermometer and maintained throughout the experiment between 36.5°C and 37.5°C by a homeothermic blanket system for rodents (Harvard Instruments, Edenbridge, Kent, U.K.). The rats were placed in a stereotaxic frame and the bone overlying a segment of the dural meningeal artery was carefully drilled thin, applying cold saline (4°C) during the drilling until the artery was clearly visible. Since drilling of the skull induces vasodilatation, we allowed the animal to recover for 1 hour before proceeding with the experimental protocol. The drilled area was covered with mineral oil to prevent drying and to facilitate visualization of the meningeal artery. The artery was captured with an intravital microscope (model MZ 16; Leica microsystem Ltd., Heerbrugg, Switzerland) using a cyan blue filter on a cold source of light. A zoom lens (80 to 450 times magnification) and a camera were used to display images on a standard television monitor. The blood vessel diameter (30 to 40 μm at baseline) was continuously monitored and measured with

a video dimension analyzer (Living Systems Instrumentation Inc., Burlington, VT, U.S.A.) and the effects of αCGRP, capsaicin and periarterial electrical stimulation were studied as specified below. In rats where periarterial electrical stimulation was used to evoke dilatation of the dural blood vessels, a bipolar stimulating electrode (NE 200X, Clark Electromedical, Edenbridge, Kent, U.K.) was placed on the surface of the cranial window approximately within 200 μm from the vessel of interest. The surface of the cranial window was stimulated at 5 Hz, 1 ms for 10 s (Stimulator model S88, Grass Instruments, West Warwick, RI, U.S.A.). For neurogenic dural vasodilatation, we initially started with a current intensity (monitored on an oscilloscope, model 54601A, Hewlett Packard, Palo Alto, CA, U.S.A.) of 100 μA and increased with 50 μA steps until a maximal level of dilatation was achieved, usually at 200 μA. The resulting data were displayed and recorded using a WINDAQ data acquisition system (Version 2.54; DataQ Instruments Inc., Akron, OH, U.S.A.).

Experimental protocol

After a stable haemodynamic condition for at least 60 min, baseline values of mean arterial blood pressure (MAP) and heart rate were determined. Then, the animals were divided into three groups (n=25, 24 and 20) which received αCGRP (1 μg/kg, i.v.), capsaicin (10 μg/kg, i.v.) and periarterial electrical stimulation (150-250 μA), respectively. 30 min were allowed to elapse after each of these treatments for the recovery of baseline diameter. Each of these groups was subsequently subdivided into 4 subgroups (n=5-7) which were given (after 30 min) i.v. cumulative doses of, respectively, ketamine (10, 18 and 30 mg/kg), MK801 (0.2, 0.5, 1 and 3 mg/kg), GYKI52466 (0.5, 2 and 5 mg/kg) and LY466195 (0.03, 0.1 and 0.3 mg/kg). Each dose of antagonist was administered 5 min before a subsequent treatment with α-CGRP, capsaicin or periarterial electrical stimulation. The selected doses of ketamine,[422] MK801,[250] GYKI53466[251] and LY466195[260] have previously been shown to be effective in blocking their respective receptors. The ionotropic glutamate receptors nomenclatures is in accordance to the most recent BJP's guide to receptors and channels.[423] The duration of each experiment was approximately 2.5 hours after stabilisation. The Ethical Committee of our institution, dealing with the use of animals in scientific experiments, approved the protocols of the present investigation.

Data presentation and statistical evaluation

All data are presented as mean±SEM. The peak increases in diameter of the dural meningeal artery are expressed as percent change from baseline. The changes in MAP and heart rate are expressed as, respectively, absolute values (mmHg) and percentage change from baseline. The difference between the variables within one group of animals was compared by using a one-way repeated measures analysis of variance followed by the Dunnet's test.[424] Statistical significance was accepted at $P<0.05$ (two-tailed).

Drugs

The compounds used in the present study were obtained from the sources indicated: capsaicin, MK801 hydrogen maleate, GYKI52466 hydrochloride, 2-hydroxypropyl-β-cyclodextrin 45% (HBC) (Sigma Chemicals Co., Steinheim, Germany); rat α-CGRP (NeoMPS S.A., Strasbourg, France); nembutal (Ceva Sante Animale B.V., Maassluis, The Netherlands); ketamine hydrochloride (Alfasan, Woerden, The Netherlands); LY466195 (Eli Lilly and Company, Indianapolis, IN, U.S.A.). Capsaicin (1 mg/ml) was dissolved in a mixture of tween 80, ethanol 70% and water (1:1:8); GYKI52466 (20 mg/ml) was dissolved in 45% HBC, whereas the other compounds were dissolved in isotonic saline. All compounds were stored in aliquots at -80°C, until required. Just before use, the stock solutions were further diluted to the appropriate concentration in isotonic saline for injection. The doses of all compounds refer to their respective salts.

Results

Effect of αCGRP, capsaicin and periarterial electrical stimulation on dural diameter, mean arterial blood pressure and heart rate

I.v. administration of 1 μg/kg αCGRP or 10 μg/kg capsaicin increased dural artery diameter by, respectively, 103±7% (n=25) and 77±6% (n=24), whereas periarterial electrical stimulation (150 μA-250 μA) increased dural artery diameter by 78±5% (n=20). Repeated treatment with αCGRP, capsaicin or periarterial electrical stimulation for 4 times produced reproducible increases in the dural artery diameter (data not shown).

At the beginning of the experiments, the average baseline MAP from all animals was 96±2 mmHg. There were no significant differences between the baseline values before and after the experiments in most groups ($p>0.1$), except in those of capsaicin with ketamine (Figure 1; right middle panel) and electrical stimulation with MK801 (Figure 2; right lower panel). The MAP was decreased after infusion of αCGRP, but not after infusion of saline when the dilatation of the dural artery was maximal in the MK801, GYKI52466 and LY466195 treated groups; these decreases were similar after giving these antagonists (Figure 2-4; right upper panels). αCGRP significantly decreased MAP in the ketamine group at the highest dose (Figure 1; right upper panels). In general, capsaicin and periarterial electrical stimulation did not significantly affect MAP in most groups, except in that of capsaicin with GYKI52466 (Figure 1-4; right middle and lower panels). Heart rate was not affected by any of the above treatments (data not shown), except for the ketamine treated group (see below).

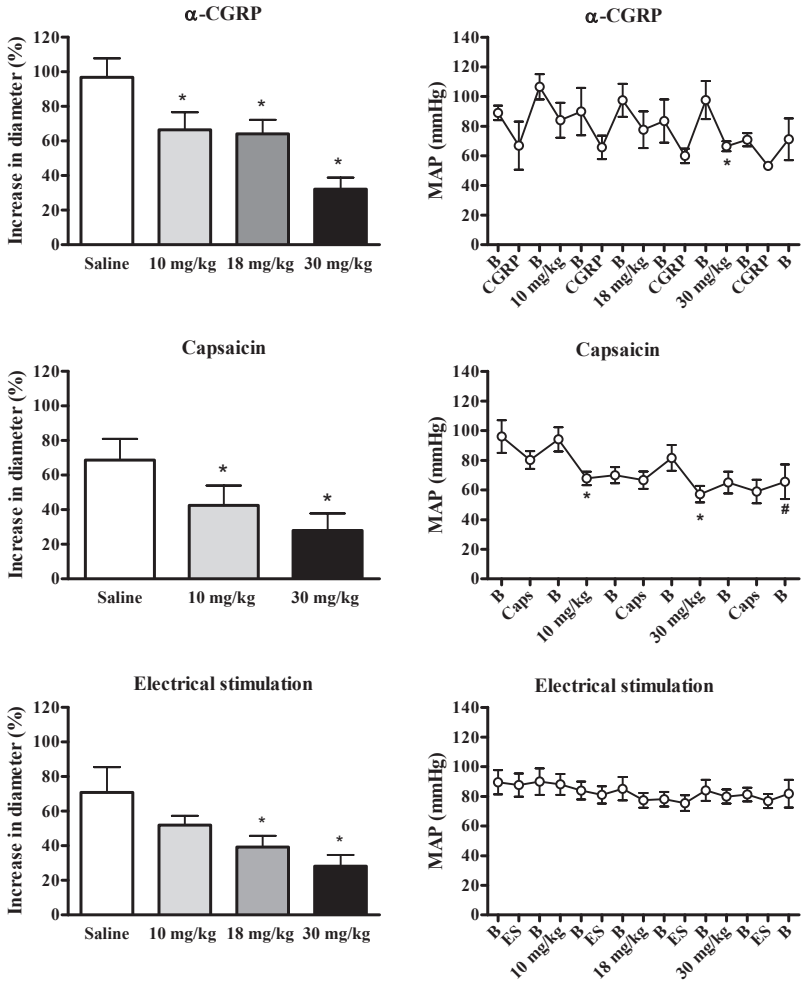


Figure 1: Effect of increasing doses of ketamine on the dural vasodilatation (percentage of increase in diameter, left panels) and mean arterial blood pressure (mmHg, right panels) induced by αCGRP (upper panels, n=6); capsaicin (middle panels, n=6) and periarterial electrical stimulation (lower panel, n=6). B, baseline; Caps, 10 μg/kg capsaicin i.v.; ES, periarterial electrical stimulation. * $p<0.05$ compared to the control or the corresponding baseline; # $p<0.05$ compared to the baseline at the beginning of the experiment.

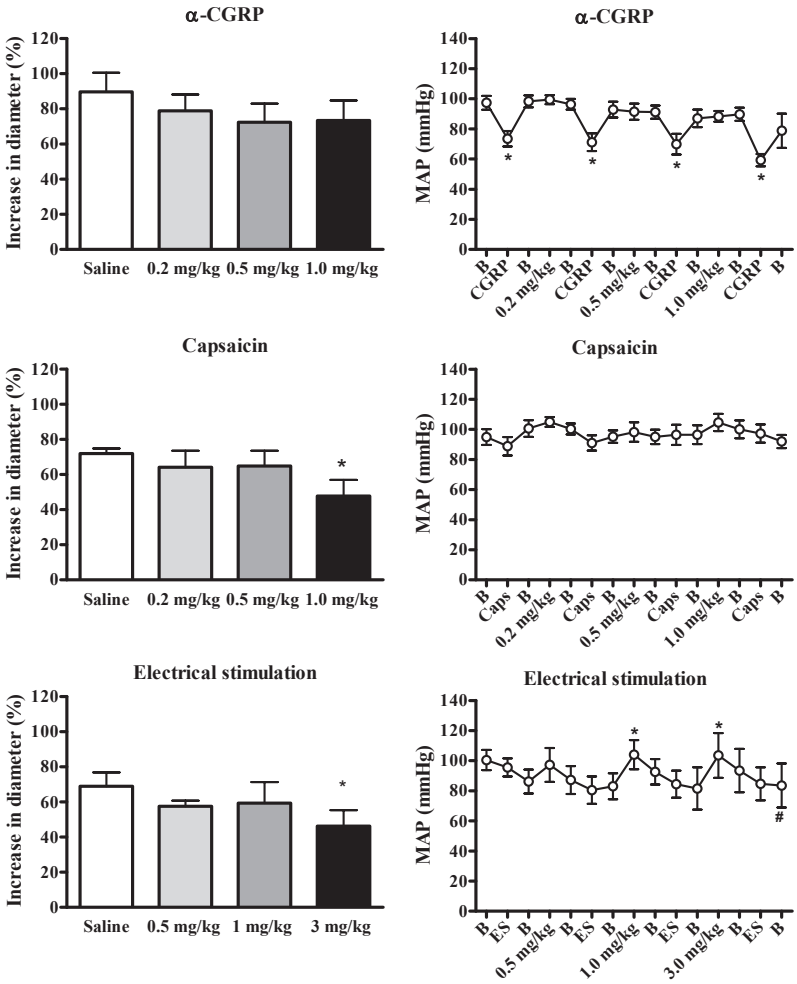


Figure 2: Effect of increasing doses of MK801 on the dural vasodilatation (percentage of increase in diameter, left panels) and mean arterial blood pressure (mmHg, right panels) induced by αCGRP (upper panels, n=8), capsaicin (middle panels, n=7) and periarterial lectrical stimulation (lower panels, n=5). B, baseline; Caps, 10 μg/kg capsaicin i.v.; ES, periarterial electrical stimulation. * $p<0.05$ compared to the control or the corresponding baseline; # $p<0.05$ compared to the baseline at the beginning of the experiment.

Effect of the NMDA receptor antagonists ketamine and MK801 on dural artery vasodilatation, mean arterial blood pressure and heart rate after treatment with α-CGRP, capsaicin and periarterial electrical stimulation

Ketamine induced a dose-dependent attenuation of the vasodilator responses to αCGRP (10 mg/kg: 66±10%; 18 mg/kg: 64±8%; 30 mg/kg: 32±7%, Figure 1; left upper panel) and capsaicin (10 mg/kg: 42±11%; 30 mg/kg: 28±10%, Figure 1; left middle panel) compared to the control groups (97±11% and 69±12% for αCGRP and capsaicin, respectively). Ketamine also produced a significant attenuation of the vasodilatation in the electrical stimulation group at the doses of 18 mg/kg (39±6%) and 30 mg/kg (28±6%) compared to control (71±15%, Figure 1; left lower panel).

In contrast, the doses used of MK801 did not block the vasodilator responses to α-CGRP (Figure 2; left upper panel), although it significantly attenuated the vasodilator responses to both capsaicin (at 1 mg/kg; 43±9%, Figure 2; left middle panel) and electrical stimulation (at 3 mg/kg; 46±9%, compared to the control (72±3% and 69±8% respectively, Figure 2; left lower panel). The NMDA receptor antagonists did not affect dural artery diameter *per se* (data not shown).

Ketamine significantly decreased MAP at the highest doses used in the αCGRP and at all doses in the capsaicin treated groups (Figure 1; right upper and middle panel). In the electrical stimulation group, ketamine did not decrease MAP at any of the doses tested. Moreover, MK801 did not change MAP in the αCGRP and capsaicin treated groups, while it increased the MAP in the electrical stimulation treated group (Figure 1; right lower panel). Ketamine significantly decreased heart rate at all doses tested (10 mg/kg: -10±3%; 18 mg/kg: -14±4%; 30 mg/kg: -16±4%), while MK801 did not affect heart rate (data not shown).

Effect of the AMPA receptor antagonist GYKI52644 on dural artery vasodilatation, mean arterial blood pressure and heart rate after treatment with αCGRP, capsaicin and periarterial electrical stimulation

GYKI52466, at all doses tested, did not significantly modify the vasodilatation to capsaicin or periarterial electrical stimulation (Figure 3; right middle panel and lower panel) but, at 5 mg/kg, attenuated the vasodilatation to exogenous αCGRP (92±20%) compared to the control group (124±23%, Figure 3; right upper panel). The effects of GYKI52466 on dural artery diameter were not different from that of its vehicle (HBC; volume corresponding to that at 5 mg/kg GYKI52466) and did not affect the dural diameter *per se* (data not shown).

Interestingly, the vehicle of GYKI52466 (HBS) significantly increased MAP in the α-CGRP, capsaicin and electrical stimulation groups (Figure 3; left panels). This increase is also present at the highest doses of GYKI52466 in the capsaicin group (Figure 3; left middle panel) and at all doses of GYKI52466 in the electrical stimulation group (Figure 3; left lower panel). Heart rate was not attenuated by this compound (data not shown).

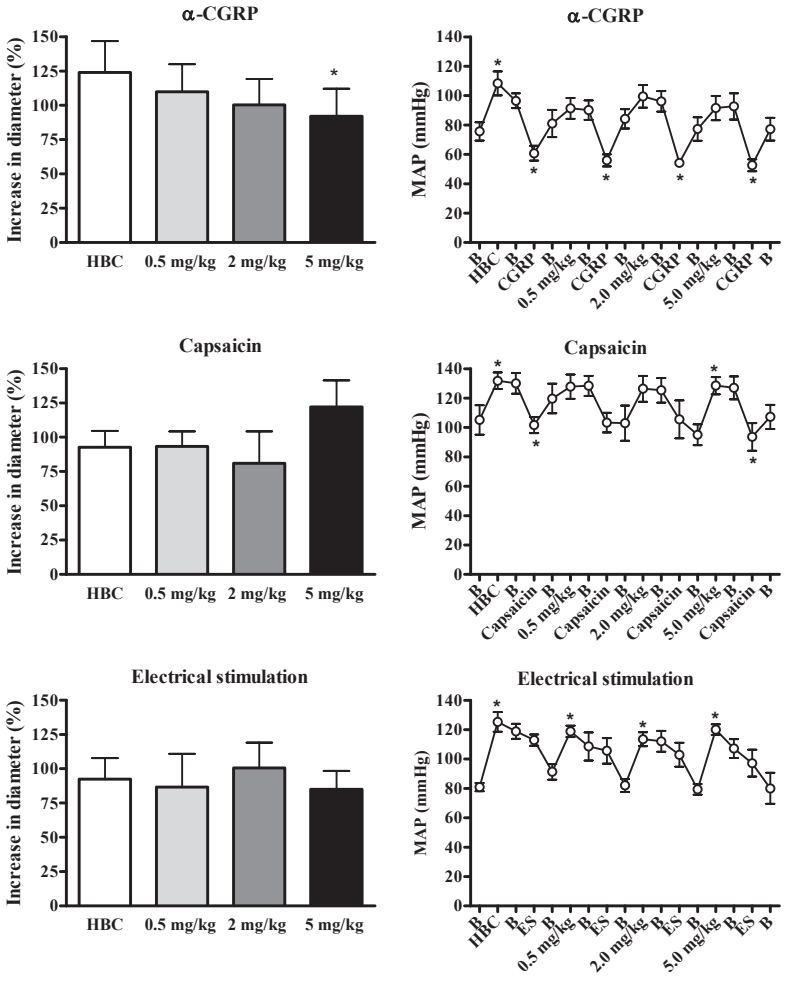


Figure 3: Effect of increasing doses of GYKI52466 on the dural vasodilatation (percentage of increase in diameter, left panels) and mean arterial blood pressure (mmHg, right panels) induced by αCGRP (upper panels, n=5), capsaicin (middle panels, n=5) and periarterial electrical stimulation (lower panels, n=4). 2-hydroxypropyl-β-cyclodextrin (HBC) is the vehicle control of GYKI52466. B, baseline; Caps, 10 μg/kg capsaicin i.v.; ES, periarterial electrical stimulation. * $p<0.05$ compared to the control or the corresponding baseline; # $p<0.05$ compared to the baseline at the beginning of the experiment.

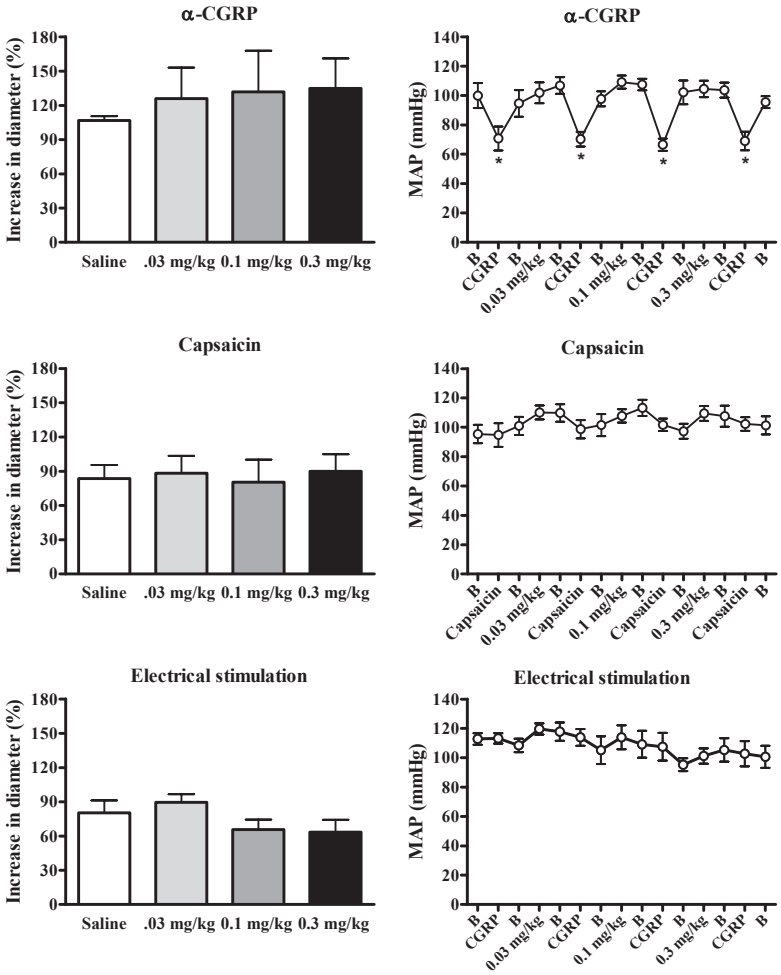


Figure 4: Effect of increasing doses of LY466195 on the dural vasodilatation (percentage of increase in diameter, left panels) and mean arterial blood pressure (mmHg, right panels) induced by αCGRP (upper panels, n=6), capsaicin (middle panels, n=6) and periarterial electrical stimulation (lower panels, n=5). B, baseline; Caps, 10 μg/kg capsaicin i.v.; ES, periarterial electrical stimulation. * $p<0.05$ compared to the control or the corresponding baseline; # $p<0.05$ compared to the baseline at the beginning of the experiment.

Effect of the kainate receptor antagonist LY466195 on dural artery vasodilatation, mean arterial blood pressure and heart rate after treatment with αCGRP, capsaicin and periarterial electrical stimulation

At all doses used, LY466195 did not affect the vasodilatation induced by αCGRP, capsaicin or electrical stimulation (Figure 4; left panels). Moreover, LY466195 did not affect dural artery diameter per se (data not shown). MAP (Figure 4; right panels) and heart rate (data not shown) were not changed in the presence of LY466195.

Discussion and conclusions

The present study investigated the effects of the NMDA receptor antagonists ketamine and MK801, the AMPA receptor antagonist GYKI52466, and the kainate receptor antagonist LY466195 on rat dural vasodilatation induced by endogenous and exogenous αCGRP, using intravital microscopy.[59] The release of endogenous CGRP was induced by either chemical stimulation with capsaicin or periarterial electrical stimulation. Moreover, normal physiological conditions were kept stable throughout the experiment.

Apart from the implications discussed below, our results confirm that 1 μg/kg α-CGRP, 10 μg/kg capsaicin or 150 μA-250 μA periarterial electrical stimulation induced dural vasodilator responses in the rat, as previously reported by others.[63,425] Moreover, the maximal responses on the vasodilatation induced by αCGRP and capsaicin were not different from those in our previous study.[75] However, the vasodilatation induced by electrical stimulation needed a higher voltage to cause the maximal vasodilatation. This difference may well be due to the difference in sex of the animals between both studies, which is in accordance with the higher density of CGRP-containing fibres in female than in male rats.[420] As expected, α-CGRP decreased the MAP due to stimulation of vascular CGRP receptors resulting in systemic vasodilatation.[169] This vasodepressor response is unrelated to cardiac effects, as our results did not show any significant change on heart rate as compared to the saline control. In contrast, electrical stimulation was given locally and, consequently, did not affect the peripheral (systemic) vascular tone.

Activation of the ionotropic glutamate receptors leads to calcium influx in neurons and induces the release of vasoactive agents.[264] Accordingly, we hypothesize that ionotropic glutamate receptor antagonists would block the influx of calcium, preventing CGRP release from periarterial nerve endings and would thus attenuate endogenous CGRP release, but not the response to exogenous αCGRP. However, our results showed that the NMDA receptor antagonist ketamine not only attenuated the vasodilatation to endogenous CGRP (i.e. in response to capsaicin and perivascular electrical stimulation), but also that to exogenous α-CGRP. Moreover, the attenuation induced by increasing doses of ketamine

on the vasodilatation to αCGRP, capsaicin or periarterial electrical stimulation is not due to tachyphylaxis, since repeated treatment (up to four times) with αCGRP, capsaicin or periarterial electrical stimulation in control experiments (without antagonists) was highly reproducible; in addition, in most cases there was no difference between the MAP value at the beginning and the end of the experiments, indicating that our model was stable during the whole duration of the experiments. However, the NMDA receptor antagonist ketamine induced a vasodepressor response in the αCGRP and capsaicin groups, which is likely due to the inhibitory effect of ketamine on heart rate,[426] which is in line with our observations.

It could be argued that the effect of ketamine on the vasodilatation to αCGRP and capsaicin may be an artefact if this high vasodepressor response might have decreased dural artery diameter. However, this possibility is highly unlikely because ketamine, as the other antagonists, did not affect dural artery diameter *per se.* Furthermore, it has previously been shown that dural artery diameter in this model is not affected by a decrease in blood pressure up to 60 mmHg;[427] in our experiments, this decrease did not reach 60 mmHg. Thus, the changes on the dural artery diameter induced by ketamine are most likely caused by a direct pharmacological effect. Since ketamine also reduced the dural artery vasodilatation to exogenous αCGRP, such an effect cannot be explained in terms of an interaction with the NMDA receptor. Although ketamine has been reported to be a selective antagonist for NMDA receptors,[428] Ho and Su[429] have shown that ketamine also attenuates sympathetic activity, which is not mediated by NMDA receptors. Further, ketamine has an effect on the μ-opioid receptors,[430] which has been shown to inhibit neurogenic dural vasodilatation in this experimental model.[431] Therefore, the effect of ketamine on neurogenic dural vasodilatation in this study might be also mediated via the μ-opioid receptor. Finally, it cannot be excluded that ketamine might also have affinity for CGRP receptors, which could then contribute to the potential antimigraine efficacy of ketamine, although no receptor binding data are available to confirm or reject this possibility. Another possibility is that ketamine might reduce cranial vasodilatation via a still unknown mechanism, since ketamine is also shown to reduce cerebral vasodilatation induced by isoflurane.[432]

Because of the limited pharmacological specificity of ketamine, we also studied the NMDA receptor antagonist, MK801. This antagonist did not affect the vasodilatation to exogenous αCGRP, but it attenuated the vasodilatation to endogenous CGRP. This may suggest that such attenuation is via blockade of NMDA receptors which, in turn, results in less release of CGRP. The effect of MK801 on the vasodilatation to capsaicin and electrical stimulation is not an effect of MK801 *per se,* nor is it an artefact effect due to the changes in MAP, since MK801 only affected the MAP in the electrical stimulation group and not in the capsaicin group. Since the vasodilatation to capsaicin is blocked by ketamine and MK801, and capsaicin stimulates neuronal vanilloid receptors, we cannot rule out that these antagonists also interact with vanilloid receptors. Clearly, further experiments which fall

beyond the scope of the current study are required to investigate this possibility.

Interestingly, the AMPA receptor antagonist GYKI52466 only attenuated the vasodilatation induced by exogenous αCGRP. Although the MAP values were increased in this group, the attenuation of the vasodilatation was not due to this effect, since MAP was also affected in the capsaicin and electrical stimulation group. This apparent increase in MAP by GYKI52466 could be rather attributed to its vehicle, namely HBC, because HBC alone also increased MAP. Since GYKI52466 only attenuated αCGRP-induced vasodilatation, but not the vasodilatation to endogenous CGRP, the possible anti-migraine effect of AMPA receptor antagonists may be unrelated to an interaction with the AMPA receptor. A possibility for this effect would be that GYKI52466 might behave like a CGRP scavenger. In this respect, Juhl *et al*.[425] have recently shown that CGRP scavengers inhibit the vasodilatation induced by exogenous αCGRP, but not the endogenously released CGRP in the same model.

The kainate receptor antagonist, LY466195, did not affect the vasodilatation in the rat dural arteries induced by either endogenous or exogenous CGRP. This suggests that LY466195 does not attenuate the release of CGRP induced by capsaicin and periarterial electrical stimulation, nor does it affect the binding of CGRP to its receptor. This is in line with an earlier finding, where the kainate receptor antagonist UBP302 did not affect the dural vasodilatation induced by electrical stimulation or exogenous αCGRP in a neurogenic dural vasodilatation model.[262] In addition, LY466195 blocked the calcium influx evoked by glutamate and attenuated the amount of c-fos positive cells after trigeminal neuron stimulation.[260] Moreover, LY466195 does not induce vasoconstriction *per se* nor does it affect the vasoconstrictor properties of sumatriptan in the rabbit saphenous vein.[260] In contrast, the antiepileptic drug topiramate, which has affinity for the kainate receptor, attenuated the vasodilatation induced by electrical stimulation and NO infusion after 15 min, but not the CGRP-induced vasodilatation in the same intravital microscopy model.[433] Interestingly, LY466195 is effective in the treatment of migraine, but it also causes mild reversible visual distortions.[272] Hence, we cannot exclude the possibility that the antimigraine efficacy of LY466195 could involve a central effect unrelated to vascular CGRPergic pathways and/or its receptors. This possibility is reinforced by other findings showing that the trigeminocervical complex and the ventroposteromedial thalamic nucleus are important sites of action for the anti-migraine effect of LY466195.[263]

From our data, we suggest that NMDA receptor antagonists could be candidates for the treatment of migraine, because of blockade of vasodilatation to endogenously released CGRP in the dural artery. However, blockade of NMDA receptors, the activation of which mediates coronary vasodilatation,[434] might also negatively affect the cardiovascular protection by CGRP.[57,173-174] In addition, frequent and recreational ketamine used is known to be associated with cognitive impairments and elevated psychopathological symptoms.[435] However, another NMDA receptor antagonist, memantine, reduces headache frequency with uncommon and generally mild side effects.[436] The effects of AMPA receptor antagonists in migraine are still

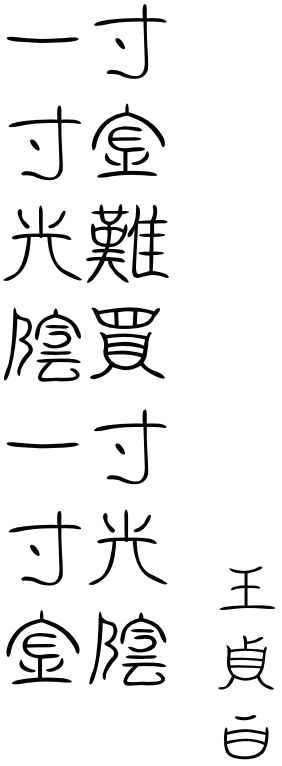
unknown. The AMPA/kainate receptor antagonist tezampanel is well tolerated and has no vasoconstrictor liability in clinical trials.[273] This finding supports our data that GYKI52466 did not affect vasodilatation induced by endogenous CGRP. However, due to the mixed AMPA and kainate receptor action of tezampanel, it is not clear which receptor is responsible for its anti-migraine effect.[273] Although GYKI52466 has not been tested clinically, our data suggest that it would not inhibit migraine via vascular mechanisms. Moreover, given the physicochemical properties of the glutamate receptor antagonists used in this study, we cannot rule out that these compounds are capable of crossing the blood brain barrier; therefore, they may have central effects which could also contribute to the pharmacological profile of the mechanisms characterized in this study.

On the basis of our results, it is tempting to suggest that the kainate receptor antagonist, LY466195, may have antimigraine properties without cardiovascular side effects. Evidently, further studies on its cardiovascular safety, which fall beyond the scope of the present investigation, are warranted, as exemplified by the small increase in MAP after its high dose. This small vasopressor effect may be due to the moderate affinity of LY466195 for the NMDA receptor.[263] More specific kainate receptor antagonists may provide a neuronal and non-vascular migraine treatment. Obviously, it has to be kept in mind that ionotropic glutamate receptors are involved in several mechanisms in the brain and spinal cord; thus, blockade of these receptors may induce neurological side effects.

In conclusion, this study demonstrates that the different ionotropic glutamate receptor antagonists affect in a differential manner the vasodilatation induced by endogenous and exogenous CGRP. The NMDA receptor antagonists ketamine and MK801 are capable of inhibiting neurovascular CGRP release. This property may represent a therapeutic mechanism of action in the treatment of migraine, but might also result in cardiovascular side effects. Since the AMPA receptor antagonist GYKI52466 did not affect CGRP release, potential antimigraine efficacy of AMPA receptor antagonists is unlikely to be related to a vascular mode of action. Similarly, the kainate receptor antagonist LY466195, which has demonstrated antimigraine efficacy, did not affect CGRP release and/or its vasodilator effects. Thus, its antimigraine action is most likely mediated via a central mechanism, not involving vascular CGRPergic pathways and/or receptors. This study extends the knowledge of ionotropic glutamate receptors in migraine, although further studies are required to explore the effect of glutamate and glutamate receptors in migraine pathophysiology.

Acknowledgements

The authors would like to thank Dr. Kirk Johnson (Lilly Research Laboratories, Eli Lilly and Company, IN, U.S.A.) for providing LY466195.



Time is money, but you cannot buy time with money
(Wang Zhen Bai)

Chapter 8

Pharmacological characterization of VIP and PACAP receptors in the human meningeal and coronary artery

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Abstract

We pharmacologically characterized pituitary adenylate cyclase activating polypeptides (PACAPs), vasoactive intestinal peptide (VIP) and the $VPAC_1$, $VPAC_2$ and PAC_1 receptors in human meningeal (for their role in migraine) and coronary (for potential side effects) arteries.

Concentration response curves to PACAP38, PACAP27, VIP and the $VPAC_1$ receptor agonist ([Lys15,Arg16,Leu27]-VIP(1-7)-GRF(8-27)) were constructed in the absence or presence of the PAC_1 receptor antagonist PACAP6-38 or the $VPAC_1$ receptor antagonist, PG97269. mRNA expression was measured using qPCR.

PACAP38 was less potent than VIP in both arteries. Both peptides had lower potency and efficacy in meningeal than in coronary arteries, while mRNA expression of $VPAC_1$ receptor was more pronounced in meningeal arteries. PACAP6-38 reduced the E_{max} of PACAP27, while PG97269 right shifted the VIP-induced relaxation curve only in the coronary arteries. In conclusion, the direct vasodilatory effect of VIP and PACAP might be less relevant than the central effect of this compound in migraine pathogenesis.

Introduction

Migraine is a neurovascular disorder associated with activation of the trigeminal cervical complex, release of vasoactive peptides and dilatation of cephalic blood vessels. This vasodilatation seems to be principally caused by calcitonin gene related peptide (CGRP), which is released after activation of perivascular trigeminal sensory nerves.[91,143] Interestingly, also other neuropeptides released after activation of the trigeminovascular complex, including pituitary adenylate cyclase activating polypeptides (PACAPs) and vasoactive intestinal peptide (VIP)[280] have recently been described to induce headache, and might play a role in the pathophysiology of migraine.[281,300-301,303]

PACAP and VIP, which belong to the secretin/glucagons/VIP peptide family, are associated with various physiological functions, including cranial vasodilatation.[283-284] Further they are identified in trigeminal sensory and parasympathetic ganglia with perivascular nerve fiber projections.[282,285-286] PACAPs exert their effects via the $VPAC_1$, $VPAC_2$ and PAC_1 receptors, while VIP induces its effects only via the $VPAC_1$ and $VPAC_2$ receptors (Table 1). These three receptors are G_s-protein coupled receptors and activate adenylate cyclase to exert their effects.[283-284] The mRNA of these receptors is found in human cerebral arteries and cranial ganglia with perivascular nerve fiber projections.[287]

	$VPAC_1$ receptor	$VPAC_2$ receptor	PAC_1 receptor
PACAP38	8.2	-	8.4-9.0
PACAP27	8.9	7.7-8.0*	8.0-8.7
VIP	8.5-9.7	7.8-8.7	6.0-6.3
$VPAC_1$ receptor agonist	9.1	4.5	-

Table 1: pK_i values of PACAP38 and VIP for the three receptors subtype. * pIC_{50} value. For references, see the database of the IUPHAR Committee on Receptor Nomenclature and Drug Classification.

PACAP38 and VIP have been shown *in vivo* in humans to decrease mean flow velocity in the middle cerebral artery and to induce vasodilatation in the superficial temporal artery in a similar magnitude.[281,300-301,303] Moreover, PACAP38 induced migraine-like headache in migraine patients and headache in healthy volunteers.[281] However, VIP only induced mild headache in migraine patients and healthy volunteers.[300-301] Since PACAP38 displays a higher affinity for the PAC_1 receptor, activation of this receptor may result in migraine-like headaches and, accordingly, antagonism of the PAC_1 receptor may be a putative target for migraine treatment. On this basis, and in an attempt to extend the knowledge of the mechanisms of these neuropeptides in migraine pathophysiology, the present study set out to investigate in both human meningeal and coronary arteries: (i) the vasorelaxation induced by VPAC/PAC

receptor agonists in the absence or presence of VPAC and PAC receptor antagonists; and (ii) the mRNA expression of VPAC/PAC receptors.

Materials and methods

Isolated human arteries

Human meningeal arteries (7 male, 6 female, age 42-75 years, internal diameter 500-750 μm) were obtained perioperatively from patients undergoing neurosurgical procedures at Erasmus Medical Center, Rotterdam. The right proximal (5 male, 4 female, age 32-63 years, internal diameter 2-3 mm) and distal (16 male, 8 female, age 17-61 years, internal diameter 500-1500 μm) coronary arteries were obtained from heart beating organ donors who died of noncardiac disorders less than 24 hours before the tissue was taken to the laboratory. The hearts were provided by the Rotterdam Heart Valve Bank after donor mediation by Bio Implant Services Foundation / Eurotransplant Foundation (Leiden, The Netherlands) following removal of the aortic and pulmonary valves for homograft valve transplantation (large and small epicardial arteries). Medical histories of the patients or donors were not available due to ethical restrictions. Blood vessels were placed in Krebs bicarbonate buffer solution for meningeal arteries (composition in mM: NaCl 119, KCl 4.7, $CaCl_2$ 1.25, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11.1; pH 7.4) or for coronary arteries (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 8.3; pH 7.4) aerated with 5% CO_2 in O_2 (carbogen) and transported to the laboratory for investigation.

Functional experiments

The arteries were cut in small segments of about 2-4 mm each and suspended on stainless steel hooks in 15-ml organ baths (proximal coronary segments) or Mulvany myographs (meningeal and distal coronary segments) containing oxygenated Krebs bicarbonate solution at 37°C. After equilibration for at least 30 min, with change of solution twice at 15-min intervals, changes in tension were recorded with a Harvard isometric transducer. The vascular segments were stretched to a stable tension of about 15 mN for the proximal segments (the optimal tension as determined in previous studies), or to a tension normalized to 90% of I_{100} for the distal segments the diameter when transmural pressure equals 100 mm Hg.[399] The segments were exposed to 30 mM KCl once (distal segments) or twice (proximal segments). After washout, the tissue was exposed to 100 mM KCl to determine the maximum contractile response to KCl.

The relaxant effect of human PACAP38, PACAP27 (a C-truncated 27 amino acid version of PACAP38), VIP and the $VPAC_1$ receptor agonist, ([Lys15,Arg16,Leu27] -VIP(1-7)-GRF(8-

27)), were examined by cumulative application of increasing concentrations of the peptide in the absence or presence of the PAC_1 receptor antagonist, PACAP6-38 or the $VPAC_1$ receptor antagonist, PG97269. Each segment was precontracted with 30 mM KCl before the agonists were added. Segments were exposed to a single cumulative concentration-response curve and a matched pair's protocol was used, where one segment acted as control (no antagonist present) while in other segment response to the agonist were measured in presence of the antagonist. Agonist responses were assessed in the presence of precontraction induced by 30 mM KCl following equilibration (20-30 min) with 1 μM of the antagonist. After washout the functional integrity of the endothelium was verified by analysing relaxation to substance P (1 nM) after precontraction with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 1 μM) in proximal coronary artery segments, and by relaxation to substance P (10 nM) after precontraction with the thromboxane A_2 analogue U46619 (10 nM) in distal coronary and meningeal artery segments as described previously.[109,405] Although relaxations to substance P were assessed with different concentrations in proximal (1 nM) and distal (10 nM) coronary artery segments, these different concentrations do not account for the relaxation effect, as determined in previous studies from our group.[28]

Compounds

VIP, PACAP38, PACAP27 and PACAP6-38 were obtained from NeoMPS (Strasbourg, France). The $VPAC_1$ receptor agonist ([Lys15,Arg16,Leu27]-VIP(1-7)-GRF(8-27)) and the $VPAC_1$ receptor antagonist (PG97-269) were obtained from Phoenix Europe (GmbH, Karlsruhe, Germany). All test substances were dissolved in distilled water and stored at -20ºC. Prior to using, the samples were diluted in isotonic saline to the desired concentration, which is thousand-fold higher than the final concentration in the organ bath.

Data presentation and statistical evaluation

The vasodilator responses to the agonists were expressed relative to the precontraction evoked by KCl (=100%). For each segment the maximum vasodilator effect (E_{max}) was calculated. The concentration response curves for all agonists were analysed using nonlinear regression analysis and the potency of agonists was expressed as pEC_{50} (i.e. negative logarithm of the molar concentration of agonist inducing half the maximum response) using Graph Pad Prism 4.0 (Graph Pad Software Inc., San Diego, CA, U.S.A.). The blocking potency of the antagonists was estimated by calculating EC_{50} ratios and plotting a Schild plot[400] using linear regression to get the slope value. Since only one concentration of antagonist was studied, the "apparent pK_B" values were calculated, constraining the Schild slope to unity. Data are expressed as mean±SEM. and 'n' refers to the number of patients from whom the vessels were collected. Statistical testing was performed using unpaired Student's t-test, unless indicated otherwise. P values of 0.05 or less were assumed to denote significant changes.

mRNA expression studies

For the mRNA expression study, human meningeal arteries (2 male, 8 female, age 21-65 years) and distal human coronary arteries (3 male, 6 female, age 31-62 years) were isolated and immediately submerged in a RNA stabilization solution (RNAlater; Ambion, Woodward, Austin, TX, USA) for preservation of the mRNA. The samples were stored at 4°C and the mRNA extraction procedure was carried out within one month after isolation. Arteries were homogenized using a rotor/stator homogenizer and sonification. Total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) with Proteinase K digestion. After isolation, purity and yield of total RNA was assessed by spectrophotometry. Yield was estimated by absorbance at 260 nm and purity of isolated RNA was estimated by the ratio between 260 nm/280 nm absorbance. RNA was stored at -80°C, and converted to cDNA within one week.

Gene	Gene NCBI GenBank Acc. No	Forward primer (3'-5')	Reverse primer (5'-3')	Position on mRNA transcript	Size (base pairs)
β-actin	NM_001101	GCAAAGACCTG TACGCCAACACA	GCTCAGGAGGA GCAATGATCTTGA	953-1087	135
$VPAC_1$	NM_004624	GCGAGTTTGGA TGAGCAGCAGA	TGGAGCTTCCT GAACAGGCTCA	495-622	128
$VPAC_2$	NM_003382	CGGAGGATGAGA GCAAGATCACGT	GCAGCTTCCTGA AGAGGCACAGAA	527-649	123
PAC_1	NM_001118	GATCTCTCAGACA TGGGAGTGGTGA	AATAATCCTGGT CCCCAGTCTCAGA	542-668	127

Table 2: Primers and target sequences used for the mRNA expression studies.

cDNA from 1 µg RNA in each batch was synthesized using Omniscript RT Kit (Qiagen, Hilden, Germany). Negative samples without reverse transcriptase were prepared for each sample to exclude genomic contamination. cDNA from human cerebellum was used as an internal control. Specific exon-/intron-spanning primers were designed using Vector NTI 10 (Invitrogen) from target mRNA sequences retrieved from NCBI Genbank. Primers and target sequences are shown in Table 2. qPCR (quantitative polymerase chain reaction) was performed with LightCycler technology (Roche, Basel, Switzerland) and SYBR Green I dye (SYBR Green JumpStart Taq ReadyMix (Sigma, St. Louis, Missouri, USA)). β-actin was used as a non-regulated reference gene. The approach was relative quantification with calibrator normalization, with nonlinear efficiency correction. The same batch of human cerebellum cDNA was used as calibrator for all experiments. The expression level for each receptor was thus calculated in respect to the expression level in human cerebellum cDNA. Each capillary sample contained 1 µl cDNA sample, 10 µl of the SYBR Green Mix, 300

nM of primer pair, and volume adjusted with sterile water to 20 µl. After 30 seconds of denaturation of each sample at 94°C, the qPCR program was allowed to run for 45 cycles. Each cycle consisted of 0 second at 94°C (denaturation), 7 seconds at 60°C (annealing) and 5 seconds at 72°C (extension). Melting curve analysis was performed automatically after each run. qPCR products were verified by inspection of melting curves for indications of primer-dimer formation or multiple products, and by agarose gel analysis. The band for each receptor was excised from the gel, and the DNA purified using QIAquick qPCR Purification Kit (Qiagen, Hilden, Germany). Each product was sent for sequencing at Eurofins MWG Operon (Ebersberg, Germany), and yielded the expected sequence. For calculating the normalized ratio, the calibrator was set at 10,000 units for all targets.

Results

Vascular responses in human meningeal arteries to PACAP38 and VIP in the absence or presence of PACAP6-38 or PG97269

The contraction induced by 100 mM KCl was 11±2 mN (n=9) and the average endothelium-dependent relaxant response to substance P (10 nM) was 64±5% of the precontraction induced by 10 nM U46619. PACAP38 and VIP both induced relaxation in human meningeal arteries with an E_{max} of 34±12% of precontraction with 30 mM KCl (n=7, Figure 1; left panel) and 40±10% (n=6, Figure 1; right panel) respectively. The pEC_{50} values of PACAP was significantly less (<6.9±0.1) than that of VIP (7.4±0.2). The concentration response curves to PACAP38 and VIP in the presence of PACAP6-38 or PG97269 did not differ from the control responses (Figure 1).

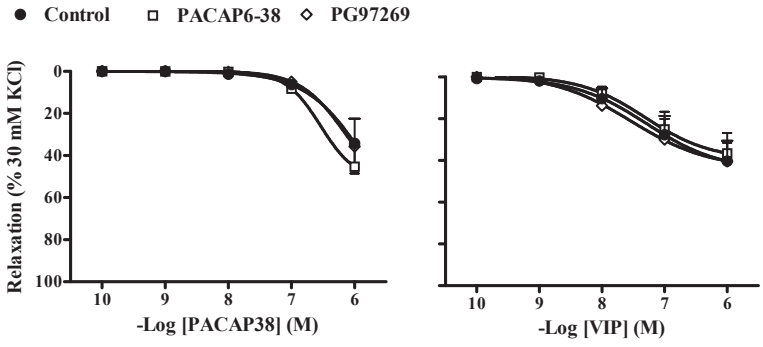

Figure 1: Relaxant effect of PACAP38 (left) and VIP (right) on human meningeal arteries that were precontracted with 30 mM KCl in the absence or presence of 1 µM of PACAP6-38 or PG97269. Values given represent mean±SEM, number of subjects n=6.

Vascular responses in human coronary arteries to PACAP38, PACAP27, VIP and the $VPAC_1$ receptor agonist in the absence or presence of PACAP6-38 or PG97269

In *proximal* coronary segments, the contraction induced by 100 mM KCl was 47±6 mN and the average endothelium-dependent relaxant response to substance P (1 nM) was 14±6% of the precontraction induced by 1 µM $PGF_{2\alpha}$ (n=4). VIP induced a relaxation with an E_{max} value of 17±6% of precontraction with 30 mM KCl (n=7) in the proximal coronary arteries (data not shown), whereas the other agonists used in this study had no effect.

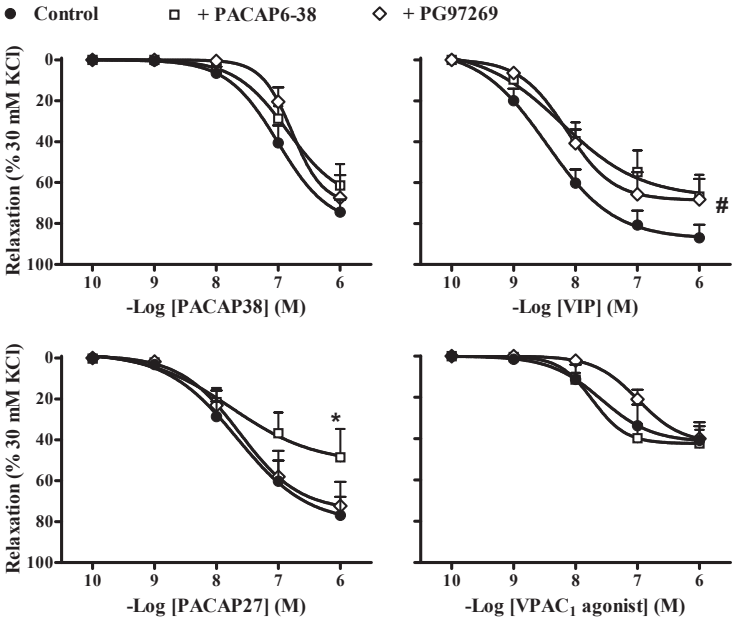

Figure 2: Relaxant effect of PACAP38 (upper left), PACAP27 (lower left), VIP (upper right) and VPAC agonist (lower right) on human distal coronary arteries that were precontracted with 30 mM KCl in the absence or presence of 1 µM of PACAP6-38 or PG97269. * PACAP6-38 reduced the E_{max} of the relaxation to PACAP27 (p=0.05, lower left). # PG97269 significantly reduced the potency of the relaxation to VIP (p=0.05, upper right). Values given represent mean±SEM, number of subjects n=6.

In *distal* coronary segments the contraction induced by 100 mM KCl was 11±1 mN (n=14) and the average endothelium-dependent relaxant response to substance P (10 nM) was 78±3% of the precontraction induced by 10 nM U46619. PACAP38 induced a relaxation with an E_{max} of 75±6% of precontraction with 30 mM KCl (n=5, Figure 2; left upper panel), which was similar to that of PACAP27 and VIP (E_{max}: 77±9% and 87±7%, respectively n=6, Figure 2; left lower and right upper panel). In contrast, the relaxation to the $VPAC_1$ receptor agonist (41±9%, n=6) was significantly lower than that of the other agonists tested (Figure 2; right lower panel). The pEC_{50} value of VIP (8.4±0.1, n=6) was significantly higher than that of PACAP38, PACAP27 and the $VPAC_1$ receptor agonist (7.1±0.2, 7.7±0.2 and

7.5±0.2, respectively, n=4-6).

The PAC_1 receptor antagonist PACAP6-38, but not the $VPAC_1$ receptor antagonist PG97269, significantly reduced the E_{max} of the relaxation to PACAP27 (Figure 2: left lower panel). In contrast, PG97269, but not PACAP6-38, produced a significant reduction of the potency of the concentration response curve to VIP (apparent pK_B value of 5.9±0.1; Figure 2; right upper panel). In addition, the relaxation induced by the $VPAC_1$ receptor agonist seemed also to be blocked by PG97269; however, this was not significant (p=0.50, Figure 2; right lower panel). Both antagonists did not affect the relaxation induced by PACAP38 (Figure 2; left upper panel) and did not affect the vascular tone *per se* (data not shown).

Detection of $VPAC_1$, $VPAC_2$ and PAC_1 receptor mRNA in human meningeal and coronary arteries

qPCR products were verified by agarose gel analysis, which showed that each qPCR product yielded only one band (Figure 3). The relative mRNA expression of the $VPAC_1$, $VPAC_2$ and PAC_1 receptor in human meningeal and coronary arteries was analysed with qPCR, with cerebellum as a reference tissue (Figure 4). The overall expression profile of the three receptors is similar in the two arteries, with the $VPAC_1$ receptor being the only one present in significantly higher quantities in human meningeal arteries (Figure 4; left panel). There seems to be a tendency of higher mRNA expression of the $VPAC_2$ and PAC_1 receptor in the human meningeal artery compared to human coronary artery, but due to high individual variability, the difference did not reach statistical significance (Figure 4; middle and right panel). The mRNA expression of $VPAC_1$ receptor in the human meningeal artery was comparable with the cerebellum, while in the human coronary artery, $VPAC_1$ receptor mRNA expression was much lower than the cerebellum (Figure 4; left panel). Similarly, The PAC_1 receptor mRNA expressions of both arteries are also lower than that of the cerebellum (Figure 4; right panel). In contrast, mRNA expression of $VPAC_2$ receptor was higher in the analysed arteries than in the cerebellum (Figure 4; middle panel).

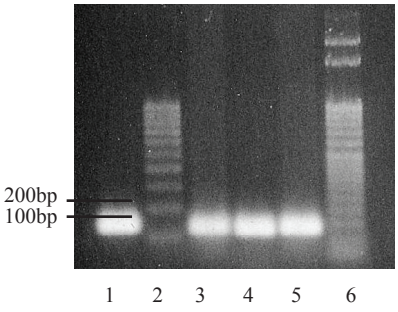

Figure 3: qPCR products verified by agarose gel analysis, 1: β-actin, 2: 100BP ladder, 3: PAC_1, 4: $VPAC_2$, 5: $VPAC_1$, 6: 50 BP ladder.

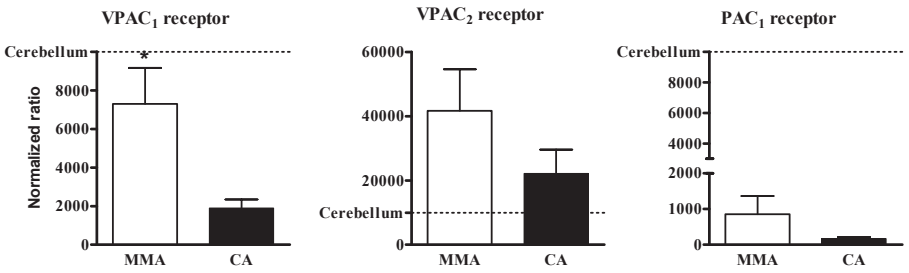

Figure 4: mRNA expression of $VPAC_1$ (left panel) $VPAC_2$ (middle panel) and PAC_1 receptor (right panel) in human meningeal arteries (MMA, n=10) and human coronary arteries (CA, n=9) expressed as normalized ratios. Values given represent mean±SEM * p=0.05.

Discussion

Several lines of evidence have recently suggested the potential role of PACAPs and VIP in the pathophysiology of migraine.[280-282] It is to be highlighted that PACAP27 and PACAP38 interact with $VPAC_1$, $VPAC_2$ and PAC_1 receptors, whereas VIP and the $VPAC_1$ receptor agonist do not interact with PAC_1 receptors. Since PACAP38, but not VIP, induces migraine-like headaches in migraine patients,[281,301] and only PACAPs interact with the PAC_1 receptor, it could be suggested that the PAC_1 receptor is involved in migraine pathophysiology. In an attempt to shed further light on this hypothesis, the present study investigated in human meningeal arteries for the first time (i) the vasodilator responses to VPAC/PAC receptor agonists in the absence or presence of VPAC and PAC receptor antagonists; and (ii) the mRNA expression of VPAC/PAC receptors. Although it has been suggested that cranial blood vessel dilatation during migraine is just an epiphenomenon[3,26,437] there is also ample evidence for a prominent role of cranial blood vessels in the pathophysiology of migraine.[144,328,438] Futhermore, meningeal arteries are of special interest in migraine research as sensitization of dural afferent nociceptors has been proposed to be a pain mechanism during migraine attacks.[439]

In the current study, similar experiments were carried out in human coronary arteries, which allowed us to investigate the potential cardiac side effects of antagonists blocking the VPAC/PAC receptors, as investigated for other antimigraine drugs.[109]

Apart from the implications discussed below, our study shows that all VPAC/PAC receptor agonists induced vasorelaxation with different efficacy and potency in the human meningeal and coronary arteries. In the proximal coronary artery, only VIP was able to induce a small dilatation of the artery, which is in accordance with a previous study in human epicardial coronary artery,[440] while in the distal artery, all the tested agonists induced vasodilatation. Such a difference between proximal and distal coronary artery in VIP-, but

not PACAP-induced vasodilatation has also been observed in the rabbit coronary artery.[297] Moreover, in this study, PACAP38 and VIP both induced vasodilatation with a lower potency and efficacy in meningeal arteries than in coronary arteries. Possible explanations for these regional differences in vascular responses are tissue-dependent factors such as the levels of receptor protein expression and coupling efficiency of the receptors.[283] For example, the potency of PACAP and VIP in meningeal artery is considerably lower than that described earlier in human lenticulostriate and posterior cerebral artery.[295] Different vasodilator responses to PACAP and VIP between several tissues were also described in the rabbit posterior cerebral artery and coronary artery,[297] as well as in the rat basilar artery and middle cerebral artery.[298] Since the $VPAC_1$ receptor has been suggested to be primarily responsible for vasodilatation,[441-442] a higher $VPAC_1$ receptor protein expression would be expected in the human coronary artery. This seems to be in contrast with our mRNA expression data, showing a higher mRNA expression of the $VPAC_1$ receptor in the human meningeal than in the coronary artery. However, it should be kept in mind that mRNA expression of a given receptor does not necessarily correlate with its receptor protein expression. Unfortunately, the antibodies we tried were not selective in our hands; therefore we were not able to study protein expression of the receptors involved.

VIP was more potent than PACAP38 in both blood vessels. This observation points to $VPAC_1$ and $VPAC_2$ receptor activation as being primarily responsible for vasodilatation in these arteries, a finding consistent with the low mRNA expression level of the PAC_1 receptor. Moreover, lower potency of PACAP on vasodilatation was also found in the rabbit posterior cerebral artery[297] and the rat basilar artery,[298] while this difference was not seen in the rat middle meningeal artery.[298] This supports the regional differences in vascular responses induced by PACAP and VIP, may probably be assigned to species differences and/or expression of the receptors in vasculature.

In the antagonists experiments, the $VPAC_1$ receptor antagonist PG97269 and the PAC_1 receptor antagonist PACAP6-38 did not affect the relaxation to PACAP38 and VIP in human meningeal arteries; this may suggest that such responses are not primarily mediated by $VPAC_1$ and PAC_1, but rather via $VPAC_2$ receptors, as is the case in pig urinary bladder neck.[443] However, the $VPAC_2$ receptor antagonist, PG99465, did not affect vasorelaxation to VIP on the dural artery of rat in an intravital model[441] and to both peptides in cranial arteries *in vitro*,[298] which may suggest limited vascular involvement of the $VPAC_2$ receptor in cranial arteries. Moreover, PG99465 has also been described as a $VPAC_1$ receptor agonist,[284] which limits the use to investigate the effect of the $VPAC_2$ receptor. Another theoretical possibility could be that if two receptors (that is, VPAC and PAC) are involved in the vasorelaxant responses and only one receptor was blocked in each experiment, then activation of the other (unblocked) receptor would overshadow the blockade produced by one antagonist. Receptors will not be very effective in affecting vasorelaxation of the meningeal arteries in migraine.

In the human coronary artery, PG97269 blocked the relaxation to VIP and slightly attenuated the relaxation to the $VPAC_1$ receptor agonist. This suggests that the vasodilatation response is partly mediated via the $VPAC_1$ receptor. The PAC_1 receptor antagonist PACAP6-38 did not affect the relaxation to PACAP38, VIP or the $VPAC_1$ receptor agonist, but it reduced the maximal response by PACAP27 at a concentration of 1 μM. Reduction of maximal response suggests that PACAP6-38 might act as a non-competitive or irreversible antagonist on the PAC1 receptor. However, this is unlikely since PACAP6-38 did not reduce the maximal response induced by PACAP38. This discrepancy might be explained by the fact that that vasodilatation induced by PACAP27 and PACAP38 is probably not via the same VPAC/PAC receptor. It has been shown that N-terminus of PACAP has a higher affinity for the $VPAC_2$ receptor than the $VPAC_1$ receptor,[444] while the C-terminus of PACAP has a higher affinity for the $VPAC_1$ receptor than the $VPAC_2$ receptor.[445] This suggests that PACAP38 generally have a preference to act on the VPAC2 receptor and PACAP27 on the $VPAC_1$ receptor. Since the vasodilatation induced by PACAP27 is blocked by PACAP6-38 and not by PG97269, might suggest that vasodilatation induced by PACAP27 in human coronary arteries is mediated mainly via the PAC_1 receptor. In addition, PACAP38 induced vasodilatation is not blocked by PACAP6-38 and not by PG97269, suggesting that the vasodilatation effect of PACAP38 in human coronary artery is most likely mediated via the $VPAC_2$ receptor. More selective and potent agonists and antagonists would be helpful in unequivocal characterization of these receptors.

Based on the above discussion, it can be suggested that vasodilatation induced by PACAP and VIP differ not only in species but also in region of the arteries from the same species. This is probably due to the selective activation of the three type of the VPAC/PAC receptor, which might be tissue dependent. Moreover, several splice variants of the PAC_1 receptor, which are to a certain extent tissue specific, have been described to affect cellular function by altering receptor pharmacology and signaling.[283] This supports the evidence that the effect of PACAP and VIP differ in tissues. It is unlikely that differences between the $VPAC_1$, $VPAC_2$ and PAC_1 receptors between species do account for the different responses, since these receptors are quite well conserved. The homology in amino acid identity between rat and human receptors is: 83.9%, 84.9% and 86.7% for $VPAC_1$, $VPAC_2$ and PAC_1 receptors, respectively, with consensus position figures being 89.5% for $VPAC_1$, 89.3% for $VPAC_2$ and 90.5% for PAC_1. Such homology is considered as highly identical sequences, major differences in functionality would be unlikely.

As previously described, PACAPs and VIP are less potent in human meningeal arteries as compared to CGRP (pEC_{50}=8.7±0.1).[163] This low potency of both PACAP and VIP seems not to be in line with the observation that PACAP38, but not VIP, induces migraine-like headaches in migraine patients.[281,301] Furthermore, following PACAP38 infusion, migraine-like attacks occur several hours after the peak vasodilatation, and therefore migraine

development does not seem to be well correlated with dilatation of cephalic vessels. Thus, this suggests that the vasodilator properties of the PACAPs, VIP and their receptors' vascular action may be less relevant in migraine. And that the migraine-like headache induced by PACAP38 may involve other types of modulation in the perivascular space, rather than a vascular mechanism, as also previously suggested.[281,437] It has been suggested that migraine-like headache induced by PACAP38 involves sensitization of peripheral sensory trigeminal fibers, since throbbing headache, which had been related to sensitization, has been reported after PACAP38 infusion.[281] Moreover, activation of peripheral sensory trigeminal fibers by mast cell degranulation and direct sensitization of central and second order trigeminal neurons have been discussed as a possible mechanism for the migraine-like headache induced by PACAP38.[281] It is noteworthy that the mRNA expression levels of the $VPAC_2$ receptor are higher whereas those of the PAC_1 receptor are much lower in the analysed arteries in comparison to cerebellum, used as a calibrator. This may indicate that PAC_1 receptors are primarily expressed in neuronal tissues, while both VPAC receptors are of greater importance in blood vessels.

Nevertheless, it is still possible that PACAP would indirectly induce vascular effects mediated via activation of perivascular nerves, since PACAP is found in these nerves.[282,286] Consistent with these views, the PAC_1 receptor antagonist PACAP6-38 but not the VPAC receptor antagonists blocked the vasodilatation induced by neurogenic dural stimulation.[302] Moreover, neuronal firing in the trigeminal cervical complex after salivatory nucleus stimulation is also blocked by PACAP6-38, which points to a peripheral effect.[302] Taken together, it may be suggested that the PACAP- and VIP-induced vasodilatation seen in healthy volunteers and migraine patient[281,300-301] is probably mediated by perivascular nerve activation, since direct stimulation with these peptides in human isolated meningeal artery segments induced only minor relaxations. Moreover, PACAP38-induced migraine-like headache is less likely to involve cranial vasodilatation, since only PACAP38, and not VIP, induces migraine-like headache in migraine patients, while both peptides induced cranial vasodilatation.[437] Thus, the PAC_1 receptor may play a role in activating central mechanisms involved in migraine. Interestingly, PACAP38 infusion in humans causes prolonged dilatation of superficial temporal artery and all the subjects experienced flushing, especially on the face and trunk, which lasted up to 24 hours. Therefore, it cannot be totally ruled out that PACAP38 activates other processes, including vascular mechanisms.

In conclusion, since the potency of the PACAPs and VIP in human meningeal arteries is lower than that of CGRP, the vasodilator properties of the PACAPs and VIP (and their vascular receptors), may be less relevant in migraine. In addition, the migraine-like headaches induced by PACAP38 may not involve meningeal vasodilatation, but may involve central effects of the PAC_1 receptor. This study extends the knowledge of PACAP and VIP as well as their receptors in the vascular component of migraine pathogenesis.

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讀萬卷書不如

劉彝

Traveling thousands of miles is better than reading thousands of books (Liu Yi)

Part D: Cardiovascular side effects of 5-HT receptor ligands

Chapter 9
Cranioselectivity of sumatriptan revisited

K.Y. Chan, S. Labruijere, R. de Vries, A.H.J. Danser, C.M. Villalón, and A. MaassenVanDenBrink, (In progress).

Abstract

Almost two decades ago sumatriptan was introduced, providing a great step forward in the treatment of migraine. During the following years, second generation triptan derivates were synthesized to improve the oral bioavailability. Since concerns existed about the coronary side-effect potential of the triptans, we previously performed detailed studies on the cranioselectivity of the triptans. We concluded, based on the modest contraction that these drugs caused in the human coronary artery (HCA), that triptans are generally safe, but should be contraindicated in patients with coronary artery disease. All of our studies (and those of others) were performed on proximal segments of the HCA, although myocardial ischemia may originate from both large and small HCAs. Thus, we have now investigated whether the contractions to sumatriptan are different in the proximal or distal parts of the HCA. Concentration response curves to 5-hydroxytriptamine (5-HT) and sumatriptan were constructed in proximal (Ø 2-3 mm), distal (Ø 1,000-1,500 μm) and small (Ø 500-1,000 μm) HCAs (2 M, 2 F; age 37-62 years). Whereas sumatriptan induced significantly smaller contractions than 5-HT in proximal HCA, contractions to sumatriptan in distal and small HCA were significantly larger, and equalled those to 5-HT. Surprisingly, the contractions induced by sumatriptan in distal and small HCA were significantly higher than in proximal HCA and these contractions resembled those in the human meningeal artery (as previously reported by our group). The potency of sumatriptan in coronary artery segments of different diameters was similar. Thus, the above data obtained in distal HCA suggest that triptans have a limited cranioselectivity. However, in the last years triptans have been used extensively, and myocardial ischemia is an extremely rare event. Therefore, our findings reconfirm that the triptans should remain contraindicated in patients with coronary artery disease, although the risk profile definitely favours their use in the absence of contraindications.

Introduction

Sumatriptan, a tryptamine derivative with selective agonist activity at 5-$HT_{1B/1D}$ receptors, was introduced two decades ago[446] and has provided a great step forward in the treatment of migraine.[122] In preclinical studies, this drug has been shown to induce constriction of cranial arteries, including the canine external carotid bed and porcine carotid arteriovenous anastomoses.[212,447] Moreover, especially when parenterally administered, sumatriptan is effective and well-tolerated in most patients.[124,446] Thus, by making such a drug that selectively activates 5-$HT_{1B/1D}$ receptors, without activity at most of the other 5-HT receptor types, the vast array of unwanted effects seen with exogenous 5-HT (e.g. platelet aggregation, bronchoconstriction, generalized vasoconstriction and various gastrointestinal effects) can be avoided. Indeed, sumatriptan has clearly proven to be an effective and well-tolerated medicine when used properly.[122]

However, the presence of 5-HT_{1B} receptors on coronary arteries raised concerns about the potential coronary side effects of sumatriptan. Indeed, up to 15% of patients consistently report chest symptoms, including chest pressure, tightness and pain, often mimicking pectoral angina.[44,125,448] On the basis of these symptoms, our group carried out extensive preclinical studies using sumatriptan and other triptans to corroborate their cranioselectivity and any cardiovascular risk potential.[52,109] The results showed that compared to the human meningeal arteries where the triptans will, at least partly, exert their therapeutic effect, the contraction induced by these agents in the human coronary artery at therapeutic concentrations is rather limited. However, it must be emphasized that: (i) all of these studies were performed on proximal segments of human coronary arteries (due to limited experimental options); and (ii) myocardial ischemia may originate from both large and small human coronary arteries.[449-450]

Thus, parallel studies using proximal and distal segments of the coronary artery would provide valuable information on the effects of any drug. Interestingly, our group has recently reported that calcitonin gene-related peptide (CGRP) is capable of inducing differential effects in the proximal and distal segments of the human coronary artery, as well as in coronary arterioles.[165] In this respect, CGRP was more potent to induce vasorelaxation in distal than in proximal coronary artery segments or coronary arterioles.[165] Considering the above findings, the present study was designed to investigate whether the contractions to sumatriptan are different in the proximal and distal parts of the human coronary artery.

Materials and methods

Tissue preparation

The right coronary artery was obtained from 4 heart-beating organ donors; all donors died of noncardiac disorders less than 24 hours before the tissue was taken to the laboratory (2 male, 2 female; age 31-62 years). The hearts were provided by the Rotterdam Heart Valve Bank after donor mediation by Bio Implant Services Foundation / Eurotransplant Foundation (Leiden, The Netherlands) following removal of the aortic and pulmonary valves for homograft valve transplantation. The hearts were stored at 0 to 4°C in a sterile organ protecting solution (UW, EuroCollins or HTK-Bretschneider) immediately following circulatory arrest. Proximal (internal diameter: 2-3 mm), distal (internal diameter 1,000-1,500 μm) and small (internal diameter 500-1,000 μm) portions of the right coronary artery were dissected, placed in oxygenated (95% O_2 and 5% CO_2) Krebs bicarbonate solution (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 8.3; pH 7.4) and stored overnight at 4°C. No attempt was made to denude the endothelium of the artery segments. The ethics committee dealing with human experimentations at the Erasmus Medical Center Rotterdam approved the study protocol.

Isometric tension measurements

The artery was cut in small segments of about 2-4 mm each and suspended on stainless steel hooks in 15-ml organ baths (proximal segments) or Mulvany myographs (distal segments and small segments) containing oxygenated Krebs bicarbonate solution at 37°C. After equilibration for at least 30 min, with change of solution twice at 15-min intervals, changes in tension were recorded with a Harvard isometric transducer. The blood vessel segments were subsequently stretched to: (i) a stable tension of about 15 mN for the proximal segments; or (ii) a tension normalized to 90% of l_{100} for the distal segments the diameter when transmural pressure equals 100 mm Hg.[399] Then, the vessels were exposed to 30 mM K^+ once (distal segments) or twice (proximal segments). After washout, the tissue was exposed to 100 mM K^+ to determine the maximum contractile response to K^+. At the end of the experiments, the functional integrity of the endothelium was verified by observing the relaxation to substance P (1 nM) after precontraction with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 1 μM) in proximal coronary artery segments, and by relaxation to substance P (10 nM) after precontraction with the thromboxane A_2 analogue U46619 (10 nM) in distal segments.

Determination of agonist and antagonist potency

After stabilization, concentration response curves to 5-hydroxytriptamine (5-HT) and sumatriptan were constructed paired parallel design,[109] in logarithmic steps from 1 nM up to 100 μM. Contractile responses were expressed as percentage of the contraction induced by 100 mM KCl.

Analysis of data

Curves that cover the full sigmoidal range were analyzed by means of a computerized curve fitting technique to obtain pEC_{50} -log of molar concentration of an agonist needed to reach half of the maximal effect, i.e. -logEC_{50},[451] and E_{max} (maximal response) values. E_{max} values are expressed as percentage of contraction to 100 mM K^+ in the respective blood vessel segment. All data are presented as mean±SEM. Statistical testing was performed using unpaired Student's t-test, unless indicated otherwise. At every concentration, ANOVA was conducted between all groups, followed by posthoc Bonferonni's multiple comparisons test. P values of 0.05 or less were assumed to denote significant changes.

Compounds

5-Hydroxytryptamine, sumatriptan succinate, prostaglandin $F_{2\alpha}$ (tris salt), U46619 (9,11-dideoxy-11α,9α-epoxy-methano-prostagladin $F_{2\alpha}$) and substance P acetate were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Results

Basic properties

In *proximal* coronary artery segments, the contraction to 100 mM K^+ was 43±7 mN; the average endothelium-dependent relaxation to substance P (1 nM) was 34±10% of the precontraction to 1 μM $PGF_{2\alpha}$. The contraction to 100 mM K^+ was in distal coronary artery segments 25±5 mN and in small coronary artery segments 15±3 mN (n=4). The average relaxation to substance P (10 nM) for these arteries was 58±17% and 88±11%, respectively, of the precontraction to 10 nM U46619.

Contractile responses to 5-HT and sumatriptan

In *proximal* coronary segments, 5-HT induced concentration-dependent contractions which were larger than those induced by sumatriptan at the highest concentration (E_{max} 48±8% vs. 7±5%, n=3-4, P<0.05, Figure 1), whereas the potency did not differ between 5-HT and sumatriptan (pEC_{50} 6.61±0.22 and 5.92±0.50, respectively). In contrast, in the *distal* and *small* coronary segments, the contractions induced by 5-HT (E_{max}=67±29%, pEC_{50}=7.04±0.30

and E_{max}=53±17%, pEC_{50}=7.30±0.33, respectively, Figure 1 left panel) and sumatriptan (E_{max}=54±43%, pEC_{50}=6.75±0.25 and E_{max}=57±24%, pEC_{50}=6.98±0.04, respectively, Figure 1; right panel) were similar.

Importantly, the E_{max} for sumatriptan in *distal* and *small* coronary artery segments was significantly higher than that in *proximal* human coronary artery segments. This was not the case for 5-HT.

Predicted contraction at therapeutic plasma concentrations

For each diameter of the coronary arteries, contractions at the maximal free (i.e., unbound to plasma proteins) therapeutic plasma concentrations (free C_{max}: 112-157 nM[56] attained with the therapeutic doses of sumatriptan (100 mg) were predicted from the concentration response curve. The predicted contraction at free C_{max} (range) tended to be higher in distal (35±32%) and small (34±13%) coronary artery than in proximal coronary artery (1.4±1.3%), but the difference was not statistically significant (Figure 2).

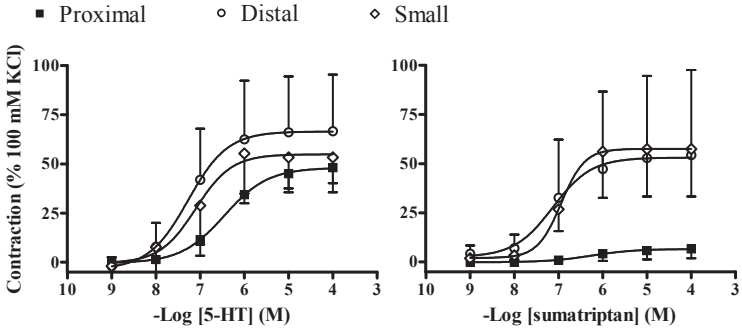

Figure 1: Contractile responses to 5-HT (left panel) and sumatriptan (right panel) (expressed as % of the response to 100 mM K^+, n=4) in the human isolated proximal, distal and small coronary arteries. Data are expressed in mean±SEM.

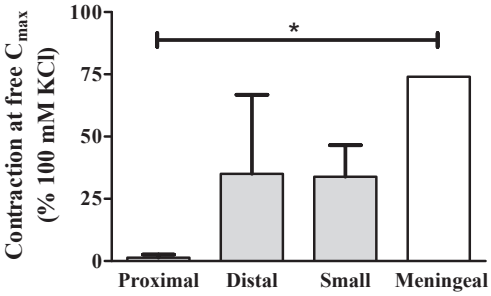

Figure 2: Predicted contraction at free C_{max} of sumatriptan (100 mg) expressed as % of the response to 100 mM K^+, n=3 in human proximal, distal and small coronary arteries (this study) as well as in human meningeal arteries. Data from MaassenVanDenBrink *et al.*.[52]

Discussion

The present study basically set out to investigate whether the contractions to sumatriptan are different in proximal and distal parts of the human coronary artery. Apart from the implications discussed below, our results clearly show that: (i) the contraction to sumatriptan is more pronounced in distal and small segments than in proximal segments of the human coronary artery; and (ii) there were no significant differences in the contractile responses to 5-HT in the distal, small and proximal segments of the human coronary artery. The difference between responses in proximal and distal coronary artery segments could be explained by differences in 5-HT_{1B} receptor expression in the coronary arteries of different diameter. To further investigate this hypothesis, the receptor expression should be analyzed at the mRNA and/or protein level. Alternatively, the different responses to sumatriptan may also be caused by different receptor coupling efficiency between the proximal and the distal coronary arteries. Thus, extensive investigations on sumatriptan- and 5-HT-induced signal transduction pathways at the various locations are also required.

Surprisingly, the contractions to sumatriptan in the distal and small human coronary arteries (present results) resemble those in the human meningeal artery.[52] This finding suggests that at therapeutic concentrations (as used in our study), sumatriptan does not only induce contraction in human meningeal artery, but also induces contraction in human distal coronary arteries. Indeed, at the maximal free plasma concentration of therapeutic concentration of sumatriptan (100 mg), contraction was induced in distal and small coronary arteries. Therefore, based on these data, the triptans would be considered to have limited cranioselectivity. However, the two decades of clinical experience with the triptans have demonstrated that the use of these agents, taking the contraindications into account, is very safe. Indeed, in cardiovascularly healthy subjects, coronary artery flow is not reduced up to a decrease in diameter of about 80%.[452] It is now clear that myocardial ischemia is an extremely rare event after the use of these drugs. Moreover, it has recently been reported that the number of adverse events was not increased when patients were both treated with cardiovascularly active drugs and triptans.[132] Due to its safety profile over many years, sumatriptan is now available without prescription in Britain.[122] However, our earlier observations in the proximal human coronary artery,[109] in conjunction with our current findings in the distal human coronary artery, clearly indicate that the triptans should remain contraindicated in patients with cardiovascular diseases.

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What you do not wish yourself, do not extend it to others
(Confucius)

Chapter 10

Functional characterization of contractions to tegaserod in human isolated proximal and distal coronary arteries

K.Y. Chan, R. de Vries, F.P.J. Leijten, H. Pfannkuche, A.J. van den Bogaerdt, A.H.J. Danser, A. MaassenVanDenBrink (2009), European Journal of Pharmacology, 619; 61–67.

Abstract

Tegaserod, a 5-HT_4 receptor agonist, has been used to treat idiopathic constipation and constipation-predominant irritable bowel disease. It has recently been suggested that tegaserod has an affinity for 5-HT_{1B} receptors, which mediate vasoconstriction. As some patients have experienced cardiac ischemia during treatment with tegaserod, we assessed contractions to tegaserod in healthy and diseased human isolated coronary arteries and compared the results with those obtained using sumatriptan, an established 5-HT_{1B} receptor agonist.

Proximal and distal human coronary arteries were divided into sets of healthy and diseased tissues based on functional endothelial responses. Concentration-response curves to tegaserod and sumatriptan were constructed to assess their contractile potential. Tegaserod's antagonist properties at 5-HT_{1B} receptors were studied by constructing concentration-response curves to sumatriptan in the absence or presence of tegaserod (1 μM).

Sumatriptan induced concentration-dependent contractions, which were greater in distal than in proximal coronary artery segments. In the proximal segments, tegaserod induced contractions only at concentrations of 10 μM or higher, while in distal segments contractions were generally absent. Tegaserod did not antagonize sumatriptan-induced contractions. There was no difference between the results obtained in healthy and diseased coronary arteries.

In conclusion, tegaserod induced contractions in human proximal coronary arteries at concentrations 1,000 times higher than C_{max} (6 mg bid). Hence, tegaserod does not exhibit a relevant vasoconstrictor potential in the human coronary artery. Further, tegaserod did not behave as an antagonist at 5-HT_{1B} receptors. Additional studies may be warranted to investigate the use of 5-HT_4 agonists in patients with cardiovascular risk factors.

Introduction

Serotonin (5-hydroxytryptamine, 5-HT), a monoamine neurotransmitter, exerts its multiplicity of physiological effects through seven different types of 5-HT receptors 5-HT_1 to 5-HT_7.[453] Some of these seven 5-HT receptors have been divided further into subtypes. Except for the 5-HT_3 receptor type, all 5-HT receptors are G-protein-coupled receptors. 5-HT receptors play an important role in the central nervous system, the cardiovascular system and the gastrointestinal tract.[453] While the largest serotonin stores in the body are found in the enterochromaffin cells of the gastrointestinal mucosa,[454] it is also synthesized and stored in central and peripheral serotonergic neurons.

The 5-HT receptors that are present in the gastrointestinal tract are 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4 and 5-HT_7 receptors. With regard to gut function, the 5-HT_3 and 5-HT_4 receptors have been studied most extensively.[455] They are also drug targets in the treatment of gastrointestinal disorders. One of these drugs is tegaserod, a partial agonist at 5-HT_4 receptors with stimulating effects on gastrointestinal motility and visceral antinociceptive activity. It has been used in the treatment of constipation-predominant irritable bowel syndrome (IBS-C), which is characterized by abdominal discomfort or pain in association with altered bowel habit.[456-457]

Recently, studies have shown that tegaserod has binding affinity for human 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors pKi values of 7.5, 8.4 and 7.0, respectively,[458] as well as for human 5-HT_{1B} receptors pKi 7.2.[459] Both 5-HT_{1B} and 5-HT_{2A} receptors are found on arterial smooth muscle cells, where they mediate vasoconstriction.[453,460-461] Indeed, 5-HT_{1B} agonists constrict human coronary arteries, both *in vitro*[109,462] and *in vivo*.[463] In addition, 5-HT_{2B} receptors may mediate vasodilatation,[212] while the 5-HT_{2C} receptor is not expressed in the vascular system, but only in the brain.[453] In view of the affinity of tegaserod for 5-HT_{1B} and 5-HT_{2A} receptors, tegaserod might contract human coronary arteries, thereby increasing the risk of cardiovascular ischemia, especially in patients with coronary artery disease. Indeed, cases of cardiac ischemia have been reported during treatment with the drug.[464-465] Results of pooled safety analyses of 29 placebo-controlled clinical studies (11,614 patients treated with tegaserod and 7,031 treated with placebo), revealed a small but statistically significant imbalance in the number of patients having a cardiovascular ischemic event (myocardial infarction, stroke, unstable angina) during the clinical trials (13 tegaserod versus 1 placebo, p=0.024) (Novartis Pharmaceuticals Corporation. Press Release; March 30, 2007). Following a review of these data with health authorities, the marketing and sales of tegaserod (Zelnorm®/ Zelmac®) were suspended in the USA, and at least 20 other countries. To address the vasoconstrictor potential of tegaserod, we characterized its effect in human coronary arteries, using both healthy and diseased vessels. We compared the effects of tegaserod with those of the 5-$HT_{1B/1D}$ receptor agonist sumatriptan, a known coronary vasoconstrictor.[109] Further, we investigated the presence of 5-HT_4 receptor mRNA in the human coronary artery.

Methods and Materials

Tissue preparation

The right coronary artery was obtained from 20 heart-beating organ donors, including 4 donors with a warm ischemia period less than 20 min. All donors died of noncardiac disorders less than 24 hours before the tissue was taken to the laboratory (10 male, 10 female; age 17-63 years, mean age ± SEM: 44 ± 3 years). The hearts were provided by the Rotterdam Heart Valve Bank after donor mediation by Bio Implant Services Foundation/ Eurotransplant Foundation (Leiden, The Netherlands) following removal of the aortic and pulmonary valves for homograft valve transplantation. The hearts were stored at 0 to 4°C in a sterile organ protecting solution (UW, EuroCollins, or HTK-Bretschneider) immediately following circulatory arrest. Proximal (internal diameter 2-3 mm) and distal (internal diameter 600-1,000 μm) portions of the right coronary artery were dissected, placed in oxygenated (95% O_2 and 5% CO_2) Krebs bicarbonate solution (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 8.3; pH 7.4) and stored overnight at 4°C. No attempt was be made to denude the endothelium of the artery segments. The ethics committee dealing with human experimentations at the Erasmus Medical Center Rotterdam approved the study protocol.

Isometric tension measurements

The artery was cut in small segments of about 2-4 mm each and suspended on stainless steel hooks in 15-ml organ baths (proximal segments) or Mulvany myographs (distal segments) containing oxygenated Krebs bicarbonate solution at 37°C. After equilibration for at least 30 min, with change of solution twice at 15-min intervals, changes in tension were recorded with a Harvard isometric transducer. The vessel segments, stretched to a stable tension of about 15 mN for the proximal segments (the optimal tension as determined in previous studies), or stretched to a tension normalized to 90% of l_{100} for the distal segments (the diameter when transmural pressure equals 100 mm Hg),[399] were exposed to 30 mM K^+ once (distal segments) or twice (proximal segments). Subsequently, the functional integrity of the endothelium was verified by observing relaxation to substance P (1 nM) after precontraction with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 1 μM) in proximal coronary artery segments, and by relaxation to substance P (10 nM) after precontraction with the thromboxane A_2 analogue U46619 (10 nM) in distal segments. Coronary arteries were classified as healthy or diseased according to their functional response to substance P after precontraction with $PFG_{2\alpha}$ or U46619 as described previously;[109,405] the hearts with a relaxant response to substance P above the median value were classified as 'healthy', the hearts with a relaxant response to substance P below the median value were classified as 'diseased'. After washout, the tissue was exposed to 100 mM K^+ to determine the maximum contractile response to K^+.

Determination of agonist and antagonist potency

After stabilization, concentration response curves to tegaserod and sumatriptan (dissolved in either water or the vehicle of tegaserod, DMSO, to correct for the effect of the different solvents of sumatriptan and tegaserod), were constructed paired parallel design,[109] in logarithmic steps from 1 nM up to 100 μM. Contractile responses were expressed as percentage of the contraction induced by 100 mM K^+. In addition, contractions to sumatriptan were studied in the presence of tegaserod (1 μM) or its vehicle, which was administered 30 min before construction of the concentration response curves to sumatriptan. The maximum contraction to sumatriptan obtained in the control concentration response curve (i.e., in the absence of tegaserod) was sometimes extremely low and thus could not be used to quantify a potential rightward shift of this concentration response curve. Since U46619 may augment contractions to sumatriptan in human isolated coronary artery segments where the maximal response is small,[466] a threshold concentration U46619 (1-30 nM; titrated to about 5-10% of contraction to 100 mM K^+, determined in quarter log steps) was administered simultaneously with tegaserod or its vehicle in some experiments. In addition, the possibility of an augmentation of the contractile responses to tegaserod by U46619 was investigated by constructing concentration response curves to tegaserod in the absence or presence of U46619 (1-5 nM) in both proximal and distal coronary artery segments. To assess whether such potentially augmented responses to tegaserod were mediated via the 5-HT_{1B} receptors, the contractile responses to tegaserod in the presence of U46619 were also studied after pre-incubation of the vessels for 30 min with the 5-$HT_{1B/1D}$ receptor antagonist, GR55562 (1 μM), or its vehicle.

Isolation and detection of the 5-HT_4 receptor mRNA from coronary arteries

Tissue samples of the left ventricle, right atrium and distal coronary artery were isolated from two donor hearts (2 F, age 61 and 64 years), snap frozen in liquid nitrogen and stored at -80°C until use. Total RNA was isolated using TRIzol® reagent (Invitrogen Corporation, California, U.S.A) with subsequent chloroform-isopropanol extraction according to the manufacturer's instructions. 0.5 μg/μl random primers (Promega Corporation, Madison, U.S.A.) was added to 1 μg total RNA, and was used for first-strand cDNA synthesis. Total RNA was denatured at 65°C for 10 min and quickly chilled on ice for 2 min cDNA was synthesised in a reaction volume of 25 μl containing 50 mM Tris-HCl (pH 8.3; 75 mM KCl; 3 mM $MgCl_2$), 10 mM DTT (dithiotheitol), 0.5 mM deoxiribonucleotide triphosphate (dNTPs) and 200U Superscript II Rnase H^- Reverse Transcriptase (Invitrogen Corporation, California, U.S.A). The reactions were carried out for 60 min at 42°C, extended for another 10 min at 70°C and then cooled at 4°C; the cDNA thus synthesised was stored at -20°C until used as a PCR template. PCR was carried out in 50 μl of reaction mixture containing 0.3 μM of each primer, 1 unit of Platinum *Taq* DNA Polymerase (Invitrogen Corporation, California, U.S.A), 0.2 mM dNTP; 1.5 mM $MgCl_2$ and 1× PCR buffer (Invitrogen Corporation, California, U.S.A). Amplification for

5-HT_4 receptor mRNA detection was carried out by using the following protocol: 5 min at 95°C, 40 denaturing cycles at 95°C for 1 min, annealing at 65°C for 1 min and extension at 72°C for 1 min in a DNA Thermal Cycler PTC-100 (MJ, Research Inc., Watertown, MA, U.S.A.). Oligonucleotide primers (Invitrogen Corporation, California, U.S.A) for RT-PCR: upper primer, common: ON191, 5′-GTGATGATGAGCGCTACCGAAGAC-3′; lower primer, h5-$HT_{4(a)}$ specific: ON194, 5′-GAGCCATGTCCTCAATCAGAAGCA-3′; lower primer, h5-$HT_{4(b)}$ specific: ON167, 5′-AGAATCTGGGAAGAGGGAGTGTTGGGAAAT-3′.[467] The quality of cDNA was verified by PCR amplification of the house keeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) EMBL ACCESSION number NM_002046[468] using specific oligonucleotide primers: upper primer: 5'-ATCCCATCACCATCTTCCAG-3', lower primer: 5'-CCTTCCACGATACCAAAGTTG-3'. Reactions were performed using the following protocol: 5 min at 95°C, 30 denaturing cycles at 95°C for 30 seconds, annealing at 59°C for 30 seconds. and extension at 72°C for 1 min. Samples were subsequently analyzed on 1.3% agarose gels containing ethidium bromide and the PCR products were separated by electrophoresis along with a PCR 100 base pair Low Ladder (New England Biolabs Inc., Ipswich, U.S.A.). The gel was visualized under UV light and digitally photographed.

Analysis of Data

Curves that cover the full sigmoidal range were analyzed by means of a computerized curve fitting technique to obtain pEC_{50} -log of molar concentration of an agonist needed to reach half of the maximal effect, i.e. –log EC_{50},[451] and E_{max} (maximal response) values. E_{max} values are expressed as percentage of contraction to 100 mM K^+ in the respective vessel segment. If E_{max} was not reached, the contraction obtained at the highest concentration of agonist (100 μM) was considered as E_{max}. In case E_{max} was zero, these results were not considered for analysis of pEC_{50} values. pEC_{50} and E_{max} values were averaged for the respective agonists. All data are presented as mean±SEM. Statistical testing was performed using unpaired Student's t-test, unless indicated otherwise. P values of 0.05 or less were assumed to denote significant changes.

Chemical compounds

Tegaserod hydrogen maleate and sumatriptan succinate were supplied by Novartis Pharma AG. Prostaglandin $F_{2\alpha}$ (tris salt), U46619 (9,11-dideoxy-11α,9α-epoxy-methano-prostagladin $F_{2\alpha}$), substance P acetate and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). GR55562 (3-[3-(dimethylamino)propyl]-4-hydroxy-N-[4-(4-pyridinyl)phenyl]benzamide) was a gift from GlaxoWellcome (Stevenage, Ware, Herts, U.K.). Tegaserod was dissolved in DMSO, while sumatriptan was dissolved in either distilled water[52,109] or in DMSO, vehicle of tegaserod.

Results

Basic properties

In *proximal* vessel segments, the contraction induced by 100 mM K^+ was 47±6 mN; the average endothelium-dependent relaxant response to substance P (1 nM) was 9±5% of precontraction induced by 1 μM $PGF_{2\alpha}$. Since the relaxant response to substance P turned out to be absent in eight out of the 14 arteries, these eight were classified as 'diseased', while the remaining six were classified as 'healthy'. After division in healthy and diseased coronary arteries, the contraction induced by 100 mM K^+ was 41±3 mN and 51±11 mN, and the relaxation to substance P was 20±10% and 0±0% in healthy (n=6) and diseased (n=8) coronary arteries, respectively.

In *distal* vessel segments, the contraction induced by 100 mM K^+ was 18±3 mN (n=15); the average endothelium-dependent relaxant response to substance P (10 nM) was 96±4% of precontraction induced by 10 nM U46619. In view of the consistently high responses to substance P in *distal* coronary arteries, no distinction was made between healthy and diseased coronary arteries.

Contractile responses to tegaserod and sumatriptan

In *proximal* coronary segments, sumatriptan induced concentration-dependent contractions that were slightly different for sumatriptan dissolved in water or in DMSO, the vehicle of tegaserod (E_{max} 17±9% vs. 9±7%, n=12-14, p=0.04; pEC_{50} 5.58±0.23 vs. 6.26±0.23 n=4-8, p=0.05). In contrast, in the *distal* coronary segments, no differences were observed between sumatriptan dissolved in water or DMSO. Further, the vehicle DMSO in separate experiments did not induce either a contraction or relaxation in the *proximal* and *distal* coronary segments. Finally, in a previous study[368] we did not observe a difference in contraction to sumatriptan dissolved in water or DMSO in human *proximal* coronary arteries. Therefore, we decided to pool the data of contractions to sumatriptan dissolved in water or dissolved in DMSO for further analysis.

In *proximal* vessel segments, maximal contractions to sumatriptan (12±8%, n=14, Figure 1; left panel) were not significantly different between healthy (E_{max} 20±18%, n=6) and diseased (E_{max} 6±2%, n=8) coronary artery segments (Figure 2; left panel). In contrast, the pEC_{50} (potency) values (5.82±0.19, n=8) were higher in healthy (6.20±0.25, n=4) than in diseased (5.45±0.14 n=4, p=0.04) segments. The number of experiments used for analysis of the pEC_{50} values was lower than for the E_{max} values, because in some cases a sigmoidal curve could not be fitted in view of the extremely low or even absent contractions to sumatriptan in some vessel segments. Tegaserod induced a small contraction at 10 μM of 2±1% (range 0-9%, n=14, Figure 1; left panel) of contraction to 100 mM K^+, which was not different between healthy (2±1%, n=6) and diseased (2±1%, n=8) coronary artery segments (Figure 2;

right panel). At 100 µM, the contraction to tegaserod was 20±4% (Figure 1; left panel, range 0-43%) of the contraction to 100 mM K^+, and again not different between healthy (24±5%) and diseased (17±6%) coronary artery segments. In view of the fact that tegaserod induced a discernable contraction only at the highest concentrations tested, pEC_{50} values were not determined.

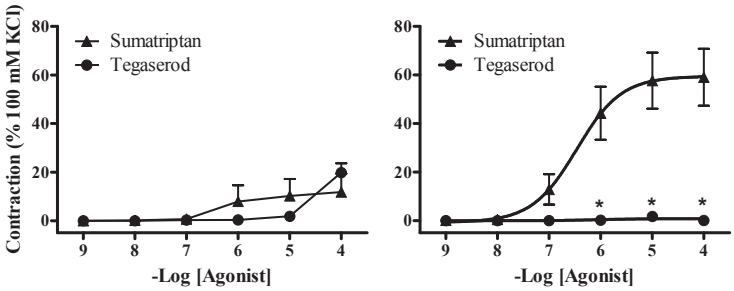

Figure 1: Contractile responses to tegaserod (n=13-14) and sumatriptan (n=11-14) (expressed as % of the response to 100 mM KCl) in the human isolated proximal (left panel, pooled data from healthy and diseased) and distal (right panel) coronary arteries. Data are mean±SEM. * p=0.0001 (contraction amplitudes of tegaserod vs. sumatriptan).

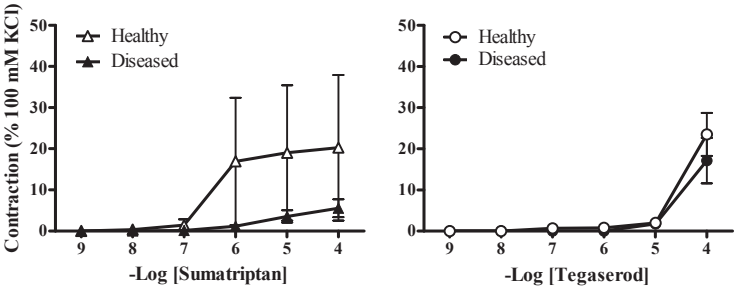

Figure 2: Contractile responses to sumatriptan (left panel) and tegaserod (right panel) in human healthy (n=6) and disease of (n=8) isolated proximal coronary artery (expressed as % of the response to 100 mM KCl) Data are mean±SEM. A: The pEC_{50} of sumatriptan is significantly higher healthy than diseased coronary arteries (p=0.04).

Antagonism of sumatriptan by tegaserod

In *proximal* vessel segments, sumatriptan induced a concentration-dependent contraction, which was not different in the absence (E_{max} 11±4%, pEC_{50} 5.69±0.20, n=7-11) or presence (E_{max} 16±5%, pEC_{50} 5.80±0.19 n=8-11) of 1 µM tegaserod (Figure 3; upper left panel). Because in some cases sumatriptan did not induce a contraction that was large enough to quantify reliably, we also performed experiments as above in the presence of the thromboxane A_2 analogue U46619 (1 nM-30 nM, titrated to about 5-10% of contraction to 100 mM K^+). Also in these experiments, contractions to sumatriptan in the absence (E_{max} 46±27%, pEC_{50} 6.06±0.14, n=3-5) or presence (E_{max} 48±29%, pEC_{50} 5.71±0.42, n=3-5) of tegaserod were similar (Figure 3; left lower panel). For these experiments, no distinction was made between

healthy and diseased vessel segments.

Similar as in the *proximal* coronary artery, we also investigated the potential antagonism of contractions to sumatriptan by tegaserod in *distal* coronary artery segments. Concentration response curves to sumatriptan were not different in the absence (E_{max} 63±15%, pEC_{50} 6.54±0.2, n=8) or presence (E_{max} 56±15%, pEC_{50} 6.90±0.3, n=8) of tegaserod (Figure 3; right upper panel). Likewise, in the experiments where vessel segments were precontracted with U46619, contractions to sumatriptan were similar in the absence (E_{max} 97±14%, pEC_{50} 6.61±0.12, n=7) or presence (E_{max} 99±11%, pEC_{50} 6.62±0.20, n=7) of tegaserod (Figure 3; right lower panel). Contractions to sumatriptan in the absence or presence of U46619 were not significantly different in either the *proximal* or the *distal* vessel segments.

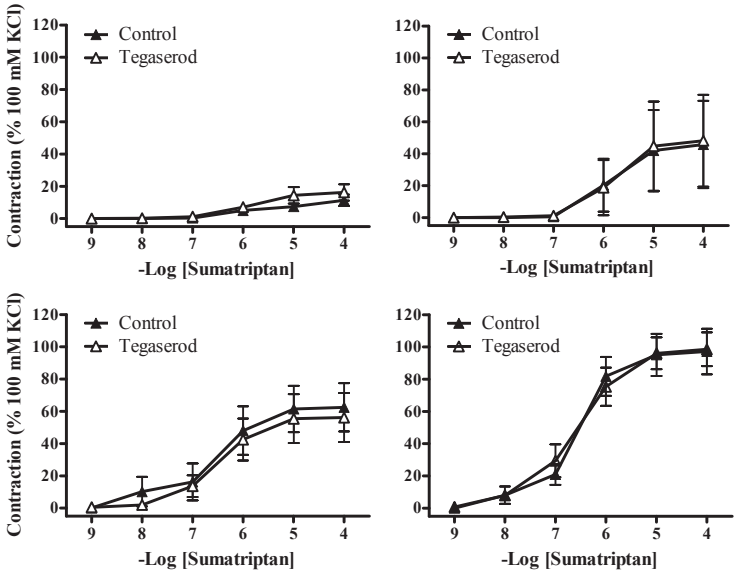

Figure 3: Contractile responses to sumatriptan (expressed as % of the response to 100 mM KCl) in the absence (control, vehicle) or presence of tegaserod (1 μM) in the human isolated proximal (upper panels) and distal (lower panels) coronary artery. Concentration response curves were constructed in the absence (left panels, n=8-11) or presence of a threshold precontraction with the thromboxane A_2 analogue U46619 (1 nM-30 nM, right panels, n=5-7). Data are mean±SEM.

In distal coronary artery segments, tegaserod (when used as a potential antagonist of the contractions to sumatriptan) seemed to induce a somewhat higher contraction in the presence of U46619 than its vehicle (13±5% vs. 8±4% respectively, p=0.01). To investigate this in further detail, we constructed full concentration response curves to tegaserod in the presence of U46619 as described below. The 5-$HT_{1B/1D}$ receptor antagonist GR55562 was used to assess whether potentially augmention to tegaserod were mediated via 5-$HT_{1B/1D}$ receptors.

Contractile responses to tegaserod and sumatriptan in the presence of U46619 and GR55562

In proximal coronary segments, tegaserod induced a contraction with an amplitude of 49±16% of contraction to 100 mM K+ (range 0-109%, n=7, Figure 4; left panel) at a concentration of 100 µM. This contraction was not different in the presence of U46619 (47±18%, n=6), GR55562 (21±16%, n=4) or U46619 + GR55562 (50±41%, n=2). In contrast, in the distal coronary segments, tegaserod did not induce any contractions, either in the absence or presence of U46619 and GR55562 (Figure 4; right panel). At a concentration of 100 µM, tegaserod induced a small relaxation of -7±3% (n=7) in the distal coronary segments, which was not statistically different in the presence of U46619 (-17±11%, n=7), GR55562 (-11±7%, n=5) or U46619 + GR55562 (-24±13%, n=5).

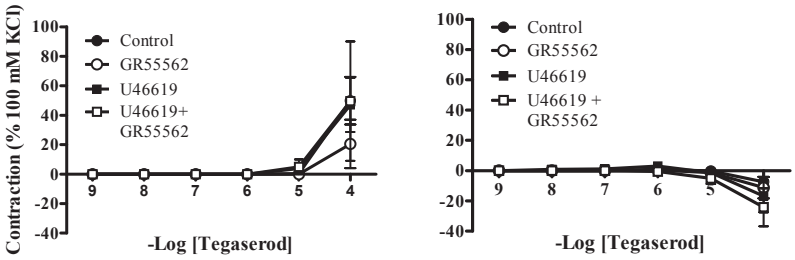

Figure 4: Contractile responses to tegaserod (expressed as % of response to 100 mM KCl) in the absence (control, n=6-7) or presence of GR55562 (1 µM, n=2-5) in human isolated proximal (left panel) and distal (right panel) coronary artery segments. Concentration response curves were constructed in the absence or presence of a threshold precontraction with the thromboxane A_2 analogue U46619 (1 nM-30 nM). Data are mean±SEM.

To verify that the concentration of GR55562 (1 µM) that we used was sufficient to indeed antagonize 5-HT_{1B} receptor-mediated contractions, we investigated contractions to sumatriptan in the absence and presence of GR55526. Sumatriptan-induced contractions (E_{max} 33±16%, n=6, Figure 5; left panel) in *proximal* vessel segments were apparently antagonized by GR55562 (E_{max} 5±5%, n=3), although differences compared to the control curves did not reach significance (probably due to the small number of experiments). In the *distal* vessel segments, sumatriptan induced a concentration-dependent contraction (E_{max} 71±26%, pEC_{50} 6.64±0.16, n=6, Figure 5; right panel), which was significantly antagonized by GR55562 (1 µM) (E_{max} 79±36%, pEC_{50} 4.99±0.08, n=3, p=0.005).

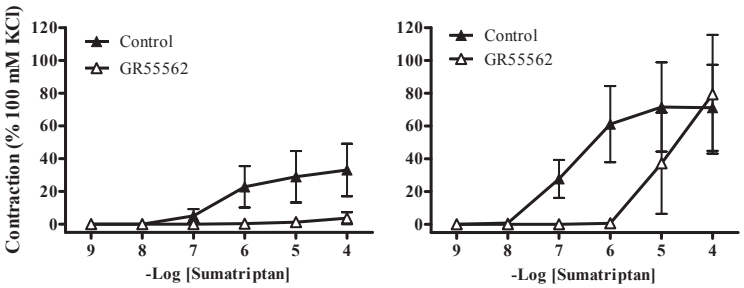

Figure 5: Contractile responses to sumatriptan (expressed as % of the response to 100 mM KCl) in the absence (vehicle, n=6) or presence of GR55562 (1 μM, n=3) in human isolated proximal (left panel) and distal (right panel) coronary artery segments. B: In the distal coronary artery, GR55562 induced a significant shift to the right (p=0.0005). Data are mean±SEM.

Detection of the 5-HT$_4$ receptor mRNA

The mRNA expression of the 5-HT$_{4(a)}$ and 5-HT$_{4(b)}$ receptors in human left ventricle, right atrium and distal coronary was analyzed by RT-PCR. PCR products of the expected size from the positive control fractions and all the test tissue fractions were sequenced. mRNA expressions were shown for the 5-HT$_{4(a)}$ and 5-HT$_{4(b)}$ receptors (Figure 6) in the human left ventricle, right atrium and left distal coronary.

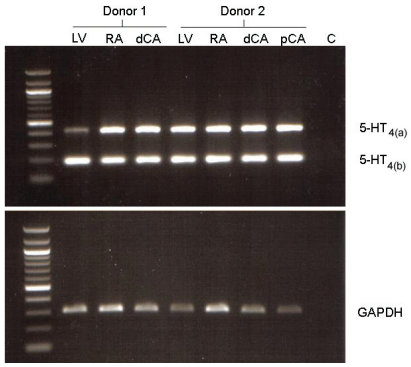

Figure 6: Reverse transcription polymerase chain reaction (RT-PCR) analysis of 5-HT$_4$ receptor splice variant expression in human left ventricle (LV), right atrium (RA) and coronary arteries (CA) tissue obtained from two heart beating donors (1 and 2). From donor 2 both a proximal (pCA) and a distal (dCA) segment were included. The bands representing the 5-HT$_{4(a)}$ (194bp) and 5-HT$_{4(b)}$ (428 bp) as well as the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) control are indicated. Lane 1 *(left)* in both panels is the 100-bp DNA ladder, lane 9 *(right)* is the negative control (C).

Discussion

In the current study, we investigated the contractile responses of proximal and distal human isolated coronary artery preparations to tegaserod and compared these to those induced by sumatriptan. The effects were also studied in the presence of the thromboxane analogue U46619 and the 5-$HT_{1B/1D}$ receptor antagonist GR55562. In addition, we studied whether the presence of tegaserod antagonized contractions to sumatriptan.

Sumatriptan induced concentration-dependent contractions in segments of both proximal and distal human coronary arteries, which were antagonized by the 5-$HT_{1B/1D}$ receptor antagonist GR55562, similar as in an earlier study.[462] As we have previously shown that these contractions are mediated via the 5-HT_{1B}, and not the 5-HT_{1D} receptor,[469] the higher responses observed in the distal preparations may suggest that the 5-HT_{1B} receptor expression or its intracellular coupling is different in coronary artery segments of different caliber. To our best knowledge, such a phenomenon has not been described before for 5-HT_{1B} receptors. However, we have previously observed greater responses to CGRP in distal coronary arteries than in proximal coronary arteries.[28] Further, 5-HT_{1B} receptor density[37] as well as contractions to sumatriptan[52] are higher in small diameter meningeal arteries than in large coronary arteries.

In proximal coronary artery segments, tegaserod induced clear, but variable, contractions at concentrations greater than 1 μM. In contrast, this contractile effect of tegaserod was less pronounced in distal coronary artery segments, where tegaserod even induced a relaxation at the highest concentration studied (100 μM) in some experiments. The fact that contractions to sumatriptan were greater in distal than in proximal coronary artery preparations, while contractions to tegaserod were smaller, argues against 5-HT_{1B} receptors mediating the contractions to tegaserod. Moreover, the 5-$HT_{1B/1D}$ receptor antagonist, GR55562 did not inhibit the contractions induced by tegaserod, reinforcing the evidence that the contractions induced by tegaserod are not mediated via the 5-HT_{1B} receptor. Thus, our experiments confirm earlier findings in isolated blood vessels from animals that tegaserod does not have the potential to cause vasoconstriction through the 5-HT_{1B} receptors.[470] The contractions in proximal coronary arteries as well as relaxations in distal coronary arteries induced by tegaserod at concentrations higher than 1 μM could be mediated via another receptor, for which tegaserod has additional affinity. Although this study demonstrated 5-$HT_{4(a)/(b)}$ receptor mRNA in the coronary arteries, as has previously been demonstrated in human atrium and ventricle,[467] it is highly unlikely that this receptor mediated the contractions to tegaserod. 5-HT_4 receptor stimulation results in an increase in intracellular cAMP;[471] increased levels of cAMP are associated with vasodilatation[28] rather than with vasoconstriction. Further, as holds true also for the other 5-HT receptor subtypes that will be discussed below, the response in human coronary artery had a lower potency that would have been expected on the basis of its affinity for the 5-HT_4 receptor (pK_i 7.6-8.4).

Tegaserod may also bind to the 5-HT_{2A} receptor,[464] which mediates vasocontriction in arterial smooth muscle.[453] However, tegaserod was shown to be an antagonist rather than an agonist at the 5-HT_{2A} receptors, since it inhibited contractions to 5-HT in the porcine coronary artery,[472] while it did not antagonize contractions to the 5-$HT_{1B/1D}$ receptor agonist sumatriptan (current study). Thus, the contractions induced by tegaserod at concentrations higher than 1 μM are unlikely to be mediated via the 5-HT_{2A} receptor.

The slight relaxation induced by tegaserod in the distal coronary segments might be mediated via the 5-HT_{2B} receptor, since tegaserod also has affinity for this receptor[458] and this receptor may induce relaxation in coronary arteries.[212] However, similarly as described above for the 5-HT_{2A} receptor, tegaserod acts as an antagonist on the 5-HT_{2B} receptor,[458] making it unlikely that the 5-HT_{2B} receptor mediates the relaxations seen with tegaserod.

It should be kept in mind that both the contractions and relaxations induced by tegaserod in our study only occurred at high concentrations, where pharmacological selectivity is reduced, thus limiting the relevance of studies to characterize the receptors involved in these responses. Similarly, in view of clinical cases of cardiac ischemia observed after the use of tegaserod,[464-465] the contractions as well as relaxations induced by tegaserod are of limited relevance because the concentration that induced coronary contractions is about 1,000 times higher than the maximal plasma concentration observed following the recommended dose of 6 mg bid C_{max} 9 nM after the 6 mg oral dose.[457,473]

There was no difference between contractions to tegaserod obtained in healthy or diseased coronary arteries as defined by endothelium-dependent relaxations to substance P, suggesting tegaserod does not have an increased vasoconstrictor potential in diseased coronary vessels. In diseased coronary artery segments, sumatriptan was slightly less potent than in healthy segments (i.e., lower pEC_{50} values). This seems to contradict our previous study, where we observed in a large post hoc analysis that not the pEC_{50}, but the E_{max} value seemed to be reduced in diseased arteries. However, both our previous study[474] and the study described herein (with a limited number of experiments) clearly indicate that contractions to sumatriptan are not increased in diseased coronary artery segments. Moreover, if any changes occur in diseased vessels, contractions to sumatriptan rather tend to be reduced.

In distal segments, no division between healthy and diseased coronary arteries was made in view of the reproducibly high relaxant response to substance P. Although relaxations to substance P were assessed with different concentrations in proximal (1 nM) and distal (10 nM) coronary artery segments, these different concentrations do not account for the difference in relaxations observed, as determined in previous studies from our group.[28]

In experiments where the possible antagonism of contractions to sumatriptan by tegaserod was investigated using a single concentration of tegaserod (1 μM) and full concentration response curves to sumatriptan, no antagonism was observed in either proximal or distal coronary artery segments. Also in the presence of the thromboxane A_2 analogue U46619,

which augments contractions to sumatriptan when its maximal response is low,[466] no rightward shift was observed in the concentration response curves to sumatriptan. Although contractions to sumatriptan were increased from 11% to 46% in proximal coronary arteries and from 63% to 97% in distal coronary arteries, this augmentation by U46619 was not significant. This is most probably due to the high variability of contractions to sumatriptan in human coronary arteries and is in accordance with our earlier study, where a significant augmentation was only observed when the endogenous thromboxane A_2 production in coronary arteries segments was inhibited.[466] Remarkably, contractions to tegaserod seemed to be augmented by the thromboxane A_2 analogue U46619 in the human distal, but not the proximal coronary artery. In view of the potential presence of thromboxane A_2 in the circulation, especially in patients with coronary artery disease,[466] this feature may be of clinical interest. However, in a separate series of experiments, specifically designed to investigate such a possible augmentation, contractions to tegaserod were not different in the absence or presence of U46619 in either proximal or distal coronary segments.

In conclusion, tegaserod does not exhibit vasoconstrictor properties mediated via the 5-HT_{1B} receptor in human coronary arteries, nor does it have antagonist properties at the 5-HT_{1B} receptor. The contractile response in healthy and diseased coronary arteries does not seem to differ. As the concentration of tegaserod that induced coronary contractions in our study was about 1,000 times higher than the plasma concentrations observed after therapeutic dosing, the vasoconstrictor potential of tegaserod seems to be of limited relevance in clinical cases of cardiac ischemia.

Acknowledgements

We wish to express our sincere gratitude to Alice van Dijk and Corina van Tricht of the Rotterdam Heart Valve Bank (Bio Implant Services Foundation / Eurotransplant Foundation) for their help in supplying us with the human heart tissue.

千里之行始於足下

老子

A journey of thousands miles starts beneath one's feet
(Lao Zi)

Chapter 11

Inotropic effects of prokinetic agents with 5-HT_4 receptor agonist actions on human isolated myocardial trabeculae

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Abstract

Besides acting as gastrointestinal prokinetic agents, 5-hydroxytryptamine$_4$ (5-HT$_4$) receptor agonists can induce positive inotropism in human isolated atrium, but not in ventricles. We pharmacologically evaluated the gastroprokinetic 5-HT$_4$ receptor agonists tegaserod, prucalopride, R199715, cisapride, the cisapride metabolite norcisapride, and the 5-HT$_3$ receptor agonist MKC-773 on human isolated myocardial trabeculae, and compared their effects with those induced by 5-HT and 5-methoxytryptamine (5-MeOT).

Atrial and ventricular trabeculae were paced and changes in contractile force were studied in the absence or presence of the 5-HT$_4$ receptor antagonist GR113808. Partial agonism was assessed using 5-HT$_4$ receptor agonists as antagonists against 5-HT. To test the contribution of L-type calcium channels, the inotropic responses to 5-HT and 5-MeOT were studied in the absence or presence of verapamil.

Like 5-HT and 5-MeOT, cisapride and tegaserod, but not prucalopride, R19971 and MKC733, induced concentration-dependent positive inotropic responses on atrial trabeculae, which were abolished by GR113808. Concentration response curves to 5-HT were shifted to the right in the presence of prucalopride, cisapride, tegaserod and R199715, but not MKC-773. None of the agonists affected the contraction of left ventricular trabeculae. In the presence of verapamil, inotropic responses to 5-HT and 5-MeOT were attenuated. Taken together, our results show that (i) the inotropic responses to 5-HT and 5-MeOT are dependent on L-type calcium channels, (ii) tegaserod and cisapride behave as partial 5-HT$_4$ receptor agonists, while prucalopride, norcisapride and MKC-733 cause no significant effects on human atrial trabeculae, (iii) R199715 seems to behave as a 5-HT$_4$ receptor antagonist.

Introduction

Gastroprokinetic drugs activating 5-HT_4 receptors are commonly used to facilitate gastrointestinal transit in patients suffering from gastrointestinal hypomotility disorders, such as constipation or gastroparesis.[475-476] Several 5-HT_4 receptor agonists have been evaluated for the treatment of chronic constipation and irritable bowel syndrome; however, until now, none of these drugs is widely available. For instance, the benzamide derivate, cisapride, is a mixed 5-HT_4 receptor agonist/5-HT_3 receptor antagonist, which has been clinically used as a gastroprokinetic agent, but was withdrawn in the U.S.A. due to its propensity to induce cardiac adverse effects.[477-478] Also, the partial 5-HT_4 receptor agonist tegaserod, which was introduced in the U.S.A to treat irritable bowel syndrome, was withdrawn in 2007 after a meta-analysis indicating an increased number of cardiovascular ischemic events in treated patients.[479-480] The cardiac adverse events with cisapride and tegaserod are different in nature and both unrelated to 5-HT_4 receptors. Indeed, the selective 5-HT_4 receptor agonist prucalopride has been shown to be effective in the treatment of chronic constipation, while extensive clinical trials to date suggest that prucalopride is devoid of cardiac side effects as seen with cisapride and tegaserod.[481-482]

5-HT_4 receptors are widely distributed in the human central nervous system[483] and peripheral organs, including the urinary bladder,[484] gastrointestinal tract,[485] vasculature[131] and heart.[467] Several 5-HT_4 receptor splice variants have been identified, but their functional role is currently not known, in part because no selective pharmacological ligands are available.[486] In the human heart, activation of 5-HT_4 receptors exerts positive inotropic effects on atrium, which is mediated via increase in the Ltype calcium channel current (I_{ca}).[487] This 5-HT_4 receptor-induced positive inotropism is not seen in ventricle of non-failing hearts.[488-489] Moreover, tachycardia elicited by 5-HT may involve activation of the 5-HT_4 receptors in human atrial cardiomyocytes, mediating subsequent activation of the I_f pacemaker current.[490] An increase in either I_{ca} or I_f current may contribute to the 5-HT-induced arrhythmic contraction by stimulation of 5-HT_4 receptors.[491] Thus, the presence of 5-HT_4 receptors in the human heart might theoretically be of concern with regards to arrhythmia and tachycardia[492] when using 5-HT_4 agonists to treat gastrointestinal disorders.[493] However, on the other hand, 5-HT_4 receptor antagonists have been suggested to be beneficial in the treatment of heart failure.[494-495]

In this study, we analyzed the ability of the 5-HT_4 receptor agonists tegaserod, prucalopride, R199715,[496] cisapride as well as its main metabolite, norcisapride and the 5-HT_3 receptor agonist MKC-773[497] to induce positive inotropic responses on human myocardial trabeculae. We compared this with the effects of 5-HT and 5-methoxytryptamine (5-MeOT) and analysed the contractile responses to the above gastroprokinetic agents in the absence and presence of the 5-HT_4 receptor antagonist GR113808.[498] In addition, the contractile

responses to 5-HT and/or 5-MeOT were investigated in the absence and presence of (i) the L-type calcium channel blocker verapamil and (ii) each of the above gastroprokinetic agents.

Methods

Preparation of tissue

Myocardial atrial and ventricular trabeculae were obtained from 70 heart beating organ donors (31 male, 39 female; mean age 46±4 years, age between 12-65 years), who died of non-cardiac disorders (36 cerebrovascular accident, 20 trauma of the head, 14 brain hypoxia) less than 24 hours before the tissue was brought to the laboratory. The hearts were provided by the Rotterdam Heart Valve Bank after donor mediation by Bio Implant Services Foundation/Eurotransplant Foundation (Leiden, the Netherlands) after removal of the aortic and pulmonary valves for homograft valve transplantation. Immediately after circulatory arrest, the hearts were stored at 0-4ºC in a sterile organ-protecting solution. Upon arrival at the laboratory, trabeculae of approximately 1 mm thickness were carefully dissected and mounted in a 15-ml organ bath containing oxygenated (95% O_2 / 5% CO_2) Krebs buffer solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 8.3; pH 7.4 (37°C). The trabeculae were paced at 1 Hz using electrical field stimulation (5 ms, 20% above threshold) delivered by a Grass S6 Square Wave Stimulator (Quincy, MA, U.S.A.). Resting tension was set to 7.5 mN for the right atrial and 19.5 mN for the left ventricular trabeculae. Changes in contraction were recorded with a Harvard force transducer (South Nattick, MA, U.S.A.) on a flatbed recorder (Servogor 124, Goerz, Neudorf, Austria). The preparation was allowed to stabilise for 60-90 min with a wash every 15 min.

Experimental Protocol

After washing every 15 min and stabilisation for 60-90 min, the trabeculae were contracted with noradrenaline (1 nM-10 μM) to verify the viability and responsiveness of the tissue. Again, the trabeculae were washed until a stable contraction was obtained. Subsequently, concentration response curves to the 5-HT_4 and 5-HT_3 receptor agonists were constructed. At the end of the experiment, the viability of the trabeculae was checked again with 10 μM NA. Trabeculae yielding less than 0.25 mN responses to 10 μM noradrenaline either before or after the concentration response curves were excluded from further analysis.

Inotropic effects of 5-HT$_4$ receptor agonists

To investigate the inotropic effects, concentration response curves to 5-HT, 5-MeOT, the gastroprokinetic agents and dimethylsulfoxide (DMSO; vehicle for cisapride and tegaserod) were constructed (n=6 each, 1 nM-100 μM, except for cisapride and tegaserod 1 nM-10 μM due to their precipitation at 100 μM). Concentration response curves to 5-HT and 5-MeOT were also constructed in the absence and presence of verapamil (n=6 each, 100 nM, incubated for 30 min) to investigate whether their effects are dependent on L-type calcium channels.

Receptor characterization

To confirm that the inotropic effects were mediated by the 5-HT$_4$ receptor, concentration response curves to DMSO and the 5-HT$_4$ and 5-HT$_3$ receptor agonists inducing positive inotropic effects were constructed in the absence or presence of GR113808 (n=6 each, 100 nM, incubated for 30 min). To test the antagonist effects of the 5-HT$_4$ and 5-HT$_3$ receptor agonists on the inotropic response to 5-HT (partial agonism), trabeculae were incubated in the absence or presence of cisapride (10 μM), norcisapride (10 μM), tegaserod (1 μM), R199715 (100 nM), MKC-733 (10 μM), prucalopride (1 μM) or DMSO for 30 min (n=6 each); these concentrations were chosen according to their affinity for the 5-HT$_{4b}$ receptor (about two log units below pK$_i$), but with a maximum of 10 μM in view of the loss of selectivity at even higher concentrations. Subsequently, concentration response curves to 5-HT (1 nM-100 μM) were constructed.

All experiments were performed in a paired parallel set-up using trabeculae from the same heart, except for the effects of prucalopride in the absence and presence GR113808, which were performed in an unpaired set-up. All procedures and protocols of this study were approved by the ethical committee of Erasmus Medical Center Rotterdam.

Compounds

5-hydroxytryptamine (5-HT) creatinine sulphate, noradrenaline and dimethylsulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Cisapride, norcisapride, prucalopride, tegaserod, R199715 (4-amino-5-chloro-2,3-dihydro-N-[[(3S,4S)-3-hydroxy-1-(3-methoxypropyl)-4-piperidinyl]methyl]-7-benzofurancarboxamide, monohydrochloride monohydrate) and MKC-733 (7-oxo-N-[3(R)-quinuclidinyl]-4,7-dihydrothieno[3,2-b]pyridine-6-carboxamide hydrochloride) were provided by Janssen Research Foundation (Beerse, Belgium). All compounds were dissolved in distilled water, except for cisapride (10 mM) and tegaserod (1 mM), which were dissolved in DMSO and further diluted in distilled water. All solutions were freshly prepared for each experiment.

Data presentation and statistical evaluation

Contraction changes in response to noradrenaline were expressed in mN as well as percentage changes from baseline. Contraction changes in response to the gastroprokinetic agents, which were usually stable in nature, were expressed as a percentage of the mean response (before and after concentration response curves) to 10 μM noradrenaline. The concentration response curves to cisapride and tegaserod were corrected for contraction changes in response to DMSO, which induced negative inotropic effects on both right atrial and left ventricular trabeculae. The contractility after 30 min incubation with verapamil was considered as the baseline contractility to analyse the responses that were subsequently induced by 5-HT or 5-MeOT, because baseline contractility decreased after 30 min incubation with verapamil. The pEC_{50} (negative logarithm of the concentration eliciting 50% of the maximal contractile response, E_{max}) was determined by analysing the concentration response curves with Graphpad Prism software (Graphpad Prism Inc., San Diego, California, U.S.A.). The maximum response or, in case no maximum was reached, the response at the highest concentration of agonist tested, was considered as E_{max}. For the experiments on antagonism of the contractions to 5-HT by the other agonists, where the E_{max} of 5-HT was not reached, the maximum response to 5-HT (control) was considered as E_{max} for the concentration response curve to 5-HT in the presence of the gastroprokinetic agents.

Data are presented as mean±SEM. Differences between E_{max} and pEC_{50} values of 5-HT_4 receptor agonists were analysed with one-way analysis of variance, followed by Tukey's multiple comparison t-test. Student's t-test (paired or unpaired where appropriate) was used for comparison of two groups. Statistical significance was accepted at $P<0.05$.

Results

Inotropic effects of noradrenaline and tissue viability

Baseline contractile force was 1.54±0.12 mN in the right atrial trabeculae (n=54 hearts) and 2.56±0.41 mN (n=19 hearts) in the left ventricular trabeculae. Figure 1 shows that noradrenaline (1 nM-10 μM) increased the contractile force in a concentration-dependent manner in both tissues. The responses to the highest concentration, before exposure to the 5-HT_4 receptor agonists, amounted to 2.97±0.16 mN (254±20% of baseline) in the right atrial trabeculae and 5.56±0.86 mN (247±20% of baseline) in the left ventricular trabeculae.

The increase in contractile force to 10 μM noradrenaline (administered at the end of the experiment) was similar to that obtained initially, indicating that the viability of the tissues remained unaltered during the experiment. This increase, after exposure to the serotonergic ligands, amounted to 2.88±0.18 mN (242±19% of baseline) in right atria and 4.79±0.74 mN (282±42% of baseline) in left ventricles (Figure 1).

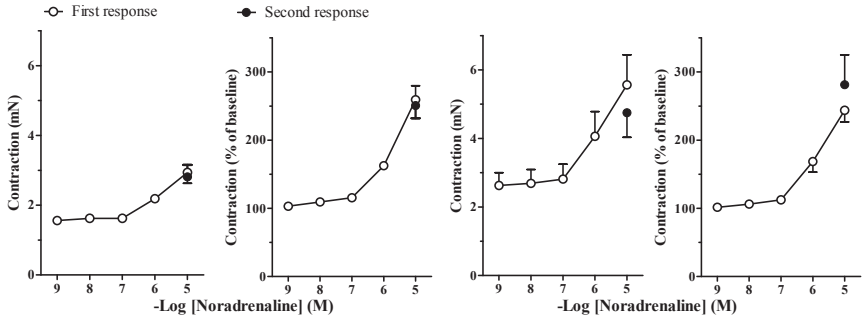

Figure 1: Contractile responses, expressed in mN (left panels) or as percentage of baseline contraction (right panels), to noradrenaline in human isolated right atrial (upper panels, n=50) and left ventricular (lower panels, n=13) trabeculae. Data are shown as mean±SEM.

Positive inotropic effects of 5-HT, 5-MeOT and the 5-HT$_4$ receptor agonists

5-HT and 5-MeOT increased contractile force in right atrial trabeculae in a concentration-dependent manner; the maximum response obtained at 100 µM was, respectively, 133±32% and 127±58% of the contraction to 10 µM noradrenaline; pEC_{50} values, with the response obtained at 100 µM considered as E_{max}, were 5.73±0.22 and 4.83±0.36, respectively (Figure 2; left panel). Cisapride and tegaserod also contracted atrial trabeculae; the maximum response amounted to 51±25% (cisapride, at 1 µM) and 41±13% (tegaserod, at 10 µM), pEC_{50} values were 6.93±0.23 and 6.85±0.31, respectively (Figure 2; right panel). Prucalopride tended to increase right atrial contractility, but the maximum response (31±16%, observed at 10 µM) was not significantly different from baseline. This may be explained by the large variability of the maximum response elicited by prucalopride (range: 0-125% of the contraction to 10 µM noradrenaline). Norcisapride, R199715 and MKC-733 did not affect atrial contractility (Figure 2; right panel).

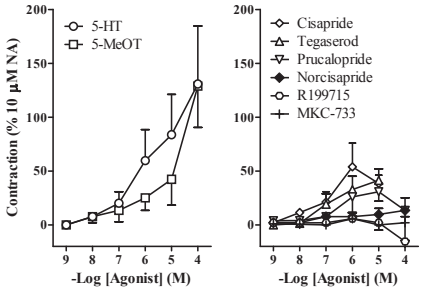

Figure 2: Concentration response curves to 5-HT, 5-MeOT (left panel), cisapride, tegaserod, prucalopride, norcisapride, R199715 and MKC-733 (right panel) on human isolated right atrial (n=6 each, 1 nM-100 µM, except for cisapride and tegaserod because of precipitation at >10 µM). Data are shown as mean±SEM.

Furthermore, the maximum inotropic effects to 5-HT (134±33%) and 5-MeOT (143±41%) were significantly attenuated by verapamil to, respectively, 85±22% and 84±24% (Figure 3). Verapamil did not change the pEC_{50} of the two tryptamines. In contrast to atrial trabeculae, none of the 5-HT_4 receptor agonists, including 5-HT and 5-MeOT, affected the contractility of left ventricular trabeculae (data not shown).

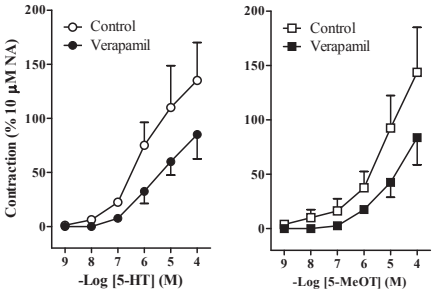

Figure 3: Concentration response curves to 5-HT and 5-MeOT in the absence or presence of verapamil (n=6 each, 100 nM) on human isolated right atrial trabeculae. Data are shown as mean±SEM.

Effect of the 5-HT_4 receptor antagonist GR113808

In the presence of GR113808 (100 nM), the contractions to 5-HT, 5-MeOT, cisapride, tegaserod and prucalopride were completely blocked at concentrations up to 10 µM (Figure 4). 5-HT induced a small inotropic effect only at 100 µM. No pK_b was determined because the effects of all compounds were abolished by GR113808. Since none of the agonists affected the contraction of left ventricular trabeculae, no experiments with GR113808 were performed in this tissue.

Effect of the 5-HT_4 receptor agonists on the inotropic responses to 5-HT

Cisapride, prucalopride, tegaserod and R199715, but not DMSO (data not shown), norcisapride or MKC-733, produced a rightward shift of the concentration response curves to 5-HT (Figure 5). The pEC_{50} values were significantly decreased from 5.98±0.23 to 3.46±0.22 (10 µM cisapride), from 6.09±0.39 to 3.61±0.17 (10 µM prucalopride), from 6.28±0.20 to 3.29±0.46 (1 µM tegaserod) and from 6.13±0.20 to 4.07±0.41 (100 nM R199715).

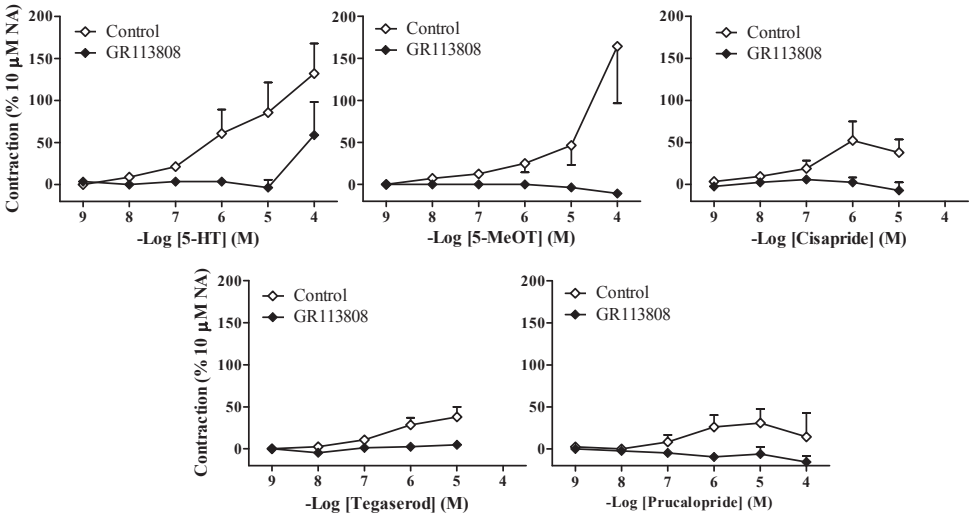

Figure 4: Concentration response curves to 5-HT, 5-MeOT, cisapride, tegaserod and prucalopride in the absence or presence of 100 nM GR113808 on human isolated right atrial trabeculae (n=6, each). Data are shown as mean±SEM.

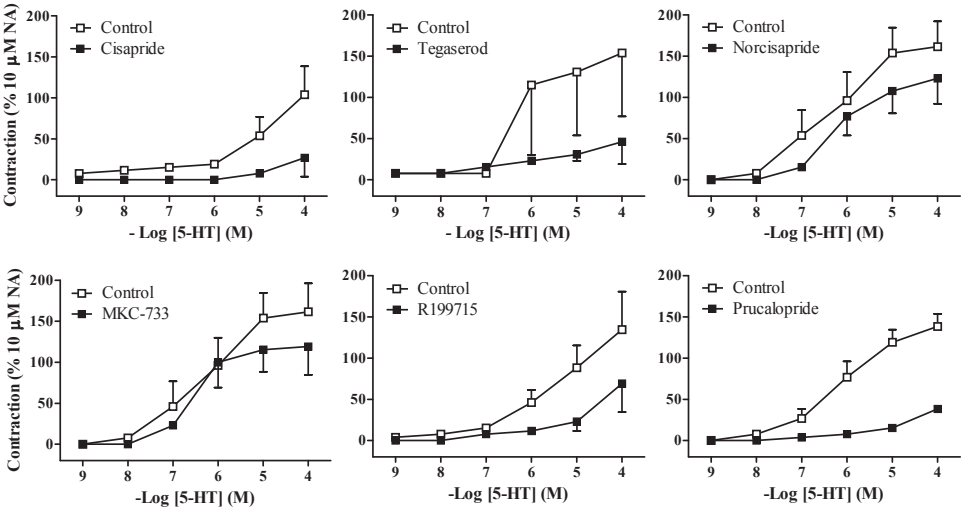

Figure 5: Concentration response curves to 5-HT in the absence or presence of cisapride (10 µM), tegaserod (1 µM), norcisapride (10 µM), MKC-733 (10 µM), R199715 (100 nM) and prucalopride (1 µM) (n=6 each) on human isolated right atrial trabeculae. Data are shown as mean±SEM.

Discussion

General

Several lines of pharmacological evidence have previously shown that activation of 5-HT_4 receptors induces contractile responses in isolated human right atria, but not in ventricles.[489,499] The present study confirms these findings and, apart from the implications discussed below, demonstrates that the gastroprokinetic agents cisapride, tegaserod and, albeit non-significantly, prucalopride (but not norcisapride, R199715 and MKC-733), are capable of producing positive inotropic responses in human right atria, which is in line with previous predictions based on a porcine model.[496,500]

Contractile responses to 5-HT and 5-MeOT on human myocardial trabeculae

5-HT and 5-MeOT increased contractility of right atrial, but not left ventricular trabeculae, as previously shown by Jahnel *et al.*,[488] although reverse transcription-polymerase chain reaction showed that 5-HT_{4a} and 5-HT_{4b} receptor mRNA is present on both human atrium and ventricle.[467] This apparent discrepancy may be explained, amongst other possibilities, by differences in translation from mRNA to protein, density of 5-HT_4 receptors and expression of 5-HT_4 receptor subtypes or differences in coupling efficiency between the human 5-HT_4 receptors in atrium and ventricle.[453] Consistent with the involvement of 5-HT_4 receptors, the 5-HT_4 receptor antagonist GR113808 inhibited the contractile responses to 5-HT and 5-MeOT in our study. Interestingly, it has recently been shown that, unlike in healthy subjects, in patients with heart failure functional 5-HT_4 receptors mediating positive inotropic effects are expressed in left ventricle.[494,501-502]

Role of calcium channels in the positive inotropic responses to 5-HT and 5-MeOT

Previous lines of evidence have shown that 5-HT and prucalopride increases the L-type calcium current ($I_{[ca]}$) in human isolated atrial myocytes.[503-504] Accordingly, we have now shown in human atrial trabeculae that 5-HT and 5-MeOT induced contractile responses were attenuated by verapamil (about 40%). This reinforces the view that activation of cardiac 5-HT_4 receptors increases the L-type $I_{[ca]}$,[487,505] presumably by phosphorylation of cyclic AMP-dependent protein kinase.[487] As previously suggested by Krobert *et al.*,[135] these positive inotropic effects may be attributed to calcium-induced calcium release from the sarcoplasmic reticulum through ryanodine channels, leading to enhanced contractility. Although not proven in the present study, it is tempting to hypothesize that the gastroprokinetic agents investigated here could also act via this mechanism. Obviously, further experiments, which fall beyond the objectives of the present investigation, will be required to confirm or refute this possibility.

Gastroprokinetic agents as antagonists of 5-HT- and 5-MeOT-induced positive inotropic responses

Similar to 5-HT and 5-MeOT, none of the gastroprokinetic agents affected the contraction of left ventricular trabeculae. In right atria, like 5-HT and 5-MeOT, both cisapride and tegaserod induced positive inotropic responses, albeit with a lower maximum response. The positive inotropic responses are consistent with results previously obtained in human[489,506] and porcine atria.[500,507] The inotropic responses to cisapride and tegaserod were abolished by GR113808, showing that the effects were mediated by 5-HT_4 receptors. Moreover, the concentration response curve to 5-HT was shifted to the right in the presence of either cisapride or tegaserod (and, as described below, by prucalopride), suggesting that both compounds bind to 5-HT_4 receptors competing with 5-HT. These results confirmed that cisapride and tegaserod behave as partial 5-HT_4 receptor agonists on human atrium. Indeed, cisapride is known to act as a partial agonist both on isolated human stomach[508] and right atrial strips.[506] In addition, tegaserod is known to act as a partial agonist on human gastrointestinal 5-HT_4 receptors.[479]

Prucalopride induced a variable increase in atrial contractility; however the maximum response was not significantly different from baseline, which is in contrast with an earlier study on human atrial trabeculae.[509] Most likely, this discrepancy may be explained by the large variability of the maximum response elicited by prucalopride in our study. In accordance with our study, Krobert *et al.*,[135] also observed that prucalopride is able to shift the concentration response curve to 5-HT, suggesting that prucalopride behaves as partial 5-HT_4 receptor agonist on human atrium.

In contrast with cisapride and tegaserod, MKC-733, which is a novel 5-HT_3 receptor agonist,[510] did not significantly increase atrial contractility, nor did it affect the contractile responses to 5-HT. The lack of these effects could be explained by its weak affinity for the 5-HT_{4b} receptor.

R199715 also did not affect *per se* atrial myocardial contractility but, consistent with its high affinity for the 5-HT_{4b} receptor, it induced a rightward shift of the concentration response curve to 5-HT. Therefore, R199715 seems to behave as a 5-HT_4 receptor antagonist in human atrium, in line with what has been described for porcine left atrium.[500] Interestingly, like 5-HT, as well as the 5-HT_4 receptor agonists prucalopride, tegaserod and R149402, R199715 concentration-dependently enhanced the electrically-induced cholinergic contractions in the porcine proximal stomach, with no influence on the resting tension.[496] This apparently contrasting pharmacological effect of R199715 could be explained by differences in receptor density and/or coupling efficiency between the tissues.[496]

The metabolite norcisapride failed to increase atrial contractility, although its affinity for the 5-HT_4 receptor is similar to that of cisapride. Moreover, it cannot be considered as a silent antagonist, since it did not affect the inotropic responses to 5-HT.

Clinical use

Cisapride has been clinically used as a gastroprokinetic agent. Oral administration of cisapride at 10 mg t.d.s., which is usually applied in clinical practice,[511] results in a total cisapride peak serum levels of 60-80 μg/L (120-170 nM), of which 98% is pharmacologically inactive, being bound to plasma proteins.[512] Thus, the free maximal plasma concentration amounts to 1-2 μg/L (2-4 nM). In our study, atrial contractility tended to increase at these concentrations, while the maximum positive inotropic effect was observed at 1 μM, which is 250-500 times higher than the therapeutic plasma concentration. Therefore, cisapride is unlikely to induce inotropic effects in patients, although this might be of relevance in patients with renal or hepatic insufficiency. However, cisapride was withdrawn from the market not due to its inotropic effect, but due to its Q-T lengthening effect.[492] This latter effect is independent of 5-HT_4 receptor and is mediated by a blockade of the human ether-a-go go related gene (HERG).[513]

Tegaserod was used to treat irritable bowel syndrome, but stands withdrawn due to the increased number of cardiovascular events.[479-480] In our study, maximum contractile response to tegaserod was observed at 10 μM, which is roughly 50,000 times the free concentration found in human plasma (total C_{max} is 9 nM, plasma protein binding is 98%)[514] after administration of the usual clinical dosage 6 mg oral dose.[457,473] This suggests that the increased number of cardiovascular events seen after tegaserod treatment may not be due to its positive inotropic effect on right atrium. Similarly, tegaserod slightly increased the funny current I_f in human atrial myocytes at a concentration of 100 nM (about 500 times free C_{max}, corresponding to 10 times total C_{max}), but this increase was not considered clinically relevant by the authors.[515] Moreover, tegaserod also has affinity for other 5-HT receptors, including the 5-HT_{1B} receptor, which mediates contraction of the human coronary artery.[462] However, coronary artery contraction is also unlikely to be responsible for the cardiovascular events after tegaserod, since the clinical dosage of tegaserod does not induce contraction in human isolated coronary arteries.[131]

In our study, prucalopride tended to increase right atrial contractility and this increase was also reported by others.[509] However, in human atrial cells, prucalopride was devoid of arrhythmic activity.[504] Several studies have shown that prucalopride is effective in chronic constipation treatment and without cardiovascular side effects. The cardiovascular safety profile has extensively been studied *in vitro* and *in vivo,* including animal studies, clinical studies in chronic constipation patients and specific additional clinical cardiovascular studies. Therefore, prucalopride seems a suitable drug for the treatment of gastrointestinal motility disorders.[482] Indeed, it has recently been approved for marketing in Europe.

In addition, since R199715 behaved as a 5-HT_4 receptor antagonist on human atria, in accordance with previous findings in pigs,[500] and enhanced the electrically-induced contractions in the porcine proximal stomach,[496] this drug might represent an alternative for

treatment of gastrointestinal motility disorders without inducing positive inotropic effects. Results of clinical trials are awaited with great interest.

Conclusion

The present study suggests that the inotropic responses to 5-HT and 5-MeOT are dependent on L-type calcium channels via activation of 5-HT_4 receptors. On human atrial trabeculae, tegaserod and cisapride behaved as partial 5-HT_4 receptor agonists. Prucalopride, norcisapride and MKC-733 caused no significant effects, while R199715 seems to behave as 5-HT_4 receptor antagonist.

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時間能說明一切

李永康

Time will tell everything
(L.W.H.)

Part E: Summary and general discussion

Chapter 12
Summarizing discussion and future perspectives

Summarizing discussion

The search for the treatment of migraine has a long history, but until now the number of effective antimigraine drugs is limited. As described in the introduction of this thesis, the currently available drugs for the treatment of migraine, i.e. ergot alkaloids and triptans, are vasoconstrictors. Although cranial vasoconstriction is likely to mediate, at least partly, their therapeutic effects; this property also induces vascular side effects. To avoid this limitation, several new targets devoid of vascular (side) effects are being explored. Although direct effects on the vascular tone are not expected for these prospective drugs, they may well induce indirect vascular effects mediated via modulation of other mechanisms that are involved in vasodilatation. In this thesis, we have investigated and discussed the potential vascular effects of these prospective antimigraine drugs that target central mechanisms.

CGRP receptor

Migraine is associated with activation of the trigeminovascular sensory nervous system and the release of the vasoactive peptide, CGRP, which is involved in sensory pain signal transmission. Based on the role of calcitonin gene-related peptide (CGRP) in the pathogenesis of migraine, CGRP receptors have been regarded as a useful target for the development of antimigraine drugs. The CGRP receptor antagonists have been shown to be effective in the treatment of migraine and they are currently under clinical development. However, the therapeutic mechanism and the effect on vascular tone are not well known. In **chapter 4**, we have shown that the CGRP receptor antagonist, telcagepant, does antagonize vasodilatation induced by CGRP without inducing vasoconstriction in cranial arteries. This suggests that the therapeutic effect of telcagepant may well, at least partly, be mediated via blockade of meningeal vasodilatation occurring during a migraine attack. Moreover, we have localized the functional CGRP receptor in these human cranial arteries. In view of potential cardiovascular side effects, we have investigated telcagepant in coronary arteries of different diameter, which is described in **chapter 5**. Interestingly, as in the cranial arteries, telcagepant does not induce direct vasoconstriction in the coronary arteries, in contrast to the triptans. Therefore, telcagepant will be a potential antimigraine drug which is unlikely to induce direct vasoconstriction under normal physiological conditions in cardiovascular healthy patients. In **chapter 5**, we also show that the vasodilator effect of CGRP and its antagonism by telcagepant entirely depend on CGRP receptors. We demonstrated this with a biochemical assay where telcagepant inhibited the increase of cAMP levels induced by CGRP, but not the increase of cAMP levels induced by forskolin. Moreover, we also confirmed that the functionally active CGRP receptors are present in the smooth muscle cells of these coronary arteries.

Although our data showed that telcagepant can antagonize CGRP-induced vasodilatation without inducing vasoconstriction, antagonism of the CGRP receptor might have indirect

cardiovascular side effects since CGRP has a cardioprotective role in pre- and post-ischemia. Indeed, in isolated rodent hearts, CGRP receptor antagonism has been reported to attenuate ischemic preconditioning-induced cardioprotection. In contrast, preclinical *in vivo* studies in multiple species with CGRP receptor antagonists have reported no intrinsic hemodynamic effect. Moreover, in a small cohort, telcagepant did not exacerbate spontaneous ischemia in patients with stable coronary artery disease. Telcagepant is currently being investigated as a migraine therapy in patients with stable vascular disease. Obviously, the absence of vasoconstriction with CGRP receptor antagonists suggests a potential cardiovascular safety advantage for CGRP antagonists when compared to the triptans.

The CGRP receptor antagonists are developed as an acute treatment of migraine. However, they might also be used as prophylactic treatment, for example in patients with menstrual migraine. In human studies, menstrual migraine has been associated with fluctuations of plasma estrogen levels. Stable estrogen levels by estrogen supplements are associated with a decrease in the number of migraine attacks. Treatment with estrogen supplements around the first day of estrogen cycle only shifted the cycle and postponed the migraine attack until no estrogen was supplied. Since menstrual migraine can be predicted, treatment with CGRP receptor antagonists before and during the first days of the estrogen cycle might avoid menstrual migraine attacks. This short term prophylactic treatment has been shown to be effective in the treatment of menstrual migraine with frovatriptan.[516] How estrogen affects menstrual migraine is not well understood. Changing estrogen levels may regulate the synthesis and receptor expression of CGRP and indeed CGRP release is increased in the presence of high levels of 17β-estradiol. Moreover, ovariectomy and estrogen replacement influence the CGRP levels found in root dorsal ganglion.[420-421]

To investigate the possible role of estrogen in migraine, our group has studied the neurovascular effect of different sex hormones. We have previously shown that high levels of estrogen increase vasodilatation induced by periarterial electrical stimulation, which suggests that high levels of estrogen influence the release of CGRP. Since a rapid decrease in estrogen levels seems to be involved in menstrual migraine,[517] we investigated the effects of estrogen withdrawal on the vascular response to CGRP. In **chapter 6**, we demonstrated that a rapid decrease of plasma estrogen concentrations does not influence the effect of exogenous CGRP-induced vasodilatation. This suggests that CGRP receptor expression in dural artery is not changed after a decrease in estrogen levels. However, more analyses have to be done to support this finding, which will be discussed latter.

As described earlier, CGRP and its mRNA are increased in the lumbar root dorsal ganglion of estrogen-treated rats,[421] and a decrease in estrogen levels might influence the CGRP content in nerve endings and therefore change the vasodilatation induced by endogenous CGRP. Since a decrease of estrogen is associated with menstrual migraine, the release from perivascular nerves might be more pronounced after a fall of estrogen levels

than when the estrogen levels are stable. Therefore studies investigating an increase of dural vasodilatation after estrogen withdrawal are now ongoing. In these studies, CGRP receptor expression will also be quantified, both at the mRNA and the protein level. To support the experimental design, plasma estrogen levels and estrogen cycles will also be determined. In the future, the effect of estrogen withdrawal on coronary and peripheral arterial function will be investigated using the Langendorff set-up and Mulvany myographs.

Other nonvascular targets

The central and vascular effects of prospective drugs, which were supposed to avoid vascular side effects, are described in the introduction. In this thesis, we have shown that targeting the ionotropic glutamate receptor as well as the PACAP and VIP receptors will (in)directly affect the vascular tone.

The glutamate receptor antagonists have been demonstrated to attenuate mechanisms that are putatively involved in the pathophysiology of migraine, including inhibition of trigeminovascular nociception in the trigeminocervical nucleus. However, as glutamate receptors play an important role in the mediation of excitatory synaptic transmission, they may also induce vascular effects since activation of ionotropic glutamate receptors on neurons leads to the release of vasoactive substances. Indeed, in **chapter 7**, we have shown that the NMDA receptor antagonists are able to reduce vasodilatation by blocking the release of vasoactive peptides from perivascular nerves. Interestingly, the kainate receptor antagonist, LY466195, affects vasodilatation neither directly nor indirectly. Therefore, we suggest that the therapeutic mechanisms of kainate receptor antagonists do not involve the perivascular release of CGRP or the vascular CGRP receptor pathway. It is more likely that kainate receptor antagonists have a central effect. Indeed, as suggested by Andreou and Goadsby,[259] the therapeutic effect of kainate receptor antagonists might be due to blockade of the glutamergic neurotransmission through kainate receptors in the ventroposteromedial thalamic nucleus. However, since ionotropic glutamate receptors are involved in various physiological process, targeting of these receptors may not only affect vascular tone (in the case of NMDA and possibly AMPA receptors), but it may also induce other neuronal side effects.

Recently, a new approach based on targeting sodium channels in specific nociceptive neurons, has been described for the treatment of pain. Blockade of voltage-gated sodium channels prevents the generation and propagation of action potentials[518] and therefore the nociceptive information. In this approach, the permanently charged sodium channel blocker N-ethyl-lidocaine is transported into the cell through the pore of the activated TRPV1 receptor channel. Co-administration of capsaicin, which is a TRVP1 receptor agonist, with N-ethyl-lidocaine results in selective blockade of sodium channels and inhibition of excitability in nociceptors.[519-520] However, in view of pain treatment in migraine, this approach

might still have an effect on the vascular tone, since activation of the TRPV1 receptor induces the release of vasoactive peptides, which results in vasodilatation. In contrast, blockade of the sodium channels will prevent the release of vasoactive peptides by activation of these receptors. Therefore, this approach might be effective in the treatment of migraine, although it might affect vascular tone both directly and indirectly.

The other central targets for prospective antimigraine drugs we have investigated in this thesis are the PACAP and VIP receptors. PACAP and VIP have both been shown to induce cranial vasodilatation and headache in humans. Both peptides are released by parasympathetic efferent nerves to regulate cerebrovascular tone and hemodynamics of the brain. It is also known that these peptides activate and sensitize intracranial sensory nerve fibers leading to the perception of pain. Interestingly, PACAP, but not VIP, also induced migraine-like headache, which makes PACAP a more interesting target than VIP in view of the treatment of migraine. In **chapter 8**, we have shown that this mechanism is unlikely to be related to cranial vasodilatation, since PACAP induced only a limited cranial vasodilatation, while VIP, which does not induce migraine-like headache, induced a more pronounced vasodilatation than PACAP. Moreover, PACAP was also less potent than VIP to induce vasodilatation in human coronary artery. Our results suggest that PACAP induces migraine-like headache mediated via a central mechanism involving the PAC receptor. The PAC receptor may be considered as a potential antimigraine target with a limited direct vascular effect. Since vasodilatation induced by PACAP might be mediated via activation of perivascular nerves, in-depth analysis of the role of this receptor in the migraine pathogenesis will provide more information about the possible indirect vascular effects.

Based on these studies, we have shown that neuronal targets may also be involved in the regulation of vascular tone. Many prospective antimigraine drugs with a putatively selective neuronal mechanism of action may display indirect vascular effects, as described in **chapter 2**. In contrast to the currently available antimigraine drugs (ergots and triptans), these drugs do not directly induce vasoconstriction, but they may inhibit either vasodilatation induced by neuropeptides (e.g., CGRP receptor antagonists) or the release of such peptides (e.g., some glutamate receptor antagonists). It remains to be elucidated whether the indirect vascular effects of the prospective antimigraine drugs discussed contribute to the therapeutic efficacy of these compounds. Alternatively, the vascular effects could be relevant in view of the cardiovascular side-effect potential of the drugs. Thus, the probably improved cardiovascular safety profile of the new generation antimigraine drugs may be of clinical relevance for patients in which the triptans are contraindicated. Obviously, antimigraine drugs with a completely non-vascular mode of action will clinically be an advantage over compounds with mild (in)direct vascular effects. Further, it should be kept in mind that, when the prospective drugs with mild (in)direct vascular effects would be used chronically as prophylactic instead of acute antimigraine drugs, the cardiovascular side effect profile may

be less favorable than expected for acute use. Clearly, further research is warranted before any definite statements can be made on this topic.

Cardiovascular effects of 5-HT ligands

Sumatriptan was the first triptan introduced for the treatment of migraine. The selective agonist activity at the 5-$HT_{1B/1D}$ receptors that induced vasoconstriction of cranial arteries was described as the therapeutic mechanism of the triptans. In the last decade, it has been suggested that inhibition of the release of neuropeptides in perivascular nerve terminals of the trigeminal system and direct inhibition of neuronal activity, reducing central pain transmission via activation of the 5-HT_{1D} and the 5-HT_{1F} receptors, may contribute to the therapeutic action. However, treatment with triptans raised concerns about potential coronary side effects, since 5-$HT_{1B/1D}$ receptors are also present in coronary arteries. Our group has shown that the contractions induced by triptans at therapeutic concentrations are limited in human coronary arteries. In these studies, only proximal arteries were used due to limited experimental options, while myocardial ischemia may originate from both large and small human coronary arteries. Therefore, parallel studies using proximal and distal segments of the coronary artery, which is now also available, would provide more information on the cardiovascular effect of the drugs. Interestingly, as described in **chapter 9,** these experiments showed that the contractions induced by sumatriptan in distal human coronary arteries are significantly higher than in the proximal human coronary arteries, while no regional differences were seen in the contraction induced by 5-HT. A similar difference of responses in proximal and distal coronary arteries was also seen in CGRP-induced vasodilatation in proximal and distal coronary arteries, which might be a result of efficiency of intracellular coupling and not due to receptor density, since CGRP receptor expression did not differ in the coronary arteries of different diameter **(chapter 5)**. However, further analyses, like immunohistochemistry or Western blotting, are needed to explain the discrepancy seen in the sumatriptan responses. Surprisingly, contractions to sumatriptan in the distal coronary arteries resembled those in the human meningeal arteries, suggesting that triptans have a limited cranioselectivity. However, the two decades of clinical experience with the triptans have now demonstrated that the use of these agents, taking the contraindications into account, is very safe. Moreover, it is now clear that myocardial ischemia is an extremely rare event after the use of these drugs and it has recently been reported that the number of adverse events was not increased when patients were treated with both cardiovascularly active drugs and triptans. Therefore, our findings reconfirm that the triptans should remain contraindicated in patients with coronary artery disease, although the risk profile definitely favour their use in the absence of contraindications.

Except triptans, other 5-HT subtype ligands have been suggested to induce cardiovascular side effects. For instance, the 5-HT_4 receptor agonist, tegaserod, which is used for the treatment

of gastrointestinal diseases, has affinity for the 5-HT_{1B} receptor. This might potentially explain the cardiac ischemia observed in some patient treated with tegaserod. However, in **chapter 10**, we have demonstrated that tegaserod does not induce vasoconstriction, nor does it inhibit the vasoconstriction induced by sumatriptan. This suggests that tegaserod does not act as an agonist, nor as an antagonist, at the 5-HT_{1B} receptor in proximal and distal human coronary artery. Therefore, cardiovascular side effects induced by tegaserod do probably not involve coronary vasoconstriction. On the other hand, activation of 5-HT_4 receptors has been associated with the contractile responses in isolated human right atria via the 5-HT_4 receptor, which might cause cardiovascular side effects. In **chapter 11**, we have investigated the inotropic responses of different 5-HT_4 receptor agonists in human isolated myocardial trabeculae. We have shown that the 5-HT_4 receptor agonists, cisapride and tegaserod, induce inotropic responses. All 5-HT_4 receptor agonists that we investigated behaved as partial 5-HT_4 receptor agonists. Based on our data, we suggest that the clinical cases of cardiac ischemia occurring after tegaserod treatment are less likely to be mediated via positive inotropic effects on right atrium and/or coronary vasoconstriction via the 5-HT_{1B} receptor.

Future perspectives

Although the underlying cause of migraine is not clear, a wide variety of targets for the treatment of migraine exists. Understanding how current and prospective antimigraine drugs work will shed further light on the pathophysiology of migraine, and might pave the way for even better treatment modalities without (cardiovascular) side effects.

Prospective antimigraine drugs

The therapeutic mechanism of telcagepant is, at least partly, likely to be mediated via inhibition of vasodilatation by blockade of the vascular CGRP receptor. Telcagepant has a high affinity for the human CGRP receptor, which suggests that a low dose of telcagepant will already have therapeutic effect. However, clinical trials have been shown that a 1,000 times higher concentration than expected from its pharmacodynamic behaviour is needed to reach the therapeutic effect. This led to discussions about the therapeutic site of action of telcagepant. Therefore, it is interesting to know where exactly telcagepant acts on the artery. To study this, telcagepant should be applied to the artery luminally and abluminally in separate experiments. This can be investigated with the pressure myograph model, where the diameter of the vessel can be measured. This model allows the application of compounds luminally or abluminally, in contrast to the myograph technique described in the current thesis, where the compound reaches both the luminal and abluminal side of the artery simultaneously. Moreover, this study has to be performed with both meningeal and coronary arteries, to allow conclusions

on both the potential therapeutic effects and the cardiovascular side effects. In addition, the differences between meningeal and coronary arteries could be further investigated by analyzing the receptor-coupling in these arteries. This can be done by performing cAMP level measurements and analyzing other downstream second messengers including myosin light-chain kinase phosphorylation in the absence or present of telcagepant.

Interestingly, it has been suggested that telcagepant might enter the brain to induce central therapeutic effects due to supposed leakage of the blood brain barrier during migraine. Therefore, telcagepant might affect the release of neuropeptides from perivascular neurons. This can be investigated by measuring dural vasodilatation changes in the absence or presence of increasing doses of telcagepant in the closed cranial window setup. Differences between vasodilatation induced by endogenous CGRP or exogenous CGRP will provide a better understanding of the central effects of telcagepant and the role of the CGRP receptor in migraine.

The effect of ionotropic glutamate receptors in migraine is still elusive. Due to their vascular effects, NMDA receptor antagonists seem to be less favorable than kainate receptor antagonists for the treatment of migraine. As described in the summary and discussion, the transport of sodium channel blockers into the cell by activating TRPV1 channels in nociceptive neurons provides another opportunity for the treatment in migraine based on targeting NMDA receptors. However, both TRPV1 and NMDA receptors have been associated with vascular effects. Activation of TRPV1 receptors induced vasodilatation, and inhibition of NMDA receptors indirectly prevented vasodilatation. Therefore, simultaneous activation of the TRPV1 receptor and blockade of the NMDA receptor might have no net vascular effect. To study the vascular effects of this combination, animals should be treated with capsaicin together with the sodium channel blocker, QX-314. Next, the effect on basal dural vascular tone and the vasodilatation induced by exogenous as well as endogenous CGRP can be investigated in the closed cranial window setup.

The role of the 5-HT_{1B} receptor in migraine has been described extensively and the current antimigraine drugs, triptans, were discovered based on the knowledge about this receptor in the cranial vasculature. Interestingly, the 5-HT_{1F} receptor agonist, LY573144, and the 5-HT_7 receptor antagonist, SB269970, have been described as prospective drugs for the treatment of migraine. However, these compounds might also have affinity for the other 5-HT receptors and the effects of these compounds on vascular tone are not well known yet. To investigate their direct vascular properties, functional experiments in human arteries with selective receptor agonists and antagonists are warranted. Moreover, the effects of these compounds on vasodilatation induced by CGRP should be studied. Obviously, quantification of the CGRP receptor expression, both at the mRNA and the protein level, in human arteries would complement such studies. To investigate any indirect vascular effects of these compounds, dural vasodilatation induced by periarterial electrical stimulation in the closed

cranial window setup in the absence and the presence of increasing doses of these compounds should be determined. Such investigations will not only provide data about the potential therapeutic and cardiovascular side effects of the new 5-HT (ant)agonizing drugs, but they will also expand the understanding of the role of the 5-HT receptor family in migraine.

Genetic factors

Familial hemiplegic migraine (FHM) is a rare autosomal dominant form of migraine. Mutations in three different genes have been found in FHM: the CACNA1A gene, encoding the $Ca_v2.1$ (α_{1A}) subunit of voltage-dependent P/Q-type calcium channels,[78] the ATP1A2 gene, encoding the α_2 subunit of the Na^+/K^+ pump[77,521] and the SCN1A gene encoding the sodium channel α subunit.[522] P/Q-type calcium channels,[523] the Na^+/K^+ pump[524] as well as sodium channels[522] are mainly expressed in the central nervous system, but they are also present in peripheral tissues, including arterial smooth muscle cells.[525-526] P/Q-type calcium channels in perivascular sensory nerve endings may modulate release of neuropeptides such as CGRP[527-529] and, conceivably, Na^+/K^+ channels and sodium channels may also modulate CGRP release indirectly via the Na^+/Ca^{2+} exchanger. However, the role in neurovascular vasodilatation responses is not yet elucidated. To study the neurovascular effect of mutations as present in FHM patients, knock in mice, expressing mutations in the P/Q type calcium channel (Cacna1a R192Q and Cacna1a S218L) as well as expressing mutations in the Na^+/K^+-ATPase (Atp1a2 T345A) that are observed in migraine patients could be investigated using the closed cranial window model. The vascular effect of these mutations could be further investigated by characterization of vascular responses to endothelium-dependent and endothelium-independent vasodilators, including CGRP, as well as vasoconstrictors and receptor protein analysis in isolated blood vessels of transgenic and control animals. This animal model will not only provide more knowledge about FHM, but it also serves as a relevant model for investigating novel drugs targeting ion channels.

Female sex hormones

Several lines of evidence have suggested that female sex hormones are involved in menstrual migraine, but the exact mechanism is still elusive. To study this in further detail, we have investigated the effect of estrogen withdrawal on the dural vasodilatation induced by exogenous CGRP in the closed cranial window setup. Additional analyses are now required, as described in **chapter 6**. Moreover, the effect of estrogen withdrawal on vasodilatation induced by endogenous CGRP has to be investigated in this model. In addition, since not only estrogen is of importance in the menstruation cycle, a decrease in the progesterone levels alone or in combination with a decrease in estrogen levels might also play a role in menstrual migraine. Therefore, further studies focusing on the effect of progesterone or the removal of these female sex hormones in the same experimental setup should be performed.

Estrogen not only induces the expression of receptors by binding to the nuclear estrogen receptor, but it can also act on membrane receptors thereby exerting direct vascular effects. These two different effects might explain the contribution of estrogen to migraine after a rapid change in plasma estrogen levels. The high level of estrogen might influence receptor expression, while the change of estrogen could be responsible for the direct effects. Therefore, the net vascular effect of the different pathways of estrogen receptor activation has to be explored further. To this end, human arteries should be incubated with different concentrations of estrogen. Short and long time incubations have to be performed using the same concentrations. The functional vascular effects of endothelium-dependent and endothelium-independent vasodilators, including CGRP, as well as vasoconstrictors, could then be studied with Mulvany myographs. The receptor-coupling efficiency could be analyzed by second messenger (including cAMP) measurements. Moreover, CGRP expression should be determined at the mRNA and the protein level. Obviously, differences in the effect of estrogen between meningeal and coronary vessels, as well as sex differences of the donors of the arteries should be taken into account to fully understand the role of estrogen in migraine. The relation of female sex hormones and CGRP can be further explored in human studies. The differences in plasma CGRP levels between males and females as well as during the menstrual cycle in females can be analyzed. Vascular responses to vasodilators in the same group can be studied using the laser Doppler dermal perfusion model. Moreover, females using different types of hormones in contraceptives could also be included in these studies. The molecular pathway how the female sex hormones affect the release of CGRP or expression of CGRP receptor could be investigated by using neuronal cell lines treated with hormones. These cells can be analyzed for the CGRP peptide and CGRP receptors as well as the detection of downstream signaling protein by Western blotting and immunochemistry. Female sex hormones also seem to play a role in FHM, since the prevalence of FHM, as well as sporadic hemiplegic migraine is about 2.5 times higher in women than in men.[530-532] This is supported by the findings that female sex hormones enhance neuronal excitability in an interaction with ion channel mutations.[533-534] To study the role of female sex hormones in these mutations, the neurovascular effect, vascular responses to endothelium-dependent and endothelium-independent vasodilators, including CGRP, as well as vasoconstrictors and receptor protein analysis in isolated blood vessels between male and female transgenic mice as previous described could be compared. Moreover, ovariectomy or hormone treatment of the transgenic mice could be used to study the influence of hormones levels. Plasma levels of CGRP and female sex hormones in these mice have to be measured and using the laser Doppler method we could study microvascular vasodilatation in response to capsaicin in the transgenic mice.

Although studies on FHM have resulted in the discovery of three genes that may also be of relevance for the more common forms of migraine,[535-536] mutations in these genes seem to only account for a limited part of migraine inheritance.[537-538] Recently, epigenetic mechanisms have been proposed to play a role in migraine.[537-539] Female sex hormones are known to induce epigenetic mechanisms.[540-541] Moreover, the changes in migraine prevalence between men and women are dependent on female sex hormones. Therefore, female sex hormones may also affect the prevalence of migraine via epigenetic mechanisms. The role of female sex hormones in epigenetic mechanisms could be studied by inhibition of DNA methyltransferase (DNMT) by 5-aza-2'-deoxycytidine and histone deacetylase (HDAC) by trichostatin A in hormone-treated wildtype or transgenic animals. In these animals, the neurovascular effect could be investigated with the closed cranial window model. Using isolated blood vessels of these animals, the vascular effect of isolated blood vessels to vasodilators as well as constrictors could be studied. The DNA methylation status should be measured by a cytosine extension assay and histone acetylation using acetyl-specific histone antibodies in trigeminal ganglion and nucleus, dural arteries and thoracic aorta. In addition to these global changes in DNA methylation and histone acetylation, individual genes of interest such as the estrogen receptors and CGRP receptor components should be studied by bisulfite sequencing and chromatine immunoprecipitation assays of the respective genes. In addition, expression of these genes should be studied by immunohistochemistry and Western blotting. Using the transgenic mice, which are not treated with hormones, interactions between genetic and epigenetic mechanisms could be studied. Finally, DNA methylation or histone acetylation could also be determined in human isolated meningeal artery, sex differences and hormones treatment could than be compared.

Closed cranial window model

Although the closed cranial window model already provides the opportunity to measure the change in dural artery via endogenous and exogenous stimuli, some optimization of this model will provide advantages for future investigations. For instance, the administration route of exogenous compound should be altered to avoid an effect on blood pressure. This can be done by cannulation of the carotid artery instead of the femoral artery, as described by others.[542] Moreover, local application of exogenous compounds into the brain is also possible using microiontophoresis.[262] This technique avoids the blood pressure effect and reduces the amount of the compound that needs to apply. Moreover, stimulation of the trigeminal ganglion might be of interest since it would provide a model that is closer to a migraine attack than perivascular electrical stimulation. In addition, this set up also need improvements to allow a proper investigation of mice. Although we have already described mouse studies with our set up, subtle differences in vascular responses are more difficult to discriminate in mice than in rat due to the size of the dural arteries in mice. The resolution of our system could

be improved by using a digital instead of an analog output system for the measurements of the dural artery. The higher resolution obtained will lead to a better signal-noise ratio in the measurements.

Since central mechanisms mediated via the trigeminocervical complex and the ventroposteromedial thalamic nucleus have been associated with migraine, these areas of the brain should be studied in further detail with our model, e.g. in relationship with the effect of female sex hormones. This approach would involve cell firing measurements and cerebral blood fluid sampling (making use of microdialysis) for the detection of neuropeptides in these parts of the brain after treatment with hormones *in vivo*.

Nederlandse samenvatting

Migraine is een neurovasculaire aandoening die gekenmerkt wordt door hevige hoofdpijn en gepaard gaat met fotofobie, fonofobie, misselijkheid en braken. De oorzaak is nog niet helemaal bekend. Migraine lijkt geassocieerd te zijn met activatie van het trigeminovasculaire systeem en afgifte van neuropeptiden door perivasculaire zenuwen. Een bekend neuropeptide dat vrijkomt is 'calcitonin gene-related peptide' (CGRP). CGRP is een potente vaatverwijder en veroorzaakt vaatverwijding in de bloedvaten in het hersenvlies door te binden aan CGRP receptoren. Deze vaatverwijding activeert het ganglion trigeminale, dat vervolgens andere delen van de hersenen stimuleert. Dit leidt tot de symptomen van migraine.

De zoektocht naar een optimale behandeling van migraine heeft een lange geschiedenis, maar tot op heden is het aantal doeltreffende antimigraine medicijnen beperkt. Zoals beschreven in de introductie van dit proefschrift zijn de huidige specieke medicijnen voor de acute behandeling van migraine de ergot alkaloïden en triptanen. Deze middelen veroorzaken vaatvernauwing van de bloedvaten in het harde hersenvlies, wat wordt beschouwd als één van de therapeutische effecten. Echter, deze vaatvernauwende eigenschap treedt niet alleen op in de bloedvaten in het hoofd, maar ook in de rest van het lichaam zoals de kransslagaders. Hierdoor zijn de ergot alkaloïden en triptanen gecontraindiceerd voor patiënten met hart- en vaatziekten. Om deze beperking van de huidige migraine behandeling te verbeteren en ook een betere effectiviteit te bewerkstelligen worden antimigraine middelen ontwikkeld die in de hersenen zelf werken, waardoor de vasculaire bijwerking vermeden worden. Hoewel deze centraal werkende middelen geen directe effecten hebben op de vasculaire tonus, kunnen ze wel andere mechanismen activeren of remmen die indirect betrokken zijn bij vaatverwijding. In dit proefschrift worden studies naar potentiële vasculaire effecten van toekomstige antimigraine middelen die een centraal therapeutisch effect hebben bediscussieerd.

CGRP receptor

Zoals eerder beschreven speelt CGRP een rol in het ontstaan van migraine. Hierdoor worden CGRP receptoren beschouwd als een aangrijpingspunt voor de behandeling van migraine. Het karakteriseren van de CGRP receptoren heeft geresulteerd in de ontwikkeling van CGRP receptor antagonisten die effectief zijn in de behandeling van migraine, deze worden momenteel verder onderzocht in klinische studies. De therapeutische mechanismen van de CGRP receptor antagonisten en hun effecten op de vasculaire tonus zijn nog niet compleet duidelijk. In **hoofdstuk 4** hebben we aangetoond dat de CGRP receptor antagonist, telcagepant, de door CGRP geïnduceerde vaatverwijding blokkeert en geen direct vaatvernauwend effect heeft in meningeaal arteriën van de mens. Ook hebben we functionele CGRP receptoren in deze bloedvaten kunnen lokaliseren. De resultaten suggereren dat het therapeutische effect van telcagepant gedeeltelijk verklaard kan worden door blokkade van meningeale

vaatverwijding die optreedt tijdens een migraine aanval.

Met het oog op potentiële cardiovasculaire bijwerkingen hebben wij telcagepant ook in kransslagaders van verschillende diameter onderzocht, wat in **hoofdstuk 5** is beschreven. Net zoals in de meningeaal arteriën laten onze resultaten zien dat telcagepant geen direct vaatvernauwend effect heeft op kransslagaders van de mens, in tegenstelling tot de triptanen. Op basis van onze bevindingen lijkt telcagepant een potentieel antimigraine middel te zijn dat geen rechtstreeks vaatvernauwend effect heeft in cardiovasculair gezonde patiënten. In **hoofdstuk 5** hebben wij ook laten zien dat het effect van telcagepant tot stand komt door het blokkeren van de CGRP receptor. Dit hebben we aangetoond door middel van een biochemische studie waarbij het cAMP niveau gemeten wordt. Telcagepant was in deze studie selectief in staat om een toename in het cAMP niveau die door activatie van de CGRP receptoren tot stand kwam te blokkeren. We hebben ook aangetoond dat CGRP receptoren aanwezig zijn in de kransslagaders van de mens.

De CGRP receptor antagonisten zijn ontwikkeld als acute behandeling van migraine. Echter, deze middelen zouden ook als preventieve behandeling gebruikt kunnen worden, bijvoorbeeld in patiënten met menstruele migraine, waarvan het ontstaan afhankelijk lijkt van fluctuaties in de oestrogeen spiegels in het bloed. Om de mogelijke rol van oestrogeen in migraine te onderzoeken heeft onze groep het neurovasculaire effect van verschillende geslachtshormonen in ratten bestudeerd met behulp van het intravitale microscopie model. Wij hebben in het verleden aangetoond dat de vaatverwijding in de bloedvaten in het harde hersenvlies die geïnduceerd wordt door elektrische stimulatie verhoogd wordt in aanwezigheid van hoge plasmaspiegels oestrogeen. Dit suggereert dat oestrogeen spiegels de afgifte van CGRP verhogen. Gezien het feit dat de abrupte daling van oestrogeen spiegels rond de eerste dag van de menstruatie betrokken lijkt bij menstruele migraine hebben wij onderzocht wat de gevolgen van een onttrekking van oestrogeen zijn op de door CGRP geïnduceerde vasculaire effecten. In **hoofdstuk 6** demonstreerden wij dat een snelle afname van plasma oestrogeen concentraties het vaatverwijdende effect van exogeen CGRP niet beïnvloedt. Deze resultaten suggereren dat CGRP receptoren in de bloedvaten in het harde hersenvlies niet veranderd zijn na de afname van de oestrogeen concentratie. Echter, om hierover meer conclusies te kunnen trekken moeten er nog meer analyses uitgevoerd worden om deze bevinden te ondersteunen. Dit kan gedaan worden door receptor expressie te bepalen op eiwit en mRNA niveau. Verder zal deze studie aangevuld worden met experimenten waarbij er gekeken wordt naar de afgifte van CGRP door de perivasculaire zenuwen, met behulp van hetzelfde model.

Andere niet-vasculaire targets

De centrale en vasculaire effecten van toekomstige medicijnen, waarvan wordt aangenomen dat vasculaire bijwerkingen afwezig zullen zijn, zijn gedetailleerd in de introductie beschreven. In dit proefschrift hebben we beschreven dat stimulatie van de ionotrope

glutamaat receptor, evenals de PACAP en VIP receptoren, de vasculaire tonus direct of indirect kan beïnvloeden.
Glutamaat receptor antagonisten lijken effect te hebben op mechanismen die betrokken zijn bij de pathofysiologie van migraine, inclusief blokkade van trigeminovasculaire nociceptie in de nucleus cervical trigemini. Echter, omdat activering van glutamaat receptoren op zenuwen ook effect kan hebben op de afgifte van vasculair actieve stoffen, kan blokkade van deze receptoren ook effect hebben op vasculaire tonus. Inderdaad hebben wij in **hoofdstuk 7** aangetoond dat de NMDA glutamaat receptor antagonisten in staat zijn de vaatverwijding in de bloedvaten in het harde hersenvlies te remmen doordat zij de afgifte van vasoactieve peptiden uit perivasculaire zenuwen blokkeren. Echter, de kainaat glutamaat receptor antagonist, LY466195, heeft geen direct of indirect effect op de vaatverwijding. Daarom suggereren onze resultaten dat het therapeutische mechanisme van de kainaat glutamaat receptor antagonisten neuronaal gemedieerd is, waarbij de perivasculaire afgifte van CGRP of de vasculaire CGRP receptoren niet betrokken zijn. Inderdaad is door anderen beschreven dat kainaat glutamaat receptor antagonisten mogelijk de glutamaat signaaltransductie in de nucleus ventroposteromedialis thalami blokkeren. Echter, omdat ionotrope glutamaat receptoren betrokken zijn bij veel fysiologische processen kunnen bij blokkade van deze receptoren niet alleen mogelijk vasculaire bijwerkingen optreden, maar ook neuronale bijwerkingen.

Het andere aangrijpingspunt voor mogelijke antimigraine middelen met een centraal effect dat wij bestudeerd hebben zijn de PACAP en VIP receptoren. PACAP en VIP induceren vaatverwijding in hersenvaten en veroorzaken na toediening bij mensen hoofdpijn. Beide eiwitten worden afgegeven door de parasympatische zenuwen om de vaattonus en hemodynamiek in de hersenen te reguleren. Het is ook bekend dat deze eiwitten zenuwsignalen in de hersenen activeren, wat leidt tot pijnperceptie. Het is interessant dat PACAP, maar niet VIP, ook migraine-achtige hoofdpijn veroorzaakt. Dit maakt de receptoren waar PACAP op aangrijpt een belangwekkend potentieel aangrijpingspunt voor de behandeling van migraine. In **hoofdstuk 8** is de studie beschreven waarbij wij hebben laten zien dat het mechanisme voor de migraine-achtige hoofdpijn door PACAP waarschijnlijk niet gerelateerd is aan vaatverwijding. Dit is gebaseerd op onze resultaten waarbij PACAP maar een beperkt effect heeft op de verwijding van menselijke bloedvaten uit het harde hersenvlies. VIP, dat geen migraine-achtige hoofdpijn veroorzaakt, induceert daarentegen een duidelijker vaatverwijding dan PACAP. Onze resultaten suggereren dat de door PACAP geïnduceerde migraine-achtige hoofdpijn door een centraal mechanisme veroorzaakt wordt waarbij PAC receptor betrokken is. De PAC receptor kan als toekomstig potentieel aangrijpingspunt gebruikt worden dat beperkte directe vasculair effecten heeft. Er moet echter rekening gehouden worden met het mogelijk indirecte effect van de PACAP receptor op de vaattonus. Om meer kennis over de rol van deze receptor en mogelijke indirecte vasculaire effecten te krijgen zijn er verdere

studies nodig.
Met onze studies hebben wij aangetoond dat neuronale aangrijpingspunten ook betrokken kunnen zijn bij de regulatie van de vasculaire tonus. In **hoofdstuk 2** hebben wij voor verschillende toekomstige antimigraine middelen beschreven wat de mogelijke vasculaire effecten zijn op grond van de literatuur. In tegenstelling tot de huidige antimigraine middelen (ergot alkaloïden en triptanen) hebben deze toekomstige antimigraine middelen geen direct vaatvernauwend effect. Deze middelen kunnen echter wel een vaatverwijding voorkomen door het blokkeren van receptoren (bijv. CGRP receptor antagonisten), of door de afgifte van vaatverwijdende stoffen te remmen. Het moet nog onderzocht worden of de vasculaire effecten van de toekomstige antimigraine middelen bijdragen aan het therapeutisch effect, of alleen maar tot mogelijke vasculaire bijwerkingen leiden. Hoe dan ook, de verbeterde cardiovasculaire veiligheid van de nieuwe generatie antimigraine middelen is relevant voor patiënten die triptanen niet mogen gebruiken. Natuurlijk zal een antimigraine middel dat volledig vrij is van vasculaire effecten voor de veiligheid van deze patiënten beter zijn dan een antimigraine middel met een mild vasculair effect.

Cardiovasulaire effecten van 5 $HT_{1B/1D}$ receptor agonisten

Sumatriptan was de eerste triptaan die werd gebruikt voor de behandeling van migraine. Deze selectieve agonist voor de 5 $HT_{1B/1D}$ receptoren veroorzaakt vaatvernauwing in de bloedvaten in het hoofd. Dit effect werd oorspronkelijk beschouwd als het therapeutische effect van de triptanen. Echter, tegenwoordig lijken ook de remming van de neuropeptiden afgifte door perivasculaire zenuwen en remming van neuronale activiteit die centraal pijn vermindert via de 5-HT_{1D} en de 5-HT_{1F} receptor bij te dragen aan het therapeutische effect van de triptanen. Door de aanwezigheid van de 5 $HT_{1B/1D}$ receptor in de kransslagaders geeft behandeling met triptanen een verhoogde kans op coronaire bijwerkingen. Onze groep heeft eerder aangetoond dat het vaatvernauwende effect door de therapeutische concentratie van triptanen in kransslagaders beperkt is. In deze studies werden echter enkel de grote kransslagaders getest door de destijds beperkte experimentele opties, terwijl zowel grote als kleine kransslagaders vaten van belang kunnen zijn bij het ontstaan van myocardiale ischemie. Door de jaren heen zijn er mogelijkheden bijgekomen om kleine vaten te bestuderen. Daarom hebben we het vaatvernauwend effect van sumatriptan vergeleken in grote en kleine kransslagaders van de mens. Dit zal meer informatie geven over de cardiovasculaire effecten van triptanen. In **hoofdstuk 9** hebben we bevestigd dat het vaatvernauwend effect van sumatriptan inderdaad gering is in de grote kransslagaders van de mens. Het is opmerkelijk dat sumatriptan beduidend meer vaatvernauwing induceert in de kleine kransslagaders van de mens dan in de grote vaten, terwijl deze regionale verschillen niet te zien zijn wanneer de vaatvernauwing werd geïnduceerd door 5-HT. Een soortgelijk verschil van reacties in grote en kleine menselijke coronaire vaten werd eerder ook door ons gezien met vaatverwijding door CGRP. Omdat de

CGRP receptor expressie in de grote en kleine vaten hetzelfde lijkt te zijn, kan het verschil tussen grote en kleine kransslagaders waarschijnlijk niet verklaard worden door verschillen in receptor dichtheid, maar wellicht wel door verschillen in intracellulaire receptor-effect koppeling in de grote en kleine vaten (**hoofdstuk 5**). De verschillen waargenomen met sumatriptan moeten natuurlijk verder geanalyseerd worden. Eén van de studies die wij zullen uitvoeren is het bepalen van de receptor expressie met eiwit en mRNA analyses in de grote en kleine menselijke kransslagaders. Verrassend genoeg kwam het vaatvernauwend effect van sumatriptan in de kleine kransslagaders overeen met dat in de bloedvaten in het harde hersenvlies van de mens. Hierdoor is de aanname dat triptanen cranioselectief zijn niet meer correct. Echter, de klinische ervaring van twee decennia heeft laten zien dat het gebruik van de triptanen, rekening houden met de contraindicaties, heel veilig is. Onze bevindingen herbevestigen wel dat het gebruik van de triptanen gecontraïndiceerd moet blijven in patiënten met hart- en vaatziekten.

Behalve triptanen zijn er andere 5-HT liganden die ook cardiovasculaire bijwerkingen hebben. Bijvoorbeeld de 5-HT_4 receptor agonist, tegaserod. Deze stofwerd gebruikt voor de handeling van darmproblemen, maar heeft ook affiniteit voor de 5-HT_{1B} receptor. Dit zou misschien de hartproblemen kunnen verklaren die in enkele met tegaserod behandelde patiënten opgetreden zijn. In **hoofdstuk 10** hebben wij laten zien dat tegaserod niet in staat is vaatvernauwing te veroorzaken in menselijke kransslagaders. Ook remt tegaserod de vaatverwijding door sumatriptan niet. Dit suggereert dat tegaserod noch als agonist, noch als antagonist werkt op de 5-HT_{1B} receptor van menselijke kransslagaders. Daarom zijn de cardiovasculaire bijwerkingen die optreden na gebruik van tegaserod niet een gevolg van vaatvernauwing van de kransslagaders. Anderzijds leidt activatie van de 5-HT_4 receptor wel tot een samentrekking van de geïsoleerd menselijk hartspierweefsel, wat de cardiovasculaire bijwerking kan verklaren. In **hoofdstuk 11** hebben wij het samentrekkend effect van verschillende 5-HT_4 receptor agonisten onderzocht in geïsoleerd hartspierweefsel van de mens. Wij hebben aangetoond dat de 5-HT_4 receptor agonisten, cisapride en tegaserod, inotrope effecten kunnen induceren. Alle 5-HT_4 receptor agonisten die wij hebben onderzocht gedroegen zich als partiële 5-HT_4 receptor agonisten. Gebaseerd op onze resultaten en die van andere studies, denken wij dat de klinische gevallen van hartproblemen die optreden na de behandeling met tegaserod ook niet het gevolg is door het samentrekkend effect in de hartspieren.

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About the author

De auteur van dit proefschrift werd geboren op 12 december 1981 te Shatin, Hong Kong. Na het behalen van haar VWO diploma in 2001 op het Internationaal College Edith Stein, begon ze aan de studie Bio-Farmaceutische Wetenschappen aan de Universiteit Leiden. Haar eerste stage heeft zij gedaan op de afdeling Toxicologie van het Leiden Amsterdam Center for Drug Research (LACDR). Onder leiding van dr. Bob van Water deed zij *in vivo* onderzoek naar het effect van cytostatica behandeling in combinatie met het blokkeren van Focal Adhesion Kinase in borst tumor cellen van de rat. Hierna deed zij haar tweede onderzoeksstage op de afdeling Farmacologie van het LACDR, dit onderzoek is uitgevoerd in samenwerking met het bedrijf ToBBB en het Leidse Institute of Chemistry, afdeling Biochemie. Onder begeleiding van dr. Bert de Boer, dr. Pieter J. Gaillard en Prof. dr. Mathieu Noteborn heeft zij onderzoek gedaan naar een mogelijke behandeling van hersentumoren. In dit onderzoek heeft ze het specifiek tumor dodende eiwit, apoptine, gepoogd in de cel te transporteren met behulp van een carrier molekuul. Na het afronden van haar studie aan de Universiteit Leiden in 2006 heeft zij tijdelijk gewerkt als onderzoeksanalist bij ToBBB om bovengenoemde onderzoek voort te zetten. Vanaf februari 2007 is zij begonnen aan haar promotieonderzoek op de afdeling Inwendige Geneeskunde, sectie Vasculaire Geneeskunde en Farmacologie aan het Erasmus Medisch Centrum. Onder begeleiding van Prof. dr. A.H. Jan Danser als promotor en dr. Antoinette Maassen van den Brink als copromotor heeft dit geresulteerd in dit proefschrift.

Gedurende de afgelopen vier jaar heeft zij samengewerkt met de afdelingen Neurochirurgie en Thoraxchirurgie (Hartkleppenbank) van het Erasmus Medisch Centrum. Ook heeft zij de mogelijkheid gekregen om samen te werken met Prof. dr. Carlos M. Villalón van het Departamento de Farmacobiología, Cinvestav-Coapa, México, dr. Lars Edvinsson van het Department of Internal Medicine, Lund University Hospital, Lund, Zweden, en Prof. dr. Jan de Hoon van het Center for Clinical Pharmacology, University Hospitals Leuven, Leuven, België.

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Full Papers

Chan KY, Vermeersch S, de Hoon J, Villalón CM, MaassenVanDenBrink A. Potential vascular (side) effects of prospective antimigraine drugs. Pharmacology and Therapeutics, In Press.

Chan KY*, Edvinsson L*, Eftekhari S, Kimblad PO, Kane SA, Lynch J, Hargreaves HJ, de Vries R, Garrelds IM, van den Bogaerdt AJ, Danser AHJ, MaassenVanDenBrink A. Characterization of the CGRP receptor antagonist telcagepant (MK-0974) in human isolated coronary arteries. Journal of Pharmacology and Experimental Therapeutics, 2010, 334: 746-52. * both authors have contributed equally to the manuscript.

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General academic and research skills *(11 ECTS)*

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	2nd European Headache and Migraine Trust International Congress 2010, Nice, France. *Cranioselectivity of sumatriptan revisited (oral poster presentation).*
2009	Wetenschapsdagen Interne Geneeskunde 2009, Antwerpen, België. *Distinct effects of several glutamate receptor antagonists on rat dural artery diameter in an intravital microscopy model (oral presentation).*

FIGON Dutch Medicines Days 2009, Lunteren, The Netherlands. *Characterization of PACAP and VIP receptors in the human meningeal and coronary artery (oral presentation).*

2008 Wetenschapsdagen Interne Geneeskunde 2008, Antwerpen, België. *Investigation of the role of glutamate receptors in migraine using intravital microscopy (poster)*.

FIGON Dutch Medicines Days 2008, Lunteren, The Netherlands. *Distinct effects of several glutamate receptor antagonists on rat dural artery diameter by intravital microscopy model (oral presentation)*.

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WorldPharma 2010 Congress, Copenhagen, Denmark.

2nd European Headache and Migraine Trust International Congress 2010, Nice, France.

2009 5th International Meeting "Steroids and Nervous System", Torino, Italy.

18th International Headache Research Seminar, Copenhagen, Denmark.

19th ADMA Annual Meeting, Amsterdam, The Netherlands

14th International Headache Congress, Philadelphia, USA.

2008 Serotonin Satellite Club meeting 2008, Oxford, UK.

European Headache and Migraine Trust International Congress 2008, London, UK.

Grants & Awards

2009 Travel award for young scientist, 5th International Meeting "Steroids and Nervous System", Torino, Italy.

2008 Travel award of IHS Trainee and Residents Sub-committee. European Headache and Migraine Trust International Congress London, UK.

Prize for the highest Scoring Abstract in the poster session Migraine- Basic Experimental Studies, European Headache and Migraine Trust International Congress London, UK.

2007 The second prize of the FIGON poster prize, FIGON Dutch Medicines Days, Lunteren, The Netherlands.

List of abbreviations

5-HT	5-Hydroxytryptamine
5-MeOT	5-Methoxytryptamine;
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
cAMP	Cyclic adenosine monophosphate
CGRP	Calcitonin gene-related peptide
CLR	Calcitonin-like receptor
CNS	Central nervous system
CSD	Cortical spreading depression
DHE	Dihydroergotamine
eNOS	Endothelial nitric oxide synthase
GTN	Glyceryl trinitrate
i.p.	Intraperitoneal route of administration
i.v.	Intravenous route of administration
iNOS	Inducible nitric oxide synthase
L-NAME	Nω-nitro-l-arginine methyl ester
MAP	Mean arterial blood pressure
MCA	Middle cerebral artery
NMDA	N-methyl-D-aspartate
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
PACAP	Pituitary adenylate cyclase activating polypeptide
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
RAMP1	Receptor activity modifying protein 1
SSS	Superior sagittal sinus
TRPV1	Transient receptor potential cation channel, subfamily V, member 1
VIP	Vasoactive intestinal peptide
VPM	Ventroposteromedial thalamic nucleus

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