Pharmacological analysis of α_{1L}-adrenoceptors

ISBN 90-9014273-8

PHARMACOLOGICAL ANALYSIS OF α_{1L}-ADRENOCEPTORS

Farmacologische analyse van α_{1L}-adrenerge receptoren

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof.dr.ir. J.H. van Bemmel
en volgens besluit van het college voor promoties

De openbare verdediging zal plaatsvinden op
donderdag 30 november 2000 om 13.30 uur

door

Wiro Bartholomeus Stam
geboren te Goirle

Promotiecommissie

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Financial support by the Dr Saal van Zwanenberg foundation and the Netherlands Heart Foundation for the publication of this thesis is gratefully acknowledged

Publication of this thesis was financially supported by Pfizer bv

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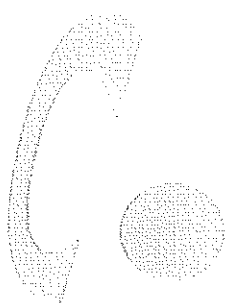
Chapter 1

Introduction



α_1-Adrenoceptor and vasopressin receptor subclassification

Development and current state

Historical background of α-adrenoceptor classification

It is well established that noradrenaline, released in response to sympathetic stimulation, as well as adrenaline, the hormone released from the adrenal medulla, interact with specific receptors (*adrenoceptors*) that are located in the membrane of the vascular smooth muscle cells. Ahlquist [1] proposed a division of adrenoceptors into α- and ß-adrenoceptors, on the basis of different agonist potency orders in vascular smooth muscle preparations. The α-adrenoceptor mediated vasoconstriction and the ß-adrenoceptor mediated vasodilation. This subdivision in α and ß, was subsequently supported by the development of selective ß- and α-adrenoceptor antagonists [2].

During the 1970's it became clear that there were subtypes of the α-adrenoceptor. At first the α-adrenoceptors were subclassified on an anatomical basis into prejunctional α_2-adrenoceptors and postjunctional α_1-adrenoceptors [3]. Berthelsen & Pettinger [4] noted that this anatomical classification was not completely satisfactory and suggested to reclassify α-adrenoceptors on a functional basis. According to this scheme α_2-adrenoceptors mediated inhibitory responses (like inhibition of the release of neurotransmitter and renin etc.), whereas α_1-adrenoceptors mediate excitatory responses (like the vasoconstriction). However, shortly thereafter it became clear that in some vascular preparations not only α_1- but also α_2-adrenoceptors caused vasoconstriction [5-7]. From that time a classification scheme of α-adrenoceptors into α_1 and α_2 evolved that is neither anatomical nor functional, but is based on the relative potency of selective agonists and antagonists [8]. Examples of selective α_1-adrenoceptor agonists are phenylephrine, methoxamine and cirazoline, while UK 14,304, BHT 920 and clonidine behave as selective α_2-adrenoceptor agonists. In the case of antagonists, prazosin is regarded as α_1-selective and rauwolscine and yohimbine as α_2-selective. Noradrenaline and phentolamine are examples of a relatively nonselective α-adrenoceptor agonist and antagonist, respectively.

Because of the differences in the receptors it has been suggested that a classification of adrenoceptors into three groups: α_1, α_2 and ß is more appropriate that the historical division into two (α and ß) classes [8]. The rationale behind this reasoning is threefold. First, differences in affinities of selective compounds for these three classes are large (3 to 4 orders of magnitude). Second, the amino acid sequences are more consistent with three rather than two major types. Third, the three classes couple to different second messenger systems. ß-Adrenoceptor subtypes, stimulate adenylyl cyclase resulting in the generation of cAMP, α_1-adrenoceptors are believed to stimulate the phosphoinositide metabolism and α_2-adrenoceptors inhibit adenylyl cyclase and decrease cAMP levels [9].

The evolution of α_1-adrenoceptor subclassification since the early 80's

(see Figure 1 for historical milestones)

At present radioligand binding and molecular cloning studies identified four different α_2-adrenoceptor subtypes (α_{2A}, α_{2B}, α_{2C} and α_{2D}) (see [8, 10]). However, it is currently believed that the α_{2D}-adrenoceptor represents a species variant of the human α_{2A}-adrenoceptor [8, 11].

The subclassification of α_1-adrenoceptors by radioligand binding and molecular cloning has occurred in a quite complementary fashion (see Figure 1 for the historical milestones). However, this has not yet resulted in a classification of α_1-adrenoceptors, with functional identified subtypes studies being fully in congruence with the outcomes from radioligand binding and molecular cloning studies (see [8]).

Radioligand binding and molecular cloning studies. In radioligand binding studies the first suggestion for receptor heterogeneity came from studies performed by Battaglia *et al.* [12], who reported shallow competition curves of [^{3}H]prazosin binding in rat cerebral cortex for both phentolamine and WB-4101. Further studies by Morrow and co-workers [13, 14] confirmed and extended these findings and concluded that [^{3}H]prazosin labelled two α_1-adrenoceptor subpopulations. Subsequently, these authors designated the binding site with high affinity for WB-4101 and phentolamine as α_{1A} and the binding site with lower affinity as α_{1B} (Table 1). The existence of two receptor subtypes was further substantiated by the identification of various other ligands, like 5-methyl-urapidil and (+)-niguldipine, which also discriminated between the two subtypes displaying higher affinity for the α_{1A}-adrenoceptor (Table 1; see [8, 15, 16]). Interpretation of the results obtained with receptor alkylating compounds like chloroethylclonidine (CEC), phenoxybenzamine (PBZ) and benextramine also identified two α_1-adrenoceptor subtypes. Johnson & Minneman [17] demonstrated that CEC, only partially inactivated the α_1-adrenoceptor population, whereas PBZ and benextramine eliminated the complete population. Subsequently, Han *et al.* [18] examined the effects of CEC in various tissues and suggested the existence of CEC-sensitive and a CEC-insensitive α_1-adrenoceptor subtype. Further experiments by Minneman's group demonstrated that the CEC-insensitive and the CEC-sensitive corresponded with the subtypes designated α_{1A} and α_{1B}, respectively, by Morrow and co-workers [9, 19].

The first cloned receptor subtype was isolated from a hamster vas deference cell line [20]. Using the classification tools that were identified in radioligand binding studies, this receptor was designated as α_{1B}, because of its low affinity for WB-4101 and phentolamine and its sensitivity for inactivation by CEC (Table 1; [20, 21]). A second cDNA clone coding for a novel α_1-adrenoceptor subtype was isolated from bovine brain [22, 23] was designated α_{1C}, since this novel subtype displayed properties of both α_{1A} and α_{1B}-adrenoceptors. This subtype was CEC-sensitive (α_{1B}) yet displayed antagonist binding properties characteristic of α_{1A} (high affinity for WB-4101 and phentolamine).

Initially, a third receptor, isolated from rat cerebral cortex by Lomasney *et al.* [21], was believed to correspond to the pharmacologically defined α_{1A}-adrenoceptor, because of its high affinity for WB-4101 and relative resistance to inactivation by CEC. Shortly after this finding was published, Perez and co-workers [24] reported the isolation of a nearly identical clone. However, upon pharmacological characterisation these authors concluded that the receptor displayed a unique profile (CEC-sensitive) that was not in agreement with that of the α_{1A}-adrenoceptor. Perez *et al.* [24] argued that a sequencing error in the cloned α_1-adrenoceptor by Lomasney *et al.* [21] accounted for the small sequence dissimilarity between the studies. Furthermore, the incongruity concerning CEC sensitivity could be explained by a difference in concentration and time of exposure. Consequently, Perez and colleagues proposed the existence of a fourth α_1-adrenoceptor subtype, which they designated α_{1D} [24]. This α_{1D}-adrenoceptor subtype displayed high affinity for WB-4101, was inactivated by CEC and displayed low affinity for phentolamine, 5-methylurapidil and (+)-niguldipine. Subsequently, Schwinn & Lomasney [25] suggested that the cloned α_{1A}- and α_{1D}-receptor were identical and introduced the rather confusing designation of $\alpha_{1A/D}$-adrenoceptor to distinguish it from the pharmacologically defined α_{1A}-adrenoceptor. They suggested the existence of four α_1-adrenoceptors (α_{1A}, α_{1B}/α_{1b}, α_{1C}/α_{1c}, α_{1ad})

Figure 1. Historical milestones in the search for a classification of α_1-adrenoceptor subtypes. Results from radioligand binding studies (right column), functional studies (middle) and molecular cloning studies (left column) are displayed in parallel.
Abbreviations: AA: amino acid, Aff.: affinity, AR: adrenoceptor

	radioligand binding	Functional	Molecular cloning
1982		*In vitro* assays: evidence for multiple α_1-AR on the basis of different affinities for prazosin and PBZ [35,37,38] α_{1H}: high aff. for prazosin and yohimbine; α_{1L}: low aff. for prazosin and yohimbine	
1986	α_{1A}: high aff.: Phentolamine, WB-4101 α_{1B}: low aff.: Phentolamine, WB-4101[14]		
1987	α_{1A}: **insensitive to CEC** rat kidney, hippocampus, vas deferens caudal artery α_{1B}: **sensitive to CEC** rat liver, spleen[17-19]		
1988	**5-Mu and (+) niguldipine:** α_{1A}-AR selective antagonists[15,16]		α_{1A}: **Hamster α_{1b}-AR** [20]
1990		Vascular tissue(Muramatsu)[43] α_{1A}: high aff. for prazosin ($pA_2 > 9.5$) α_{1L}: low aff. for prazosin ($8 < pA_2 < 9$) =WB-4101 α_{1N}: low aff. for prazosin ($8 < pA_2 < 9$) < WB-4101 < HV723	**Bovine α_{1c}-AR** cloned [22] High aff. for phentolamine, WB-4101 sensitive to CEC **Rat α_{1b}-AR** cloned [51] 96.8% AA homology with hamster α_{1b}
1991			**Rat α_{1a}-AR** cloned (Lomasney)[21] High aff. for WB-4101 insensitive to CEC hippocampus, vas deferens, cerebral cortex **Rat α_{1d}-AR** cloned (Schwinn) [24] High aff.: WB-4101 low aff.: phentolamine, 5-mu, (+)-niguldipine, sensitive to CEC *note: same DNA as lomasney*[21]
1992	α_{1D}: 5-mu but not WB-4101 discriminated between α_{1B} and a new subtype in rat heart and lung [27,28] that was CEC insensitive and was later defined as α_{1D}[32]		Schwinn & Lomasney [25]: **Rat α_{1b}-AR= Rat α_{1d}-AR** introduce **Rat α_{1B}-AR** **Human α_{1b}-AR** cloned [83] 98% AA homology with hamster α_{1b}-AR
1993			**Human α_{1c}-AR** cloned from prostate [84] 92% AA homology with bovine α_{1b}-AR present: heart, brain, liver, prostate absent: kidney, lung, adrenal, aorta,pituitary
1994/1995			**Rat α_{1c}-AR** cloned [31] 91% AA homology with bovine α_{1c}-AR present: heart, vas deferens, kidney, hippocampus absent: spleen, liver
		Classification proposal [43]	
	high affinity for prazosin: $pK_D > 9$ α_{1A} (=α_{1C}) α_{1B} α_{1D}* (=α_{1AD})		low affinity for prazosin $pK_D < 9$ α_{1L}
		IUPHAR [2]	
	Recognised α_1-adrenoceptores: α_{1A}, α_{1B} α_{1D} which all display high affinity for prazosin α_{1L}-adrenoceptor is not yet included		

Table 1 Binding affinities of some "important" antagonists for the cloned α_1-adrenoceptor subtypes. Data are means (n=1-11) of affinities reported elsewhere [21-24, 29, 30, 44, 53-56, 84-91]. The Bold font indicates the subtype selectivity of the antagonist.

	α_{1a}			α_{1b}			α_{1d}	
antagonist	*human*	*bovine*	*rat*	*human*	*hamster*	*rat*	*hamster*	*rat*
tamsulosin	**10.1**	**10.3**		9.1	8.9		9.9	9.7
prazosin	9.6	9.6	9.5	9.8	9.9	9.7	9.7	9.7
WB-4101	**9.4**	**9.6**	**9.0**	8.3	8.3	7.7	**9.0**	**9.2**
5-Mu	**8.7**	**8.8**	**8.4**	7.1	6.9	6.9	7.6	7.5
BMY 7378		6.5			7.1			**8.6**
Niguldipine	**8.7**	**9.1**	**8.3**	6.9	7.2	7.8	6.5	6.9
RS-17053	**9.0**	**9.5**		7.3			7.1	7.8
Rec 152739	**9.1**	**9.0**		7.6	7.3		8.4	7.6
indoramin	**8.4**	**8.2**		7.7	6.9		7.0	7.3

anticipating on the official nomenclature of α_1-adrenoceptors [26], which refers to cloned subtypes by lower-case letters, whereas the upper-case letters refer to subtypes present in tissues. At about the same time, though in radioligand-binding studies, several groups produced evidence for a tissue correlate of the $\alpha_{1A/D}$-adrenoceptor. Hiramatsu *et al.* [27] and Geng-Sheng *et al.* [28] reported that within WB4101-high affinity receptor population, obtained after treatment with CEC, 5-methylurapidil could discriminate between two α_1-adrenoceptor binding sites in rat heart and lung. Both groups proposed the existence of a new CEC-insensitive α_1-adrenoceptor subtype different from α_{1A} with low affinity for 5-methylurapidil and high affinity for WB-4101.

Various groups [29-33] then showed that the pharmacological profile of the cloned α_{1C}-adrenoceptor corresponds to that of the pharmacologically-defined α_{1A}-subtype, which could suggest that α_1-adrenoceptors can be satisfactorily classified into three subtypes (α_{1A}, α_{1B} and $\alpha_{1A/D}$). In the current classification the confusing $\alpha_{1A/D}$-adrenoceptor designation was reclassified as α_{1D}-adrenoceptor [26].

Functional studies. In sharp contrast to the radioligand binding and molecular cloning studies where prazosin became established as a non-selective antagonist, early functional studies with antagonists have resulted in a classification that is mainly based on the selectivity of prazosin. Holck *et al.* [34] reported that within the same tissue, the rabbit main pulmonary artery, prazosin displayed a ten-fold higher affinity against clonidine ($pA_2 = 9.4$) than against methoxamine ($pA_2 = 8.4$). In this tissue, a similar difference was found for yohimbine. In the same year, Digges & Summers [35] showed that prazosin displayed a similar difference in inhibiting the noradrenaline-mediated response of rat aorta ($pA_2 = 9.4$) and that of rat portal vein ($pA_2 = 8.4$). Interestingly, in this case yohimbine did not display the same discriminatory potency. Upon a review of the available data, several authors independently noted a wide variation in affinity for prazosin and yohimbine in functional studies on different tissues [36-39]. These observations led Flavahan & Vanhoutte [38] to propose the existence of two distinct α_1-adrenoceptors: an α_{1H}-receptor which displays high affinity for prazosin and yohimbine and is preferentially stimulated by clonidine, while the α_{1L}-receptor has low affinity for prazosin and yohimbine and is preferentially stimulated by methoxamine. More recently, Muramatsu *et al.* [40] extended this subclassification based on

	Official nomenclature			*unofficial*	
compound	$\alpha_{1a/A}$	$\alpha_{1b/B}$	$\alpha_{1d/D}$	α_{1L}	α_{1N}*
prazosin	high	high	high	low	*low*
WB-4101	high	low		low	*low*
HV723	medium/low	low		low	*high*
competitive antagonist	***5-mu***		***bmy 7378***		***HV723***

Table 2
Overall scheme of the proposed α_1-adrenoceptor subtypes [26, 40-43].
** The α_{1N}-subtype is printed in italics to indicate that it is not widely recognised.*

functional studies in different vascular preparations with five α_1-adrenoceptor antagonists. On the basis of the pK_B values for prazosin and yohimbine, the α_1-adrenoceptor population could be classified into three subtypes, α_{1H}, α_{1L} or α_{1N}. The α_{1L}-adrenoceptor population defined by Flavahan & Vanhoutte [38] was subdivided on the basis of the observed affinities for yohimbine and the new α_1-adrenoceptor antagonist, HV723. In summary, the α_{1H}-adrenoceptor displays a high affinity for prazosin, the α_{1L}-adrenoceptor displays a low affinity for prazosin and yohimbine, whereas the α_{1N}-adrenoceptor also displays a low affinity for prazosin, a relatively high affinity for yohimbine and, in addition, a high affinity for HV723. Subsequently, in an attempt to harmonise these functionally recognised subtypes with the classification proposed from radioligand studies at that time, Muramatsu and co-workers proposed a scheme (Table 2) which recognises four α_1-adrenoceptors based on the affinities for prazosin, WB-4101 and HV723, with α_{1H} further divided into α_{1A} and α_{1B} [41, 42]. More recently, Ford and co-workers [43] suggested a similar subclassification, however, these authors did not include the α_{1N}-adrenoceptor in their scheme (Table 2).

Current classification

In attempt to obtain consensus on the nomenclature of α_1-adrenoceptors, Ford and co-workers [43] proposed a subclassification in receptors displaying high (pK_D>9: α_{1H}) and low affinity for prazosin (pK_D<9: α_{1L}). The α_{1A}-adrenoceptor was suggested to be equivalent to the α_{1C}-adrenoceptor and the α_{1D}-adrenoceptor replaces the confusing $\alpha_{1A/D}$ designation. The cloned receptor subtypes (α_{1A}, α_{1B}, α_{1D}) were classified in the α_{1H} class. Shortly afterwards, this classification was largely adopted by IUPHAR subcommittee on nomenclature for adrenoceptors [26]. The α_{1A}-(=α_{1C}), α_{1B}- and α_{1D}- (previously called $\alpha_{1A/D}$ or α_{1A}) adrenoceptors are now officially recognised as subtypes (Table 2). Because molecular cloning and radioligand binding data on α_{1L}-adrenoceptors is lacking this adrenoceptor was not designated as a separate subtype.

Initially, Hieble and colleagues [26] identified 5-methylurapidil as a selective α_{1A}-adrenoceptor antagonist and BMY 7378 as a selective α_{1D}-adrenoceptor antagonist. Later, RS-17053, RS-100329, Ro 70-004 and KMD-3213 were also identified as selective α_{1A}-adrenoceptor antagonists [44-46]. The binding affinities for various α_1-adrenoceptor subtypes of several widely used and important antagonists are presented in Table 1. Interestingly, until now a truly selective α_{1B}-adrenoceptor antagonist could not be identified. Although the preferential susceptibility to irreversible inactivation by CEC has been used to subclassify α_{1B}-adrenoceptors, the lack of a selective competitive antagonist has impeded a precise quantitative characterisation of α_{1B}-adrenoceptors. Initially, radioligand binding experiments suggested that spiperone [29, 47] and risperidone [43, 48] might be competitive, selective

α_{1B}-adrenoceptor antagonists. However, functional studies in rat, guinea pig and mouse spleen (functional α_{1B}-adrenoceptor tissues) were not able to confirm this [49, 50]. In Chapter 4 we investigated the functional pharmacolical profile of (+)-cyclazosin a compound that behaved as an α_{1B}-adrenoceptor selective antagonist in radioligand binding assays [51].

The α_{1A}- /α_{1L}- adrenoceptor controversy

In accordance with the cloned receptors, binding affinities at native α_1-adrenoceptor subtypes yielded high affinities for prazosin; pK_i = 9.9-10.1 for α_{1A} on rat submaxillary gland [44, 52, 53], pK_i = 10.1-10.2 for α_{1B} at rat liver [44, 54] and pK_i = 9.8 at α_{1D} rat aorta [55]. Moreover, functional experiments in rat aorta and rat spleen, functional α_{1D}- and α_{1B}-tissues [26], consistently yielded high affinity estimates for prazosin: pA_2 values were 9.4-10.0 and 9.1-10.0 for rat aorta [35, 40, 52, 54, 56, 57] and rat spleen [49, 52, 57, 58] respectively.

The controversy over the existence of an α_1-adrenoceptor subtype, which displays low affinity values for prazosin, now appears to focus on tissues that were initially characterised as functional α_{1A}-tissues like for example: rat mesenteric resistance vasculature [43], rat vas deferens [8, 43], rat portal vein [35, 59, 60] and human lower urinary tract (see [61]). The α_1-adrenoceptor mediated functional response of rat vas deferens is well studied. There is, however, no agreement on the antagonising potency of prazosin. Ohmura *et al.* [42] demonstrated high and low affinity binding sites for prazosin in the prostatic as well as the epidydimal portion of the rat vas deferens. Low affinity values for prazosin (pK_B=8.2-8.6) in both portions demonstrated that α_{1L}-adrenoceptors dominate the functional response [42, 54, 57]. However, other groups suggested that the contraction of rat vas deferens is mediated by α_{1A}-adrenoceptors displaying high affinity for prazosin (pA_2=9.2-9.3; [6, 49, 58, 62, 63]. The pA_2 values of several antagonists correlated best with affinities on α_{1a} clones, the response was not affected by CEC, and was antagonised by BMY 7378 with low affinity (pA_2 = 6.7), thereby excluding α_{1B} and α_{1D}-adrenoceptor involvement [49, 62]. Interestingly, the more recently developed selective α_{1A}-adrenoceptor antagonist, RS-17053, recognises the previously defined α_{1A}-adrenoceptor (by the same group) in the prostatic and epididymal vas deferens with different affinities (pK_B = 8.3 and 9.5, respectively; [64, 65]). The low affinity estimate of 8.3 is in accordance with the affinity of RS-17053 that we estimated in the rat small mesenteric artery (SMA, Chapter 2)[66], another α_{1A}-/α_{1L}-tissue (see below). Similarly, in independent studies, α_1-adrenoceptors with low affinity (pA_2=8.4/8.5; [35, 57]) as well as high affinity (pA_2=9.2; [59, 64]) for prazosin were suggested to mediate the contraction of rat portal vein. The α_1-adrenoceptor of rat portal vein, however, displayed a 250 times lower potency for RS-17053 (pK_B = 7.1;[59]) than rat vas deferens. Interestingly, this low affinity of RS-17053 is similar to the antagonising potency that was found in human lower urinary tract and prostate (pA_2=7.3 and 7.1 [44, 64]), which is another representative of a tissue where α_{1A}- as well as α_{1L}-adrenoceptor have been implied to mediate the contraction (see [67]).

Rat mesenteric resistance vasculature: α_{1A} or α_{1L}

Although the rat isolated perfused mesentery was defined as a functional α_{1A}-adrenoceptor tissue displaying high affinity for prazosin (pA_2=9.3; [43, 68]), McPherson et al. [69] also estimated a low affinity for prazosin (pA_2=8.52) in this assay. Since small mesenteric arteries (SMAs; internal diameter 100-300 µm) are believed to contribute substantially to vascular resistance in rat [70-72], isolated SMA assays have been used widely as models of resistance vessels [70]. Högestatt & Andersson [73] and Nielsen & Mulvany [70] demonstrated that prazosin antagonises noradrenaline-mediated contractions of rat SMAs with high affinity (pA_2=9.58-9.84 and 9.23, respectively). Accordingly, it has been suggested that α_{1A}-adrenoceptors predominantly mediate noradrenaline-induced contraction of rat SMA [74, 75]. However, Schild analysis demonstrated complex antagonism by prazosin with its potency (pA_2) ranging from 8.8 to 9.6 and, therefore,

additional involvement of α_{1L}-adrenoceptors was suggested [74]. Van der Graaf *et al.* [76] found that despite significant correlation of antagonist affinity values with pK_i values at the cloned α_{1a}-adrenoceptor, the pA_2 value of prazosin in rat SMA (8.5) was more consistent with the profile of the pharmacologically-defined α_{1L}-subtype [38, 43, 76, 77]. The affinity of RS-17053 in rat SMA (pK_B=8.4; Chapter 2; [66]) was 35-fold lower than that reported by Ford et al. for antagonising pressor responses to noradrenaline in the perfused mesentery (pK_B=9.9; [44]); the latter being in agreement with functional affinity estimates for α_{1A}-adrenoceptors in rat perfused kidney (pA_2=9.8; [44]) and rat vas deferens (pA_2=9.5; [64]). Therefore, it appears that α_{1A}-adrenoceptors mediate the pressor response in rat perfused mesentery, whereas noradrenaline-induced contraction in rat isolated SMA is mediated by a different type of α_1-adrenoceptor, possibly α_{1L}.

Emerging picture

The different affinities in functional α_{1A}-/α_{1L}-adrenoceptor mediated responses indicate that RS-17053 can discriminate at least three α_{1A}-adrenoceptor subtypes in the rat. A high affinity estimate was demonstrated in epididymal rat vas deferens (pK_B = 9.5; [64]), rat perfused mesentery (pA_2 = 9.9; [44]) and rat perfused kidney (pA_2 = 9.9; [44]). Interestingly, this high affinity is similar to binding affinity of the α_{1c}-clone (pK_i = 9.5; [44]) and might represent the "classical" α_{1A}-adrenoceptor. In addition two low affinity subtypes have been defined: an intermediate affinity subtype was demonstrated in rat SMA and prostatic vas deferens (pK_B/pA_2=8.3; [65, 66]), and a low affinity subtype in rat portal vein (pK_B = 7.1; [59]). This low affinity subtype was also demonstrated in lower urinary tract tissues of humans and rabbits [44, 78, 79].

Interestingly, the classification scheme of Muramatsu et al. previously suggested two low affinity α_1-adrenoceptor subtypes, α_{1N} and α_{1L}(see Table 2), which could be discriminated by a different order of affinity for the antagonists HV723 and prazosin [40]. Although it was suggested that the low affinity receptor (pK_B for RS-17053 = ~7)

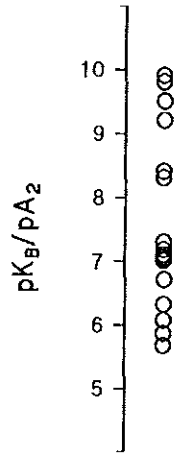


Figure 2
Range of reported affinities of RS-17053 for $\alpha_{1A/L}$-adrenoceptors in functional studies
(44,55,64-66,78,79,129-131)

present in human and rabbit prostate and rat epididymal vas deferens corresponded with Muramatsu's α_{1L}-adrenoceptor [42, 79-81], the data with HV723 on the "intermediate" low affinity (pK_B for RS-17053 =8.3) subtype are to scarce to even suggest full congruence with Muramatsu's scheme, with respect to the α_{1N} subtype [40, 76]. It should be noted, however, that the assumption of three α_1-adrenoceptor subtypes might well be an oversimplification of a range of affinities for RS-17053 that has been observed in functional $\alpha_{1A/L}$-adrenoceptor mediated responses (see Figure 2). Possibly more in line with the affinity range is the suggestion that the α_{1A}-adrenoceptor can present itself functionally in different affinity states [82]. Initially, it was shown that prazosin and RS-17053 bind with subnanomolar affinity to cloned α_{1a}-adrenoceptors, whereas their potency to functionally inhibit inositol phosphate production by the same cells was one log unit lower [82]. In Chapters 2 and 3 we further elaborate on the nature of the α_{1L}-adrenoceptor mediating the contraction of the rat SMA.

Vasopressin receptors

Each species usually has two neurohypophysial hormones: one belonging to the oxytocin family, involved in reproduction, and one belonging to the vasopressin family, involved in cardiovascular regulation. Arginine-vasopressin (AVP) is

believed to exert its action through binding to two major classes of receptors (V_1 and V_2) [92]. The V_1 receptors can be subdivided in V_{1a} and V_{1b} receptors. V_{1a} receptors, present on blood vessels and hepatocytes, mediate vasoconstriction and glycogenolysis, respectively [93]. V_{1b} receptors, present in the anterior pituitary, mediate ACTH-release [93]. An indirect vasopressor effect is established via V_2 receptors located in the renal tubule and collecting duct and they mediate an antidiuretic effect [93]. Oxytocin receptors mediate uterine contraction and milk-ejection in response to oxytocin [93]. In general, stimulation of OT and V_1 receptors results in increased production of inositol 1,4,5-triphosphate and 1,2-diacylglycerol and an increase in intracellular calcium concentration [92, 94-96], while V_2 receptors are associated with an increase in intracellular cAMP [92, 97-99]. Cloning of rat and human V_{1a} [100-102], V_2 receptor [97, 103, 104], V_{1b} [105, 106] and OT receptor [107, 108] have been reported. It should be noted that although AVP and OT have their characteristic responses they can interact and activate each other's primary receptor [109, 110].

A V_{1A} receptor mediated vasoconstrictor action is well-established in different species. AVP induced V_{1A} receptor mediated contractions of isolated human coronary [111], uterine [109], gastric [112] internal mammary [113], mesenteric [114], deferential [115] and cerebral arteries [114, 116, 117], rat small mesenteric arteries [118] and rabbit arteries [119], dog coronary resistance and femoral arteries [120, 121]. In addition to this overwhelming support for the induction of vasoconstriction by AVP, evidence for the (co-)existence of a vasodilator response to AVP in several regions of the circulation was provided. AVP was reported to dilate the rat pulmonary circulation [122, 123], canine large coronary [120, 121] and cerebral arteries [121, 124, 125] human cerebral [126] and mesenteric arteries [114]. Different pathways like stimulation of V_1 [121, 122], V_2 receptors [114, 126], in addition to an atypical pathway [114, 126] were reported to establish this vasodilator response. The recent discovery of operative cardiovascular OT receptors on a human vascular smooth muscle cell line [95] and in rat cardiac tissue [127] might result in a complex action of AVP and/or OT on the vasculature.

After characterisation of the vasopressin receptor(s) involved in the contraction of the rat small mesenteric artery and aorta (Chapter 5) we studied the interaction between AVP and noradrenaline in rat SMA (Chapter 6).

Aims and outline of the thesis

In the classical receptor concept the binding affinity is the only relevant parameter which accounts for an antagonist's capability to recognise a receptor and form a complex with it. Because this affinity is considered to be agonist and system independent, antagonist affinities for a given receptor are not expected to differ between functional and binding assays. Until recently the $\alpha_{1A/L}$-controversy was mainly based on the functional affinity estimates for prazosin, which discriminated functional α_{1A}- from α_{1L}-adrenoceptors by a one-log unit difference in affinity. This 'small' difference has not been taken serious by everyone. However, in 1996, Ford and colleagues developed a selective α_{1A}-adrenoceptor antagonist, RS-17053, which displayed more than 100-fold discriminatory potency between functional α_{1A}- and α_{1L}-adrenoceptors [44]. This finding further substantiated the α_{1A}/$_{1L}$-adrenoceptor controversy and provided a useful tool for further studies. Despite its recognised potential as a drug target in a cloning era, intensive cloning has thus far failed to identify a gene coding for the α_{1L}-adrenoceptor. This led several investigators to the belief that the α_{1L}-adrenoceptor might not exist as a separate genomic entity, but might be a conformational affinity state of the α_{1a}-gene product. However, traditional receptor

theory does not account for affinity states. Therefore, the $\alpha_{1A/1L}$-adrenoceptor controversy might pose a serious challenge for the classical concept of antagonist-receptor interaction. Though in the last decade this traditional theory has been questioned often by observations in genetically engineered systems such pressure is less common from native tissue systems. Because of the possible impact of this controversy the primary objective of this thesis was to further characterise and analyse the α_{1L}-adrenoceptor pharmacology in rat SMA. The SMA embodies a reference model for studying the $\alpha_{1A/1L}$-adrenoceptor controversy.

In **Chapter 2 and 3** we aim to determine the α_1-adrenoceptor subtype involved in the contractile response of rat SMA, and obtain further insight in its pharmacological profile. It has been suggested that the α_{1L}-adrenoceptor represents a pharmacological phenotype of the α_{1A}-adrenoceptor gene product that is determined by environmental conditions. In **Chapter 2**, by using functional pharmacological tools, we aim to identify factors that could or could not account for the observed profile. Furthermore, we investigate the possibility that α_{1A}- as well as α_{1L}-adrenoceptors are co-existing subtypes in the SMA, but the exhibition of either subtype might be favoured by experimental conditions.

Measurements of agonist affinity for α_1-adrenoceptors in functional studies displayed considerable variability within a given tissue. Variable receptor affinity rather than different subtypes, has been proposed to account for the variation in estimated agonist affinities [128]. Considering the variable receptor affinity for agonists, it is reasonable to assume that the affinity for other ligands, like antagonists, may also vary. Thus, variable receptor affinity could possibly account for the observed α_{1L}-adrenoceptor pharmacology in rat SMA. In order to investigate this variable affinity hypothesis in rat SMA we studied the agonism of noradrenaline by analysis of receptor inactivation experiments (**Chapter 3**).

Radioligand binding experiments initially proposed spiperone and risperidone as competitive, α_{1B}-adrenoceptor selective antagonists. However, functional studies were not able to confirm this. [29, 43, 47-50]. Therefore, we anticipated that also for the α_{1B}-adrenoceptor antagonist affinities measured in radioligand binding might differ from functionally measured estimates. In **Chapter 4** we aim to investigate this possibility by characterisation of the functional pharmacological profile of (+)-cyclazosin, a novel antagonist that displayed selectivity for α_{1b}-adrenoceptors in radioligand binding experiments.

In Chapters 2-4 we have focused on the pharmacological analysis of single receptor subtypes. However, the *in vivo* reality is that blood vessels are exposed to a variety of vasoactive substances, which stimulate different types of receptors simultaneously. Therefore, functional responses in *in vivo* physiological and pathophysiological situations will be the result of interactions between different receptor subtypes. Because of its importance, it was our objective to study and characterise the interaction between the α_{1L}-adrenoceptors and vasopressin receptors in rat SMA (**Chapter 6**). Vasopressin was chosen for three reasons: (1) vasopressin is an extremely potent vasoconstrictor agent and may therefore be involved in interactions even at low concentrations, (2) vasopressin has been suggested to be involved in pathological conditions and (3) the interaction between α_{1L}-adrenoceptors and vasopressin receptors has not been the subject of extensive study. In order to study the interaction thoroughly it was mandatory to first characterise the vasopressin receptor(s) that mediates vasopressin responses in rat SMA. This was the subject of study in **Chapter 5**.

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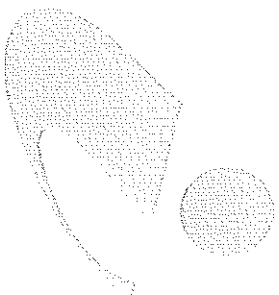
Chapter 2

Analysis of α_{1L}-adrenoceptor pharmacology in rat small mesenteric artery

Br. J. Pharmacol. (1999) 127, 661-670



Analysis of α_{1L}-adrenoceptor pharmacology in rat small mesenteric artery

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1 To illuminate the controversy on α_{1A}- or α_{1L}-adrenoceptor involvement in noradrenaline-mediated contractions of rat small mesenteric artery (SMA), we have studied the effects of subtype-selective α_1-adrenoceptor agonists and antagonists under different experimental conditions.

2 The agonist potency order in rat SMA was: A61603 >> SKF89748-A > cirazoline > noradrenaline > ST-587 > methoxamine. Prazosin antagonized all agonists with a low potency (pA_2: 8.29–8.80) indicating the involvement of α_{1L}- rather than α_{1A}-adrenoceptors.

3 The putative α_{1L}-adrenoceptor antagonist JTH-601, but not the α_{1B}-adrenoceptor antagonist chloroethylclonidine (10 μM) antagonized noradrenaline-induced contractions of SMA. The potency of the selective α_{1D}-adrenoceptor antagonist BMY 7378 against noradrenaline ($pA_2 = 6.16 \pm 0.13$) and of the selective α_{1A}-adrenoceptor antagonist RS-17053 against noradrenaline ($pK_B = 8.35 \pm 0.10$) and against the selective α_{1A}-adrenoceptor agonist A-61603 ($pK_B = 8.40 \pm 0.09$) were too low to account for α_{1D}- and α_{1A}-adrenoceptor involvement.

4 The potency of RS-17053 (pK_B/pA_2's = 7.72–8.46) was not affected by lowering temperature, changing experimental protocol or inducing myogenic tone *via* KCl or U46619.

5 Selective protection of a putative α_{1A}-adrenoceptor population against the irreversible action of phenoxybenzamine also failed to increase the potency of RS-17053 ($pA_2 = 8.25 \pm 0.06$ against A61603).

6 Combined concentration-ratio analysis demonstrated that tamsulosin, which does not discriminate between α_{1A}- and α_{1L}-adrenoceptors, and RS-17053 competed for binding at the same site in the SMA.

7 In summary, data obtained in our experiments in rat SMA indicate that the α_1-adrenoceptor mediating noradrenaline-induced contraction displays a distinct α_{1L}-adrenoceptor pharmacology. This study does not provide evidence for the hypothesis that α_{1L}-adrenoceptors represent an affinity state of the α_{1A}-adrenoceptor in functional assays. Furthermore, there is no co-existing α_{1A}-adrenoceptor in the SMA.

Keywords: A61603; α_1-adrenoceptors; BMY 7378; chloroethylclonidine; noradrenaline; resistance vessels; phenoxybenzamine; prazosin; RS-17053; small mesenteric artery (rat)

Abbreviations: 5-HT, 5-hydroxytryptamine creatine sulphate; A61603, N-[5-(4,5-dihydro-1H-imidazol-2yl)-2-hydroxy-5,6,7,8-tetrahydronaphthalen-1-yl] methanesulphonamide hydrobromide; BMY 7378, 8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4,5]decane-7,9-dione dihydrochloride; E/[A], concentration-effect; JTH-601, N-(3-hydroxy-6-methoxy-2,4,5-trimethylbenzyl)-N-methyl-2-(4-hydroxy-2-isopropyl-5-methyl-phenoxy) ethylamine hemifumarate; KHS, Krebs-Henseleit solution; RS-17053, *N*-[2-(2-cyclopropylmethoxyphenoxy)ethyl]-5-chloro-α, α-dimethyl-1*H*-indole-3-ethamine hydrochloride; SCH-23390, R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride; SKF89748-A, 1-5-methylthio-8-methoxy-2-aminotehalin hydrochloride; SMA, small mesenteric artery; ST-587, 2-(2-chloro-5-trifluormethyl-phenylimino)-imidazolin nitrate; U46619, 9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin F_2,

Introduction

Radioligand binding studies and molecular biology experiments have demonstrated the existence of at least three α_1-adrenoceptor subtypes, now referred to as α_{1A} (previously known as α_{1C}), α_{1B} and α_{1D} (previously also known as α_{1A} or $\alpha_{1A/D}$) (see Hieble *et al.*, 1995). These subtypes have been cloned and all display high, subnanomolar, affinities for prazosin. However, functional studies have provided evidence for the existence of an additional α_1-adrenoceptor subtype (α_{1L}), displaying low affinity for prazosin ($pK_B < 9$) and some other α_1-adrenoceptor antagonists, including RS-17053 (Flavahan & Vanhoutte, 1986; Muramatsu *et al.*, 1990; Ford *et al.*, 1994, 1996). The α_{1L}-adrenoceptor has no molecular correlate, but seems to mediate constriction of the human (Ford *et al.*, 1996) and rabbit (Van der Graaf *et al.*, 1997; Kava *et al.*, 1998) lower urinary tract and rabbit and guinea-pig aorta (Muramatsu *et al.*, 1990).

In rat isolated small mesenteric arteries (SMAs; internal diameter 100–300 μm), Högestatt & Andersson (1984) and Nielsen & Mulvany (1990) demonstrated that prazosin antagonizes noradrenaline-mediated contractions with high affinity ($pA_2 = 9.58$–9.84 and 9.23, respectively). Accordingly, it has been suggested that α_{1A}-adrenoceptors predominantly mediate noradrenaline-induced contraction of rat SMA (Chen *et al.*, 1996; Ipsen *et al.*, 1997). However, Schild analysis demonstrated complex antagonism by prazosin with its potency (pA_2) ranging from 8.8–9.6 and, therefore, additional involvement of α_{1L}-adrenoceptors was suggested (Chen *et al.*,

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1996). Van der Graaf *et al.* (1996) found that despite significant correlation of antagonist affinity values with pK_i values at the cloned α_{1A}-adrenoceptor, the pA_2 value of prazosin in rat SMA (8.5) was more consistent with the profile of the pharmacologically-defined α_{1L}-subtype (Flavahan & Vanhoutte, 1986; McGrath & Wilson, 1988; Ford *et al.*, 1994). Adding to the confusion was a recent report that α_{1B}-adrenoceptors mediated contraction in rat SMA (Piascik *et al.*, 1997). Thus, the α_1-adrenoceptor subtypes involved in noradrenaline-induced contractions in rat SMA are still controversial.

Using several subtype-selective α_1-adrenoceptor agonists and antagonists in the present investigation, we provide further evidence that the α_1-adrenoceptors mediating contraction of rat SMA are of the α_{1L} subtype. Since Ford and co-workers (1997) have suggested that the α_{1L} subtype may represent a particular conformational state (pharmacological phenotype) of the α_{1A}-adrenoceptor gene product, we have attempted to elaborate on the nature of the observed α_{1L}-adrenoceptor pharmacology under different experimental conditions.

Methods

Rat small mesenteric artery preparation

Male Wistar rats (250–350 g) were anaesthetized (sodium pentobarbitone, 60 mg kg^{-1}, i.p.) and killed by cervical dislocation and the mesentery was removed and placed in ice-cold modified Krebs-Henseleit solution (KHS) of the following composition (mM): NaCl 119.0, $NaHCO_3$ 25.0, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, glucose 5.5, $CaCl_2$ 2.5 and EDTA 0.026. Arterial trees were dissected and cleared from surrounding adipose tissue. As described previously (Mulvany & Halpern, 1977), from each arterial tree a ring segment (~2 mm in length) was mounted in a myograph (J.P. Trading, Aarhus, Denmark) with separated 6 ml organ baths containing modified KHS at 37°C (or at 27°C for certain experiments; see below). The KHS was continuously gassed with 95% O_2 and 5% CO_2 and tissue responses were measured continuously as changes in isometric force.

Following a 30 min stabilization period, the internal diameter of each vessel was set to a tension equivalent to 0.9 times the estimated diameter at 100 mmHg effective transmural pressure (I_{100}=200–300 μm) according to the standard procedure of Mulvany & Halpern (1977). The presence of the endothelium was then confirmed with 10 μM of methacholine after a pre-contraction with either 30 μM 5-hydroxytryptamine (5-HT) or 10 μM noradrenaline (see below). Tissues which responded with less than 60% relaxation were rejected.

In all experiments, 60 min prior to construction of each agonist concentration-effect (E/[A]) curve, cocaine (30 μM), timolol (6 μM) and SCH-23390 (10 nM) were added to the KHS to block neuronal uptake, β_1/β_2-adrenoceptors and D_1 receptors, respectively (Van der Graaf *et al.*, 1995).

Experimental designs

Single curve design After normalization and a further 30 min stabilization period, a calibration contraction (12.8±0.5 mN, n=49) was obtained to 30 μM 5-hydroxytryptamine (5-HT). After confirming the presence of the endothelium, tissues were washed for 30 min and then incubated for 60 min with antagonist or vehicle. Subsequently, a single agonist E/[A] curve was obtained by cumulative dosing at quarter-log unit concentration increments. In the experiments where the antagonism of chloroethylclonidine was investigated, tissues were pre-incubated for 30 min with 10 μM of the drug, followed by a 30-min washout period (ten solution changes).

Paired curve design After standardization of the internal diameter, the preparations were challenged five times with noradrenaline (10 μM) with washouts after each challenge. As described above, the integrity of the endothelium was assessed after the first challenge of noradrenaline. After a first agonist E/[A] curve was obtained (see Results), each tissue segment was washed (30 min) and equilibrated (60 min) with vehicle or different concentrations of antagonist. Subsequently, another agonist E/[A] curve was constructed in the presence of vehicle or antagonist.

Determination of affinity of RS-17053 under different experimental conditions

The antagonist affinity of RS-17053 was determined under the following experimental conditions.

Low bath fluid temperature Single curve design was used at a temperature of 27°C.

Protocol according to Chen et al. *(1996)* The preparations were challenged once with KCl (125 mM) and subsequently three times with a combination of KCl (125 mM) and noradrenaline (10 μM), and once more with KCl (125 mM) with washouts after each challenge. After a first agonist E/[A] curve, each tissue segment was washed for 30 min and then equilibrated for 60 min with vehicle or different antagonist concentrations as described above under Paired curve design. Subsequently, another noradrenaline E/[A] curve was obtained and the responses were expressed as percentage of the fifth noradrenaline challenge which served as calibration contraction.

Depolarization with K^+ before and after incubation of RS-17053 The single curve design was conducted except that noradrenaline E/[A] curves were obtained after partial depolarization by KCl (20 mM). This depolarization by KCl was applied either after or before incubation of the tissues with RS-17053 (0.1 μM).

Pre-contraction with U46619 (10–25 nM) The single curve design was conducted except that after incubation with RS-17053 (0.1 μM), noradrenaline E/[A] curves were obtained on top of a threshold contraction with the thromboxane A_2-mimetic, U46619 (10–25 nM).

Selective protection of α_{1A}-adrenoceptors

In a set of four experiments, after five challenges with noradrenaline (as in the paired curve design) the SMAs were incubated with RS-17053 (2 nM) for 60 min to selectively protect α_{1A}-adrenoceptors. At this concentration, RS-17053 is expected to occupy ~95% of the α_{1A}-adrenoceptor population (based on a pA_2 of 9.9 as observed in the perfused mesentery; Ford *et al.*, 1996), whereas it would occupy only ~30% of the α_{1L}-adrenoceptor population (based on a pA_2 of 8.35; see Results). In the presence of RS-17053, the alkylating agent, phenoxybezamine (1 nM), was added for 15 min followed by extensive washing (10 solution changes over 30 min). After a first A61603 E/[A] curve had been obtained, vessel segments were washed (30 min) and equilibrated (60 min) with vehicle or different concentrations of RS-17053 (10, 30 and 100 nM). Subsequently, a second A61603 E/[A] curve was obtained and

the responses were expressed as percentage of fifth noradrenaline challenge, which served as calibration contraction.

Analysis

Individual agonist curve data were fitted to the Hill equation using an iterative, least-squares method:

$$E = \frac{\alpha * [A]^{n_H}}{[A]_{50}^{n_H} + [A]^{n_H}}$$

to provide estimates of midpoint slope (n_H), midpoint location ($[A]_{50}$ estimated as logarithm) and upper asymptote (α). The effect of drug treatment on these parameters was assessed by one-way analysis of variance (ANOVA) or Student's *t*-test, as appropriate. Values of $P<0.05$ were considered to be significant.

When the minimum criteria for competitive antagonism were satisfied, that is the antagonist produced parallel rightward shift of the agonist E/[A] curves with no change in upper asymptote, antagonist affinity estimates were obtained by fitting the individual midpoint location values obtained in the absence ($\log[A]_{50}$) and presence ($\log[A]_{50B}$) of antagonist (B) to the following derivation of the Schild equation (Black *et al.*, 1985):

$$\log[A]_{50B} = \log[A]_{50} + \log(1 + [B]^h/10^{\log K_B}).$$

When the Schild plot slope parameter (b) was not significantly different from unity, then the data were re-fitted with b constrained to unity so that the antagonist dissociation equilibrium constant, K_B, could be estimated as $\log K_B \pm$ s.e. (Jenkinson *et al.*, 1995). When less than three different concentrations of antagonist were tested or the criteria of competitive antagonism were not completely satisfied, an empirical pA_2 value was estimated using the above equation, with b constrained to unity.

Combined concentration-ratio analysis

In order to test whether RS-17053 and tamsulosin acted at the same site (syntopically), a combined concentration-ratio analysis was performed according to the procedure developed by Shankley and co-workers (1988). Briefly, when two antagonists act syntopically, then their combined concentration-ratio is given by:

$$r_{B+C} = r_B + r_C - 1$$

where r_B and r_C are the concentration-ratios obtained independently in the presence of the antagonists B and C, respectively. This relationship can be re-written in terms of $\log[A_{50}]$ values of the agonist E/[A] curves in the presence and absence of antagonists B and C using the following equation:

$$S_A = \log[A_{50}]_{B+C} - \log([A_{50}]_B + [A_{50}]_C - [A_{50}]),$$

where S_A is the test statistic for the additive model. Thus, if the experimental data comply with the additive model, S_A should have a value of zero. In contrast, when two antagonists act at different sites, that is allotopically, their combined concentration-ratios multiply;

$$r_{B+C} = r_B \cdot r_C$$

and expressed in terms of log $[A_{50}]$ values;

$$S_M = \log[A_{50}]_{B+C} - \log[A_{50}]_B - \log[A_{50}]_C + \log[A_{50}],$$

where S_M is the test statistic for the multiplicative model. If the antagonists behave allotopically, S_M should have a value of zero.

Because the distributions of S_A and its standard estimator are unknown, there is no formal statistical method available to decide in which cases the additive model should be accepted or rejected. In the present study, the null hypotheses (H_0) was formulated as 'B+C act syntopically' and it was assumed that S_A and S_M and their associated standard error estimators are approximately normally distributed. Deviations of S_A and S_M from zero were tested for significance using two- and one-sided *t*-tests, respectively, and H_0 was accepted in cases when $S_A=0$ and $S_M<0$. In all other cases H_0 was rejected.

Compounds

Compounds were obtained from the following sources: A61603 (N-[5-(4,5-dihydro-1H-imidazol-2yl)-2-hydroxy-5,6,7,8-tetrahydronaphthalen-1-yl] methanesulphonamide hydrobromide): Abbott Laboratories, North Chicago, IL, U.S.A.; cocaine hydrochloride, 5-HT, methacholine bromide, l-noradrenaline hydrochloride, methoxamine hydrochloride, phenoxybenzamine hydrochloride and timolol maleate, U46619 (9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin $F_{2\alpha}$): all from Sigma, Zwijndrecht, The Netherlands; BMY 7378 (8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4,5]decane-7,9-dione dihydrochloride), chloroethylclonidine dihydrochloride, cirazoline hydrochloride and SCH-23390 (R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride): all from Research Biochemicals Incorporated, Natick, MA, U.S.A.; JTH-601 (N-(3-hydroxy-6-methoxy-2,4,5-trimethylbenzyl)-N-methyl-2-(4-hydroxy-2-isopropyl-5-methyl-phenoxy) ethylamine hemifumarate): Japan Tobacco Company, Tokyo, Japan; RS-17053 (*N*-[2-(2-cyclopropylmethoxyphenoxy)ethyl]-5-chloro-α, α-dimethyl-1*H*-indole-3-ethamine hydrochloride): Roche Bioscience, Palo Alto, CA, U.S.A.; tamsulosin: Yamanouchi Pharmaceutical Co. Ltd., Ibaraki, Tsukuba, Japan; SKF89748-A (1-5-methylthio-8-methoxy-2-aminotehalin hydrochloride): Smith Kline Beecham Pharmaceuticals, King of Prussia, PA, U.S.A.; ST 587 (2-(2-chloro-5-trifluormethyl-phenylimino)-imidazolin nitrate): Boehringer Ingelheim Ltd., Bracknell, Berkshire, U.K. Noradrenaline was dissolved in stoichiometric ascorbic acid solution. Methacholine was dissolved in ethanol. JTH-601 was dissolved in dimethyl sulphoxide as a 10 μM stock solution and further diluted in distilled water. Phenoxybenzamine was dissolved in absolute ethanol. RS-17053 was dissolved in a mixture of 10% dimethylsulphoxide, 20% propylene glycol and 70% distilled water as a 10 μM stock solution and further diluted in distilled water. SKF89748-A was dissolved in a mixture of 50% distilled water and 50% ethanol as a 20 mM stock solution and further diluted in distilled water. U46619 was dissolved initially in 20% ethanol to give a 1 mM stock solution and subsequently diluted in distilled water. All other drugs were dissolved in distilled water.

Results

Potency rank order of α_1-adrenoceptor agonists and effect of the non-selective α_1-adrenoceptor antagonist prazosin

The antagonism of prazosin (30 nM) against several agonists was studied in a paired curve design. All α_1-adrenoceptor agonists used in this investigation contracted rat SMA, displaying either full (noradrenaline, cirazoline, methoxamine, A61603) or partial (SKF89748-A, ST-587) agonism (see Table

1). The potency order (pEC_{50}) of the agonists in rat SMA was: A61603 >> SKF89748-A = cirazoline > noradrenaline > ST-587 > methoxamine. Half of the ST-587 E/[A] curves obtained were fitted with a fixed Hill slope ($n_H = 5$), since these individual curves were extremely steep. Prazosin (30 nM) antagonized the responses to all six agonists and the affinity estimates of prazosin (pA_2: 8.29–8.80), which were consistently lower than those reported at α_{1A}-, α_{1B}- or α_{1D}-adrenoceptor subtypes (Burt *et al.*, 1995; Ford *et al.*, 1996, 1997), did not differ between agonists (Table 1).

Effect of adrenoceptor antagonists, chloroethylclonidine (α_{1B}) and BMY 7378 (α_{1D})

Noradrenaline produced concentration-dependent contractions of SMAs and the individual E/[A] curves were fitted to the Hill equation to provide estimates of the midpoint location ($pEC_{50} = 5.92 \pm 0.11$), Hill slope ($n_H = 3.1 \pm 0.5$) and upper asymptote ($\alpha = 162 \pm 16\%$ of the 5-HT calibration contraction). Pretreatment of the tissues with 10 μM chloroethylclonidine, a ligand known to irreversibly inactivate α_{1B}-adrenoceptors (see Hieble *et al.*, 1995), had no significant effects on the Hill parameters of the noradrenaline E/[A] curve ($pEC_{50} = 5.71 \pm 0.09$, $n_H = 2.5 \pm 0.4$, $\alpha = 162 \pm 13\%$ of the 5-HT calibration contraction (Figure 1, left panel).

In a concentration (100 nM) that is selective for α_{1D}-adrenoceptors (see Goetz *et al.*, 1995), BMY 7378 did not shift the E/[A] curves to noradrenaline (data not shown). However, higher concentrations (1 and 10 μM) of BMY 7378 produced a significant rightward shift of the noradrenaline curve (Figure 1, right panel), and a pA_2 value of 6.16 ± 0.13 was estimated. This pA_2 value is much lower than that reported for the α_{1D}-adrenoceptor in rat aorta ($pA_2 = 8.9$; Goetz *et al.*, 1995).

Table 1 Hill parameters of different α_{1A}-adrenoceptor agonists and affinity estimates for prazosin in rat SMA ($n = 4-6$)

Agonist	*α (% of 10 μM noradrenaline contraction)*	*pEC_{50}*	*n_H*	*pA_2 prazosin*
Noradrenaline	102±8	6.32±0.11	2.3±0.3	8.50±0.1*
Cirazoline	102±3	6.85±0.08	3.6±0.8	8.44±0.06
Methoxamine	94±4	5.03±0.15	4.6±0.7	8.32±0.11
SKF89748-A	90±4	7.15±0.25	3.9±1.1	8.58±0.15
A61603	109±4	8.15±0.05	2.3±0.3	8.80±0.08
ST-587	47±11	5.56±0.20	4.3±0.4	8.29±0.13

*Reported by Van der Graaf *et al.* (1996).

Effect of selective α_{1A}-adrenoceptor antagonist RS-17053 against noradrenaline and A61603 as agonists

The selective α_{1A}-adrenoceptor antagonist RS-17053 (10–300 nM; Ford *et al.*, 1996) also produced concentration-dependent, parallel, rightward shifts of the noradrenaline E/[A] curves. The Schild plot slope parameter (1.14 ± 0.11) was not significantly different from unity and a pK_B of 8.35 ± 0.10 was estimated (Figure 2, upper panels).

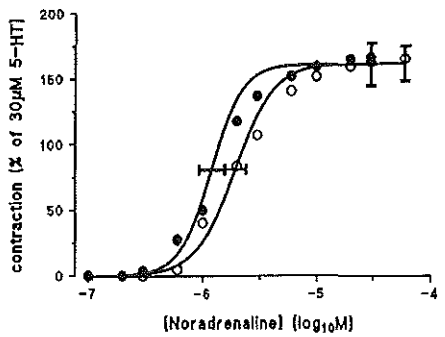
The selective α_{1A}-adrenoceptor agonist A61603 (Knepper *et al.*, 1995) behaved as a full agonist with respect to noradrenaline and the Hill parameters were: $pEC_{50} = 7.82 \pm 0.12$, $n_H = 2.60 \pm 0.21$, $\alpha = 149 \pm 6\%$ of the 5-HT calibration contraction (Figure 2, lower panels). RS-17053 (10–300 nM) also competitively antagonized the A61603-induced contractions ($b = 1.14 \pm 0.09$) and a $pK_B = 8.40 \pm 0.09$ was estimated.

Effect of putative α_{1L}-adrenoceptor antagonist JTH-601 against noradrenaline as agonist

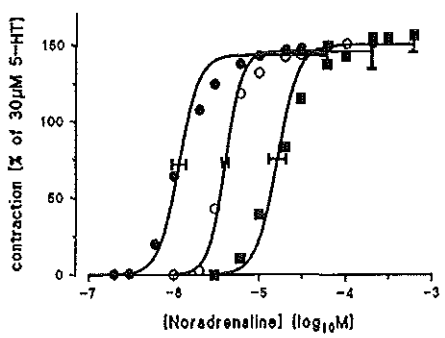
Previously, JTH-601 was demonstrated to have a ~10 times higher affinity than prazosin for the α_{1L}-adrenoceptor, whereas both compounds displayed equal binding affinities for the α_{1A} receptor subtype (Muramatsu *et al.*, 1996). In the SMA, JTH-601 (3–100 nM) produced rightward shifts of the noradrenaline E/[A] curves (Figure 3). However, the shift did not occur in a concentration-dependent manner, since the concentration-ratios obtained with 10 and 30 nM JTH-601 were practically identical (Figure 3). From the shifts obtained with 3 and 10 nM a pA_2 value of 8.34 ± 0.16 was estimated for the high affinity component.

Effect of experimental conditions on the affinity estimate of RS-17053

It was recently suggested that the α_{1L}-adrenoceptor, instead of being a distinct molecular entity, might represent a conforma-



0 (●); 10 (○) μM CEC



0 (●); 1 (○); 10 (■) μM BMY 7378

Figure 1 Concentration-effect curves to noradrenaline in rat small mesenteric artery in the absence or presence of chloroethylclonidine (left panel; $n = 3$) and BMY 7378 (right panel; $n = 4$). The lines shown superimposed on the mean data points were simulated using the Hill equation.

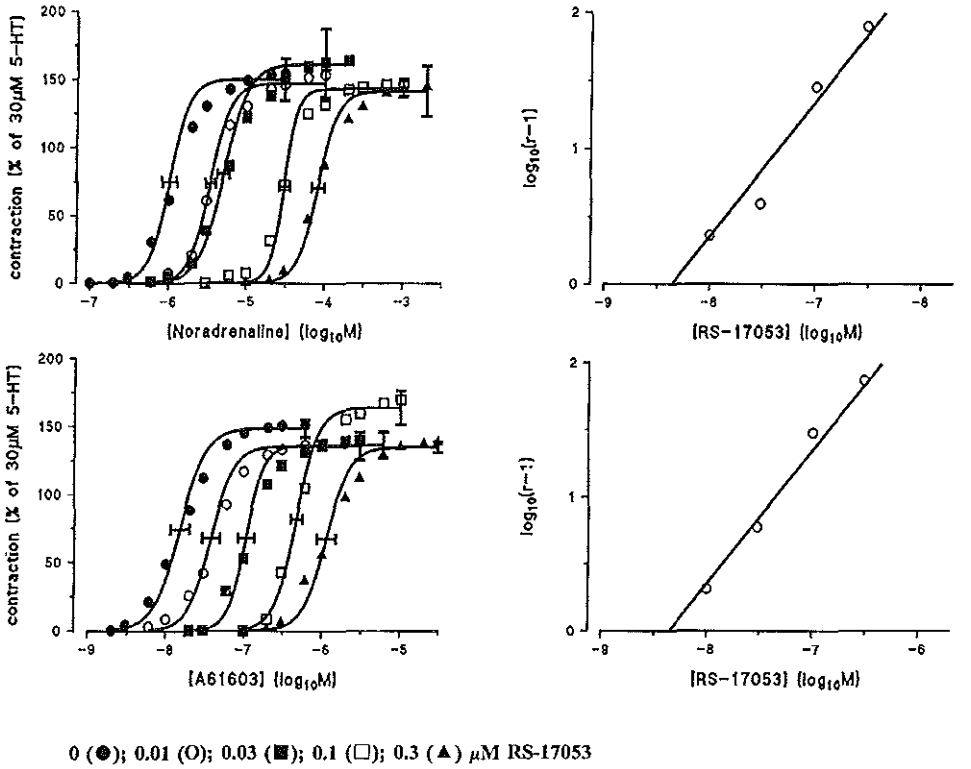


Figure 2 Left panels. Concentration-effect curves to noradrenaline (upper panel; $n=5$) and A61603 (lower panel; $n=5-6$) obtained on rat SMA in the absence or presence of RS-17053. The lines superimposed on the mean data points were simulated using the Hill equation. Right panels. Schild plots for the interaction of RS-17053 with noradrenaline (upper panel) and A61603 (lower panel). The solid lines superimposed on mean data points were simulated using the parameters obtained from the constrained model fits.

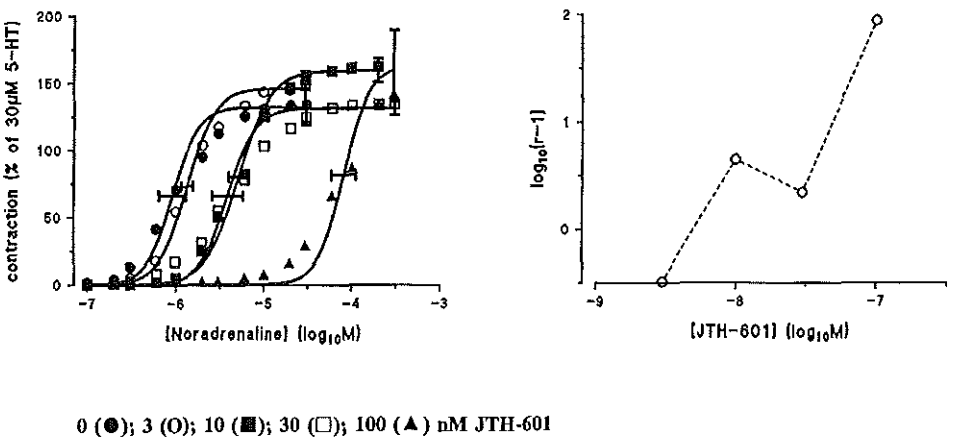


Figure 3 Left panel. Concentration-effect curves to noradrenaline obtained on rat SMA in the absence or presence of JTH-601 ($n=5$). The lines superimposed on the mean data points were simulated using the Hill equation. Right panel. Schild plot for the interaction of JTH-601 with noradrenaline.

tional affinity state of the α_{1A}-adrenoceptor and that it is possible to switch the pharmacological α_{1L}-adrenoceptor profile into an α_{1A}-profile by changing experimental conditions (Williams *et al.*, 1996). Therefore, we studied the antagonizing potency of RS-17053 under different experimental conditions (see Table 2).

Low bath fluid temperature When temperature was lowered to 27°C, noradrenaline still produced concentration-dependent contractions of the SMAs. RS-17053 (10–100 nM) behaved as a competitive antagonist (b=0.98±0.16) with an estimated affinity (pK_B=8.42) that was similar to that obtained under standard conditions (Table 2).

Protocol according to Chen et al. *(1996)* In a recent study, Chen *et al.* (1996), concluded that noradrenaline-induced contraction of the SMA involves predominantly α_{1A}-adrenoceptors. In experiments carried out according to their experimental protocol (see Methods for details), RS-17053 (10–100 nM) again caused a parallel rightward shift (b=0.95±0.23) and displayed a similar affinity as under standard conditions (pK_B=8.46; Table 2).

Depolarization with K^+ before and after incubation of RS-17053 Partial depolarization by KCl (20 mM) after pre-incubation with RS-17053 induced a threshold contraction of 4.7±0.7% of the 5-HT calibration contraction. Under these conditions RS-17053 (0.1 μM) behaved as a competitive antagonist. The pA_2 value (7.72±0.26; Table 2) was slightly lower compared to standard conditions, but due to a large between-tissue variability (95% confidence interval: ±0.63) this difference was not statistically significant. The notable large variance could indicate perturbation of the equilibrium between antagonist and receptor by 20 mM KCl. Therefore, a threshold contraction (6.1±1.1% of 5-HT calibration contraction) by partial depolarization with KCl (20 mM) was induced before the 60 min pre-incubation with RS-17053 (0.1 μM). Co-equilibration of RS-17053 and KCl (20 mM) decreased the variance (95% confidence interval: ±0.33), but did not significantly affect the affinity estimate of RS-17053 (pA_2=8.31±0.16; Table 2).

Pre-contraction with U46619 (10–25 nM) In the presence of a threshold contraction induced by 10–25 nM U46619 (14.7±0.8% of the 5-HT calibration contraction), RS-17053 (0.1 μM) unexpectedly caused a significant flattening of the noradrenaline E/[A] curve (n_H=0.9±0.1 and 1.4±0.1, respectively, with or without RS-17053; $P<0.05$). However, the estimated pA_2 value (7.87±0.33, Table 2) was not significantly different from the affinity of RS-17053 estimated under standard conditions (95% confidence interval: ±0.80).

Selective protection of α_{1A}-adrenoceptors

If the α_{1A}- and α_{1L}-adrenoceptor are distinct subtypes, both might co-exist in rat SMA, but different experimental set-ups might favour the exhibition of one over the other type. After selective protection of the putative α_{1A}-adrenoceptor population from inactivation by phenoxybenzamine (see Methods), the affinity of RS-17053 against A61603 was assessed in a paired curve design. Hill slope parameters of the first A61603 E/[A] curve were: n_H=2.3±0.5. α=69.3±4.5% of the calibration contraction, pEC_{50}=6.37±0.10. RS-17053 (10–100 nM) caused a rightward shift of the A61603 E/[A] curve. Notwithstanding a significant steepening of the A61603 E/[A] curve (n_H=3.21±0.26; $P<0.05$) with RS-17053 (100 nM), Schild analysis was performed (Figure 4). The Schild slope parameter was not significantly different from unity (b=1.04±0.16) and the estimated pA_2 (8.25±0.06) was practically identical to the potency in untreated tissues (pK_B=8.40±0.09; Figure 2).

Combination of RS-17053 and tamsulosin

The previously demonstrated susceptibility of the affinity estimate of RS-17053 but not of tamsulosin to experimental conditions (Williams *et al.*, 1996) might indicate that RS-17053 and tamsulosin act at different sites of α_{1A}-adrenoceptors. A combined concentration-ratio analysis experiment was designed to test whether RS-17053 and tamsulosin act syntopically in rat SMA. As shown in Figure 5, both RS-17053 and tamsulosin produced a parallel rightward shift of the noradrenaline E/[A] curve (concentration-ratio=17.5±8.9

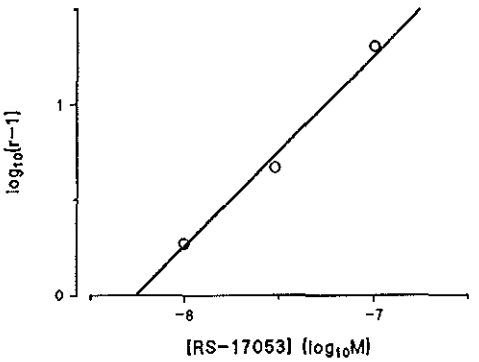
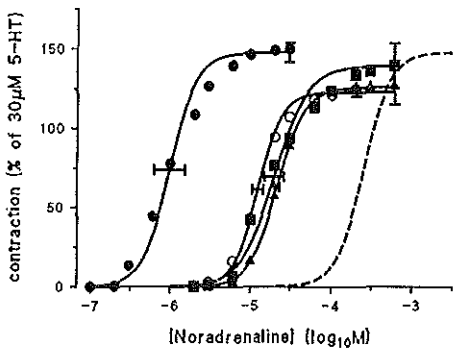


Figure 4 Schild plot for the interaction of RS-17053 with A61603 after selective protection of α_{1A}-adrenoceptors with RS-17053 (2 nM, 60 min) from inactivation by phenoxybenzamine (1 nM, 15 min); n=4 (for details, see Methods). The solid line superimposed on mean data points was simulated using the parameters obtained from the constrained model fit. Please note that the E/[A] curves have been omitted from the figure because they showed considerable variability due to unpredictable extent of receptor inactivation by phenoxybenzamine in individual segments.

Table 2 Effect of experimental protocol on the Hill equation parameters of noradrenaline and affinity estimates for RS-17053 in rat SMA

Experimental protocol (see Methods for details)	*Pre-contraction (% 30 μM 5-HT)*	*Hill equation parameters noradrenaline* α *(% of 30 μM 5-HT or 10 μM noradrenaline†)*	pEC_{50}	n_H	pK_B (pA_2) *RS-17053*
Standard*	–	162±16	5.92±0.11	3.1±0.5	8.35±0.10
Low bath fluid temperature (27°C) (n=4)	–	142±8	5.86±0.10	3.8±0.8	8.42±0.11
Protocol according to Chen *et al.* (1996) (n=3)	–	99±1†	6.01±0.16	2.9±0.07	8.46±0.09
Depolarization with K^+ after RS-17053 (n=5)	4.7±0.7	120±6	6.12±0.14	1.2±0.1	(7.72±0.26)
Depolarization with K^+ before RS-17053 (n=7)	6.1±1.1	112±4	6.53±0.09	1.6±0.2	(8.31±0.16)
Pre-contraction with U46619 (10–25 nM) (n=5)	14.7±0.8	149±8	6.65±0.17	1.4±0.1	(7.87±0.33)

Data are mean±s.e.mean. *Data from Figure 2.



0 (●); 1 nM tamsulosin (○); 10 nM RS-17053 (■); 1 nM tamsulosin + 10nM RS-17053 (▲)

Figure 5 Combined concentration-ratio analysis: concentration-effect curves to noradrenaline obtained on rat SMA in the absence or presence of 100 nM RS-17053, 1 nM tamsulosin or both 100 nM RS-17053 and 10 nM tamsulosin ($n=3$ each). The lines shown superimposed on the mean data points were simulated using the Hill equation. The dashed line shows the location of the concentration-effect curve which was predicted by assuming that the antagonists acted allotopically.

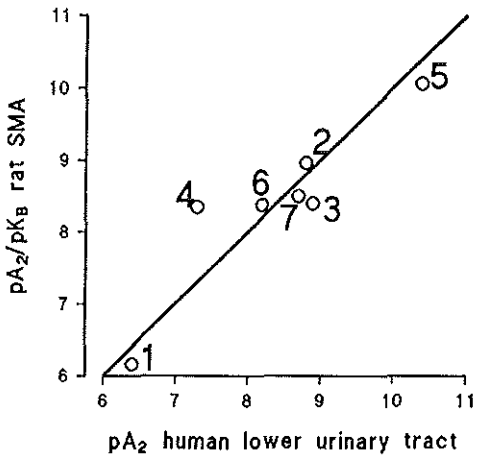


Figure 6 Relation between pA_2 estimates in human lower urinary tract (Ford *et al.*, 1996) and pK_B/pA_2 estimates in rat SMA, determined against noradrenaline (this study and Van der Graaf *et al.*, 1996) for (1) BMY 7378, (2) HV 723, (3) prazosin, (4) RS-17053, (5) tamsulosin, (6) 5-methylurapidil, (7) WB4101. The solid line represents the line of identity.

and 20.8±3.4, respectively; $pA_2=8.29\pm0.22$ and 10.06 ± 0.20, respectively). The potency of tamsulosin was in accordance with a previous reported value in rat SMA (9.8; Van der Graaf *et al.*, 1996) and with its reported affinity for α_{1L} and α_{1A}-adrenoceptors (10–10.5; Van der Graaf *et al.*, 1996). Combined concentration-ratio analysis indicated that RS-17053 (100 nM) and tamsulosin (1 nM) competed for binding to the same site, since the test statistic S_A for the additive model ($S_A=-0.16\pm0.09$) was not significantly different from 0 ($P>0.05$), whereas the test statistic S_M for the multiplicative model was significantly smaller than 0 ($S_M=-1.07\pm0.24$; $P<0.05$).

Discussion

The official nomenclature of α_1-adrenoceptors recognizes α_{1A}-, α_{1B}- and α_{1D}-adrenoceptors, which have all been cloned and which all display high, subnanomolar affinity for prazosin (Hieble *et al.*, 1995). Based on functional studies, an alternative classification scheme exists, which recognizes α_{1H}- and α_{1L}-adrenoceptors displaying high (α_{1A}-, α_{1B}- and α_{1D}-adrenoceptors) and low affinity for prazosin, respectively (Flavahan & Vanhoutte, 1986; McGrath & Wilson, 1988; Muramatsu *et al.*, 1990; Ford *et al.*, 1994). Because of the reported high (>9.2) or low (<8.5) affinity of prazosin, the involvement of either α_{1A}- or α_{1L}-adrenoceptors in rat isolated SMA, which is believed to represent resistance vessels (Mulvany & Aalkjaer, 1990; Christensen & Mulvany, 1993; Fenger-Gron *et al.*, 1995), is controversial (Högestätt & Andersson, 1984; Nielsen & Mulvany, 1990; Chen *et al.*, 1996; Van der Graaf *et al.*, 1996). The present study has further examined this controversy using prazosin and several recently discovered, selective α_1-adrenoceptor antagonists under different experimental conditions.

Involvement of α_{1L}-adrenoceptor in the contraction of rat SMA

The low affinity of prazosin ($pA_2=8.29-8.80$) in rat SMA proved to be agonist independent (Table 1) and indicated α_{1L}-adrenoceptor involvement (Muramatsu *et al.*, 1990). It may be noted that the affinity of prazosin in our experiments with intact endothelium did not differ from that found in rat SMA denuded of endothelium ($pA_2=8.5$; Van der Graaf *et al.*, 1996). The potency rank order of the agonists SKF89748-A > cirazoline > noradrenaline > ST-587 > methoxamine (Table 1) was similar to that observed for the cloned α_{1A}-subtype (Minneman *et al.*, 1994), except for SKF89748-A which was less potent than both cirazoline and noradrenaline at the α_{1A}-adrenoceptor. A lack of effect of chloroethylclonidine (10 μM), which in this concentration inactivates rat α_{1B}-adrenoceptors (Michel *et al.*, 1993; Sugden *et al.*, 1996), and the low potency of the potent and selective α_{1D}-adrenoceptor antagonist BMY 7378 (pK_i for rat cloned α_{1A}-adrenoceptors=8.2; Goetz *et al.*, 1995) excluded the involvement of α_{1B}- and α_{1D}-adrenoceptors, respectively, in the noradrenaline-induced contraction of rat SMA (Figure 1). Moreover, the affinity of another putative α_{1B}-adrenoceptor antagonist (+)-cyclazosin (Giardina *et al.*, 1996) in rat SMA ($pK_B=7.78$) did not indicate α_{1B}-adrenoceptor involvement either (Stam *et al.*, 1998).

The affinity of the selective α_{1A}-adrenoceptor antagonist RS-17053 (Ford *et al.*, 1996) against noradrenaline ($pK_B=8.35$) and against the selective α_{1A}-adrenoceptor agonist A61603 ($pK_B=8.40$) was too low (see Figure 2) to account for α_{1A}-adrenoceptor involvement (pK_i for α_{1A}-adrenoceptors in rat submaxillary gland=9.1 and pA_2 in the perfused mesentery=9.9; Ford *et al.*, 1996). Interestingly, JTH-601 caused a complex shift of the noradrenaline E/[A] curve (Figure 3) in rat SMA. However, functional data for JTH-601 on α_{1A}-adrenoceptors are required in order to assess the nature of this complex behaviour.

Is the α_{1L}-adrenoceptor a conformational state of α_{1A}-adrenoceptor?

The affinity of RS-17053 in rat SMA was 35 fold lower in the present experiments than that reported by Ford and colleagues for antagonizing pressor responses to noradrena-

line in the perfused mesentery ($pK_B = 9.9$; Ford *et al.*, 1996); the latter being in agreement with functional affinity estimates for α_{1A}-adrenoceptors in rat perfused kidney ($pA_2 = 9.8$; Ford *et al.*, 1996) and rat vas deferens ($pA_2 = 9.5$; Marshall *et al.*, 1996). Therefore, it appears that α_{1A}-adrenoceptors mediate the pressor response in rat perfused mesentery, whereas noradrenaline-induced contraction in rat isolated SMA is mediated by a different type of α_1-adrenoceptor, possibly α_{1L}. One explanation for this discrepancy is that the pressor response in the perfused mesentery to noradrenaline reflects resistance changes in distal arterioles, which were shown to co-determine vascular resistance (Fenger-Gron *et al.*, 1997).

Alternatively, the α_{1L}-adrenoceptor in the SMA assay might be a pharmacological phenotype of the α_{1A}-adrenoceptor subtype (Ford *et al.*, 1997). Functional studies in rat vas deferens (Ohmura *et al.*, 1992; Prins *et al.*, 1992; Burt *et al.*, 1995; Guh *et al.*, 1995; Chess-Williams *et al.*, 1996; Muramatsu *et al.*, 1996), portal vein (Digges & Summers, 1983; Chess-Williams *et al.*, 1996; Green *et al.*, 1996) and human lower urinary tract (see Hieble & Ruffolo, 1996), where the presence of both α_{1A}- and α_{1L}-adrenoceptor has been claimed on the basis of prazosin affinity, have now produced a range of affinities for RS-17053. The high affinity for RS-17053 in rat vas deferens ($pK_B = 9.5$; Marshall *et al.*, 1996) and perfused mesentery ($pA_2 = 9.9$; Ford *et al.*, 1996) indicated α_{1A}-adrenoceptor involvement. However, an α_{1L}-adrenoceptor displaying a 250 fold lower potency for RS-17053 was found in rat portal vein ($pK_B = 7.1$; Marshall *et al.*, 1996), human lower urinary tract ($pA_2 = 7.3$; Ford *et al.*, 1996) and prostate ($pA_2 = 7.2$; Marshall *et al.*, 1996). Interestingly, apart from RS-17053, the affinity estimates of different antagonists in the SMA are in good agreement with those determined in human lower urinary tract (Figure 6). The affinity of RS-17053 in rat SMA ($pK_B = 8.35$) is more in accordance with an intermediate affinity value demonstrated in the prostatic portion of rat vas deferens by Burt and colleagues ($pA_2 = 8.3$; 1998). Furthermore, accumulation of [^{3}H]-inositol phosphates by cells expressing the human α_{1a}-adrenoceptor was antagonized by RS-17053 with similar intermediate affinity ($pA_2 = 8.3$; Ford *et al.*, 1997). Consequently, the authors postulated that this α_{1L}-adrenoceptor was an affinity state of the α_{1A}-adrenoceptor gene product. Taken together, these observations indicate that the structurally defined α_{1A}-adrenoceptor either presents itself functionally as, or consists of, at least three different subtypes which can be discriminated by RS-17053. Indeed, in radioligand binding studies a complete switch from an α_{1L}-adrenoceptor pharmacological profile into an α_{1A}-adrenoceptor profile could be induced by changing experimental conditions, which included (i) a decrease in temperature from 37 to 20°C, (ii) the use of TRIS/EDTA buffer instead of Ham's buffer and (iii) the disruption of cells into membranes (Williams *et al.*, 1996).

Therefore, we found it of interest to study whether a switch in the state of affinity of RS-17053 can be established in functional studies with rat SMA (see Table 2). For obvious reasons, in such studies one cannot employ TRIS/EDTA buffer or cell membranes as used in the radioligand binding assay (Williams *et al.*, 1996). However, we determined the affinity of RS-17053 at a lower bath temperature. The pK_B estimate of RS-17053 in the SMA was unaffected by decreasing the temperature from 37 to 27°C. Experiments carried out according to the protocol of Chen and co-workers (1996) demonstrated simple competitive antagonism and also yielded an affinity estimate for RS-17053 similar to that obtained under standard conditions and thus incompatible with the suggested α_{1A}-adrenoceptor involvement (Chen *et al.*, 1996). Interestingly, high affinities for RS-17053 have been estimated in perfused assays, like rat kidney ($pA_2 = 9.8$ Ford *et al.*, 1996), mesentery ($pA_2 = 9.9$, Ford *et al.*, 1996) or hind limb ($pA_2 = 9.47$; Zhu *et al.*, 1997). The spontaneous development of myogenic tone in perfused vessels might be a major experimental difference with the SMA preparation (Dunn *et al.*, 1994). We induced myogenic tone in rat SMA by either partial depolarization with KCl (20 mM) or by a threshold contraction with the thromboxane A_2-mimetic, U46619. U46619 was selected, since thromboxane A_2 is produced by the endothelium, a tissue which function varies upon perfusion (Furchgott & Vanhoutte, 1989). Interestingly, the induction of myogenic tone modified the shape and location of the noradrenaline E/[A] curves similar to that observed in the rat and rabbit pressurized perfused SMA (Buus *et al.*, 1994; Dunn *et al.*, 1994), but did not affect the antagonizing potency for RS-17053 (Table 2).

Do low affinity (α_{1L}-) and high affinity (α_{1A}-adrenoceptors) sites co-exist in rat SMA

By selective inactivation of the α_{1L}-adrenoceptors with phenoxybenzamine while protecting α_{1A}-adrenoceptors, we attempted to unmask a putative α_{1A}-adrenoceptor population in rat SMA. However, Schild analysis demonstrated a single receptor again displaying low affinity for RS-17053 ($pA_2 = 8.25$). Therefore, it is unlikely that α_{1A}- and α_{1L}-adrenoceptors co-exist as distinct subtypes in rat SMA.

Observations from previous reports led to the idea that the α_{1A}-adrenoceptor antagonists, tamsulosin and RS-17053, might act at different sites at the α_1-adrenoceptor, which display differential susceptibility for affinity changes. For example, experimental conditions influenced the binding affinities of, among others, RS-17053 and prazosin, whereas that of tamsulosin and indoramin remained unaffected (Williams *et al.*, 1996). Accordingly, tamsulosin displayed similar affinities for functional α_{1A}-adrenoceptors and α_{1L}-adrenoceptors (Ford *et al.*, 1996). Combined concentration-ratio analysis, however, indicated that RS-17053 and tamsulosin compete for binding to the α_1-adrenoceptor site in rat SMA, which indicates that both α_1-adrenoceptor antagonists act syntopically.

In summary, data obtained in our experiments in rat SMA indicate that (i) the α_1-adrenoceptor mediating noradrenaline-induced contraction displays a distinct α_{1L}-adrenoceptor pharmacology, where both prazosin and RS-17053 have a low affinity; (ii) the affinity of α_{1L}-adrenoceptor for RS-17053 is not affected by changes in experimental conditions; (iii) it is unlikely that there is a co-existing α_{1A}-adrenoceptor population and (iv) tamsulosin, which does not discriminate between α_{1A}- and α_{1L}-adrenoceptors, acts at the same site as RS-17053. Overall, this study does not provide evidence for the hypothesis that α_{1L}-adrenoceptors represent an affinity state of the α_{1A}-adrenoceptor in functional assays (Ford *et al.*, 1997).

We thank Abbott Laboratories (North Chicago, IL, U.S.A.), Dr Anthony Ford (Roche Bioscience, Palo Alto, CA, U.S.A.), Japan Tobacco Company (Tokyo, Japan), Smith Kline Beecham Pharmaceuticals (King of Prussia, PA, U.S.A.), Boehringer Ingelheim (Bracknell, Berkshire, U.K.) and Yamanouchi Pharmaceuticals (Ibaraki, Tsukuba, Japan) for providing us with A61603, RS-17053, JTH-601, SKF89748-A, ST 587 and tamsulosin, respectively. We are also grateful to Dr Richard Bond (Houston, TX, U.S.A.) for suggesting receptor protection studies.

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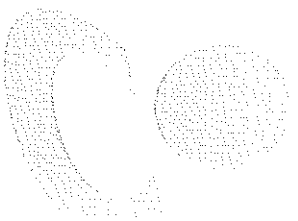
(Received January 21, 1999
Revised March 12, 1999
Accepted March 17, 1999)

Chapter 3

Analysis of receptor inactivation experiments with the operational model of agonism yields correlated estimates of agonist affinity and efficacy

J. Pharmacol. Toxicol. (1999) 41, 117-125



Analysis of receptor inactivation experiments with the operational model of agonism yields correlated estimates of agonist affinity and efficacy

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Received 12 May 1999; Accepted 24 June 1999

Abstract

The aim of this study was to evaluate whether the operational model of agonism can yield independent estimates of agonist affinity (pK_A) and efficacy ($\log \tau$) when Furchgott's method of irreversible receptor inactivation is employed. For this purpose, the interaction between noradrenaline and phenoxybenzamine was studied in rat small mesenteric artery using a paired-curve design. Phenoxybenzamine pretreatment produced a significant rightward shift and depression of the upper asymptote of the noradrenaline concentration-effect (E/[A]) curve. Although the operational model of agonism appeared to provide an adequate fit of the individual E/[A] curves, a highly significant correlation was found between the estimates of pK_A and $\log \tau$ ($r = -0.80$, $p < 0.0001$), inconsistent with the assumption that affinity and efficacy are independent parameters (best line fit: $pK_A = -0.96 \times \log \tau + 6.75$). The pK_A and $\log \tau$ estimates were not correlated with either the pEC_{50}s of the control curves or upper asymptotes of the phenoxybenzamine-treated curves. Simulations showed that the correlation between affinity and efficacy can be explained by the effect on the outcome of the analysis of random errors in the response measurements. Therefore, although in theory the operational model of agonism should provide independent estimates of agonist affinity and efficacy, this is unlikely to be the case with experimental data.

Keywords: α_1-Adrenoceptors; Affinity; Efficacy; Furchgott method; Noradrenaline; Operational model of agonism; Phenoxybenzamine; Small mesenteric artery (rat)

1. Introduction

The idea that agonist affinity and efficacy are independent properties has been a central concept of classical pharmacological receptor theory. In recent years, numerous groups have suggested that when Furchgott's (1966) irreversible receptor inactivation method is employed, agonist concentration-effect (E/[A]) curves from control and treated tissues can be fitted directly to Black and Leff's (1983) operational model of agonism to obtain estimates of affinity and relative efficacy for a variety of receptor systems in isolated tissue bioassays (see, for example, Black *et al.*, 1985a,b; Leff, 1988; Leff *et al.*, 1990; Christie *et al.*, 1992; Palea *et al.*, 1995; Sallés *et al.*, 1996; Tabernero *et al.*, 1996; Kramer *et al.*, 1997; MacLennan *et al.*, 1997a; Martin *et al.*, 1997; Pineda *et al.*, 1997; Vivas *et al.*, 1997; Watt *et al.*, 1997; Deyrup *et al.*, 1998), recombinant expression systems (Giles *et al.*, 1996; MacLennan *et al.*, 1997b) and *in vivo* animal models (Zernig *et al.*, 1996). A similar model-fitting approach has recently also been used to estimate agonist affinity and efficacy from E/[A] curves obtained in cell lines with different receptor expression levels (Wilson *et al.*, 1996; Corti *et al.*, 1997). Two kinds of experimental design are commonly used in irreversible receptor inactivation experiments which aim to estimate agonist affinity and efficacy: a single- and multiple-curve design (Leff *et al.*, 1990; Dougall, 1998). With the single-curve approach, individual E/[A] curves from control and irreversible antagonist-treated tissues are fitted simultaneously to the operational model of agonism:

$$E = \frac{E_m \cdot \tau^n \cdot [A]^n}{(K_A + [A])^n + \tau^n \cdot [A]^n} \quad (1)$$

to obtain single estimates of the maximum response achievable in the system (E_m), the slope index of the occupancy-effect function (n), and the agonist dissociation equilibrium constant (K_A) and individual estimates of the efficacy parameter (τ) for each curve. Recently, we have demonstrated that the practical utility of this simultaneous fitting proce-

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dure is limited, because the outcomes are highly dependent on between-tissue variability of the upper asymptotes of the control curves and may be unreliable even under well-controlled experimental conditions (Van der Graaf and Danhof, 1997). Therefore, it has been suggested that whenever possible a multiple-curve design should be adopted in which pre- and posttreatment E/[A] curves are obtained in the same tissue to estimate parameters that are not biased by between-tissue variability (Leff *et al.*, 1990; Van der Graaf and Danhof, 1997; Dougall, 1998). To date, however, the reliability of the multiple-curve design has not been evaluated in detail yet. Interestingly, however, Henry *et al.* (1992) have reported in a meeting abstract that the operational model of agonism in combination with the multiple-curve design yielded affinity and efficacy values that were highly correlated across different experiments, inconsistent with the basic assumption that these parameters can be estimated independently. Therefore, in the present study, we have studied the interaction between noradrenaline (NA) and phenoxybenzamine (PBZ) in rat isolated small mesenteric artery (SMA) using a paired-curve design. We applied the operational model of agonism to analyze these data and found that the model yielded highly variable and correlated estimates of affinity and efficacy, confirming the preliminary report by Henry *et al.* (1992). On the basis of simulations it is shown that this, at first sight unexpected, variability of and correlation between affinity and efficacy can be explained by the effect on the outcome of the analysis of random errors in the response measurements. This indicates that the multiple-curve design does not necessarily provide a reliable alternative for the single-curve method.

Preliminary accounts of these data were presented to the British Pharmacological Society (Van der Graaf, 1996a,b).

2. Methods

2.1. Rat isolated small mesenteric artery preparation

Male Wistar rats (225–300 g) were killed by cervical dislocation, and the mesentery was removed and placed in ice-cold modified Krebs-Henseleit solution (KHS) of the following composition (mM): NaCl 119.0, $NaHCO_3$ 25.0, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, glucose 5.5, $CaCl_2$ 2.5, and ethylenediaminetetra-acetic acid (EDTA) 0.026. Six arterial trees were dissected from each mesenteric vascular bed and cleared from surrounding adipose tissue. From each arterial tree, a ~2-mm ring segment was mounted in a small-vessel myograph (J.P. Trading, Aarhus, Denmark) with separated 6-mL organ baths (thermostatically controlled at 37 ± 0.5°C, containing the KHS and continuously gassed with 95% O_2 and 5% CO_2) as described before (Van der Graaf *et al.*, 1996). The endothelium was removed by gentle rubbing of the intimal surface with a thin, scoured, metal wire. Tissue responses were continuously measured as changes in isometric tension and displayed on potentiometric chart recorders.

2.2. Experimental protocol

Following a 30-min stabilization period, the internal diameter of each vessel was set to a tension equivalent to 0.9 times the estimated diameter at 100 mmHg effective transmural pressure (I_{100} = 245 ± 7 μm, n = 30) according to the standard procedure of Mulvany and Halpern (1977). After a further 15-min stabilization period, tissues were exposed to five concentrations of NA (10 μM), separated by 5-min washout periods. The absence of functional endothelium was confirmed by the lack of a relaxation response to 10 μM of the acetylcholine M-receptor agonist 5-methylfurmethide, added to the organ bath after the fifth exposure to NA had produced a plateau response. Effects were expressed as percentage of the response (7.8 ± 0.5 mN) produced by the fifth exposure to NA. After a 15-min washout period, tissues were incubated for 90 min with 30 μM cocaine and 6 μM timolol to block neuronal uptake and β-adrenoceptors, respectively, and a first NA E/[A] curve was obtained by cumulative dosing at third- or half-log unit concentration increments. Following a 15-min washout period, tissues were exposed to 0.1 nM (30 or 45 min) or 1 nM (10 or 15 min) PBZ and were subsequently washed for 30 min. Cocaine and timolol were then incubated as described above and a second NA E/[A] curve was obtained in each tissue.

2.3. Data analysis

2.3.1. Hill equation

Individual agonist curve data were fitted to the following form of the Hill equation, using an iterative, least-squares method [Eq. (2)]:

$$E = \frac{\alpha \cdot [A]^{n_H}}{EC_{50}{}^{n_H} + [A]^{n_H}} \qquad (2)$$

to provide estimates of midpoint slope (n_H), midpoint location (EC_{50}, estimated as a logarithm) and upper asymptote (α). The effect of drug treatment on these parameters was assessed by Student's paired t-test. Values of $p < 0.05$ were considered to be significant.

2.3.2. Estimation of NA affinity and efficacy

Agonist affinity and efficacy estimates were obtained by fitting each pair of control and PBZ-treated curves directly to the operational model of agonism [Eq. (1)], providing a common estimate of E_m, K_A and n for each pair and two τ values, $\tau_{control}$ and $\tau_{treated}$, for the control and PBZ-treated curves, respectively. K_A, $\tau_{control}$, and $\tau_{treated}$ were estimated as logarithms, because these parameters are assumed to be log-normally distributed (Leff *et al.*, 1990; Christopoulos, 1998). The estimates of E_m, pK_A (that is $-\log K_A$), n and $\log \tau_{control}$ from each pair of curves were then used to calculate means ± S.E. mean. The fitting procedures were carried out on a VAX 6000-310 computer employing the AR module (derivative-free, nonlinear regression) of the BMDP statistical software package (Dixon *et al.*, 1990). For the

simulation studies (see Christopoulos, 1998), 100 pairs of theoretical E/[A] curves were generated with the operational model of agonism and random error was added to each simulated data point with the statistical software package S-PLUS (version 4.5, MathSoft U.K.). Each pair of simulated E/[A] curves was subsequently fitted to the operational model of agonism as described above.

2.3.3. Graphical test to assess the goodness-of-fit

Black and Shankley (1990) have developed a graphical method to visualize systematic deviations of curve fits obtained with the operational model of agonism from experimental E/[A] data. Briefly, it was shown that a plot of $-\log(1-\alpha/E_m)$ against log EC_{50} data from rectangular hyperbolic agonist E/[A] curves obtained in the presence and absence of pretreatment with irreversible antagonist should yield a linear plot with unity slope. This test, however, only applies for rectangular hyperbolic E/[A] curves and could not be used for the NA E/[A] curves in the SMA, since the slopes of these curves were found to be significantly greater than unity. Therefore, an equivalent has been derived for nonrectangular hyperbolic curves from the operational model of agonism. The Hill-equation parameters, α and EC_{50}, are related to the operational model parameters E_m, τ, n, and K_A as follows (Black and Leff, 1983):

$$\alpha = \frac{E_m \cdot \tau^n}{1+\tau^n} \tag{3}$$

$$EC_{50} = \frac{K_A}{(2+\tau^n)^{1/n}-1} \tag{4}$$

From Eq. (3) and (4), for any experimental curve, Eq. (5),

$$\tau^n = \frac{\alpha}{E_m-\alpha} = \left(\frac{K_A+EC_{50}}{EC_{50}}\right)^n - 2 \tag{5}$$

which can be rearranged as follows:

$$\log\left(\left(\frac{\alpha}{E_m-\alpha}+2\right)^{1/n}-1\right) = -\log EC_{50} + \log K_A \tag{6}$$

Therefore, a plot of $\log\{(\alpha/E_m-\alpha)+2)^{1/n}-1\}$ against $-\log(EC_{50}/K_A)$ should yield a straight line with slope of unity and abscissa intercept of zero. Note that when $n = 1$, corresponding to a rectangular hyperbolic E/[A] curve, Eq. (6) simplifies to Eq. (7):

$$-\log\left(1-\frac{\alpha}{E_m}\right) = -\log EC_{50} + \log K_A \tag{7}$$

which is the equation derived by Black and Shankley (1990).

2.4. Compounds

Compounds were obtained from the following sources: cocaine hydrochloride, *l*-noradrenaline hydrochloride (NA), phenoxybenzamine hydrochloride (PBZ) and prazosin hydrochloride: Sigma, U.K.; chloroethylclonidine dihydrochloride (CEC): Research Biochemicals Incorporated, U.S.A.; timolol maleate: Merck, Sharp & Dohme, U.K.; 5-methylfurmethide iodide: James Black Foundation, U.K.

NA was dissolved and diluted in stoichiometric, aqueous ascorbic acid solution. PBZ was dissolved in absolute ethanol. Prazosin was dissolved initially in 50% ethanol to give a 2-mM stock solution and subsequently diluted in distilled water. All other drugs were dissolved in distilled water. NA solutions were made up each day. All other drug stock solutions were stored below −20°C and diluted on the day of the experiment. The maximum volume of drug solution administered to the 6-mL organ baths did not exceed 100 μL. Neither the vehicles nor the antagonists were found to produce significant effects on basal tone.

3. Results

3.1. Effects of PBZ on NA E/[A] curves

NA (10 nM–30 μM) produced concentration-dependent contraction of the SMA and the control E/[A] data ($n = 19$) were fitted to the Hill equation to provide estimates (mean ± S.E. mean) of midpoint location ($pEC_{50} = 6.69 \pm 0.07$), upper asymptote ($\alpha = 101.6 \pm 0.9\%$), and midpoint slope ($n_H = 1.45 \pm 0.08$). PBZ pretreatment produced a significant rightward shift and depression of the upper asymptote of the NA E/[A] curves (Fig. 1 and Table 1). The Hill slopes of the NA E/[A] curves obtained after PBZ treatment were always slightly lower than the slopes of the corresponding control curves, but this effect was not significant for any of the treatment groups as judged by Student's paired *t* test (Table 1).

The differences between the first and second NA curves were due to an effect of PBZ, since incubation with vehicle (10 μL ethanol) for 45 min (which was the longest incubation time used for PBZ) had no significant effects on the Hill slope parameters ($pEC_{50} = 6.63 \pm 0.10$ and 6.64 ± 0.07; $\alpha = 102.0 \pm 1.9\%$ and $102.6 \pm 2.0\%$; $n_H = 1.34 \pm 0.12$ and 1.32 ± 0.06 for the first and second curve, respectively, $n = 5$). Furthermore, the antagonistic effects of PBZ were shown to be solely due to irreversible blockade of α_1-

Table 1
Hill equation parameter estimates (mean ± S.E. mean) for noradrenaline concentration–effect curves obtained on rat small mesenteric arteries before and after treatment with phenoxybenzamine

PBZ treatment	pEC_{50}	α^a (%)	n_H	n^b
Control	6.69 ± 0.07	101.6 ± 0.9	1.45 ± 0.08	19
0.1 nM; 30 min	6.21 ± 0.06	90.3 ± 0.5	1.29 ± 0.14	3
0.1 nM; 45 min	6.32 ± 0.13	87.8 ± 4.2	1.43 ± 0.12	4
1 nM; 10 min	6.19 ± 0.14	56.3 ± 5.3	1.25 ± 0.11	8
1 nM; 15 min	6.12 ± 0.09	12.0 ± 1.4	1.27 ± 0.20	4

[a]Expressed as percentage of the fifth noradrenaline (10 μM) calibration response.
[b]Number of replicates.

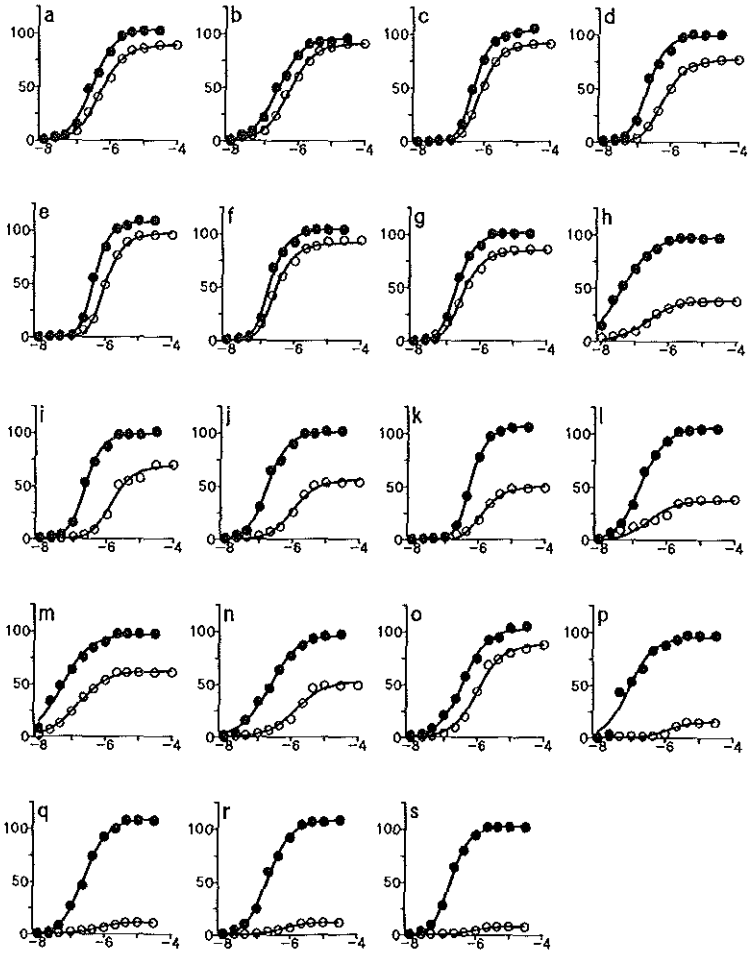

Fig. 1. Individual pairs of concentration–effect curves to noradrenaline obtained on rat small mesenteric arteries before (●) and after (○) pretreatment with phenoxybenzamine (a)–(c) 0.1 nM for 30 min; (d)–(g) 0.1 nM for 45 min; (h)–(o) 1 nM for 10 min and (p)–(s) 1 nM for 15 min. The lines shown superimposed on the experimental data points were simulated with the operational model of agonism using the individual parameter estimates given in Table 2. Abscissae: $\log_{10}$ [noradrenaline] (M). Ordinates: percentage of the response to the fifth noradrenaline (10 μM) calibration contraction.

adrenoceptors in the SMA, since co-incubation with the selective α_1-adrenoceptor antagonist, prazosin (20 nM, ~6.5 times the apparent affinity for α_1-adrenoceptors in rat SMA, Van der Graaf *et al.*, 1996), produced complete protection against the effects of 20-min pretreatment with 1 nM PBZ (pEC_{50} = 6.16 ± 0.08 and 6.22 ± 0.05; α = 105.8 ± 4.5% and 104.0 ± 0.4%; n_H = 1.88 ± 0.24 and 1.52 ± 0.05 in the absence and presence of PBZ pretreatment, respectively, n = 3). In contrast, incubation (30 min) with another irreversible α_1-adrenoceptor antagonist, chloroethylclonidine (CEC, 10 μM), had no significant effect on the NA E/[A] response (pEC_{50} = 6.31 ± 0.15 and 6.27 ± 0.09; α = 108.0 ± 3.4% and 102.2 ± 4.3%; n_H = 2.11 ± 0.12 and 1.93 ± 0.11 in the absence and presence of CEC pretreatment, respectively, n = 3).

3.2. Operational model of agonism fitting

Each pair of NA control and PBZ-treated E/[A] curves was fitted to the operational model of agonism to provide 19 individual sets of estimates of E_m, n, pK_A, log $\tau_{control}$ and log $\tau_{treated}$ (Table 2), from which mean and S.E. mean values were calculated (Table 2). Estimated E_m values were always greater than the upper asymptotes of the NA E/[A] control curves, indicating that according to the model NA behaves

Table 2
Operational model of agonism parameter estimates obtained from fitting individual pairs of noradrenaline concentration–effect curves obtained on rat small mesenteric arteries before and after treatment with phenoxybenzamine (Fig. 1)

Experiment[a]	E_m[b]	log $\tau_{control}$	log $\tau_{control}$	n	pK_A
a	120.9	0.54	0.303	1.48	6.03
b	99.5	1.18	0.846	1.22	5.43
c	115.9	0.38	0.229	2.55	6.10
d	106.3	0.64	0.226	1.93	6.13
e	112.5	0.56	0.316	2.61	5.90
f	120.4	0.26	0.159	3.19	6.76
g	123.8	0.25	0.128	2.71	6.71
h	110.5	0.91	−0.275	0.97	6.43
i	101.0	0.88	0.172	2.00	5.83
j	105.7	0.82	0.0376	1.66	5.99
k	127.8	0.20	−0.049	3.60	6.38
l	179.8	0.11	−0.379	1.52	6.67
m	113.5	0.75	0.0826	1.04	6.49
n	105.0	1.05	0.0109	1.03	5.56
o	110.1	0.98	0.502	1.23	5.46
p	99.5	1.14	−0.557	1.25	5.93
q	158.5	0.21	−0.645	1.71	6.47
r	156.7	0.20	−0.597	1.81	6.57
s	117.0	0.40	−0.486	2.24	6.52
Mean + S.E. mean	120.2 ± 5.0	0.60 ± 0.08	—	1.88 ± 0.17	6.18 ± 0.10

[a]Letters correspond to the labels in Fig. 2.
[b]Expressed as percentage of the fifth noradrenaline (10 μM) calibration response.

as a partial agonist in the SMA generating 86.3 ± 2.5% of the maximum possible response. The individual estimates were used to simulate the curves shown superimposed on the individual experimental data points in Fig. 1. The goodness-of-fit was assessed by the graphical test outlined in the Methods section. Individual values of $\log\{(\alpha/E_m-\alpha)+2)^{1/n}-1\}$ and $-\log(EC_{50}/K_A)$ were found to be highly correlated (r = 0.99, $p < 0.0001$), and linear regression yielded a slope which was not significantly different from unity (1.04 ± 0.03) and an abscissa intercept which was not significantly different from zero (−0.03 ± 0.02), indicating that the model provided an adequate description of the experimental data (Fig. 2).

However, a highly significant, negative correlation was

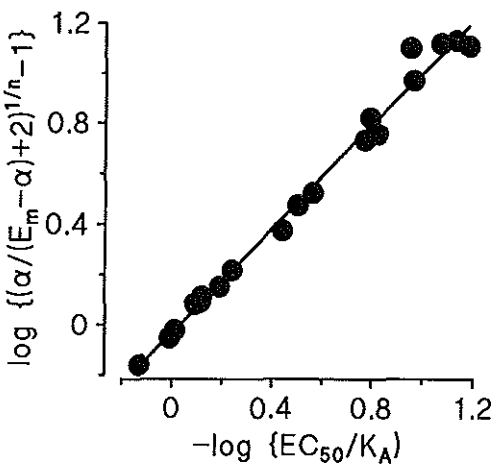

Fig. 2. Relation between individual estimates of $\log\{(\alpha/E_m-\alpha)+2)^{1/n}-1\}$ and $-\log(EC_{50}/K_A)$ obtained from model fitting of pairs of control and phenoxybenzamine-treated noradrenaline concentration–effect curves obtained on rat small mesenteric arteries.

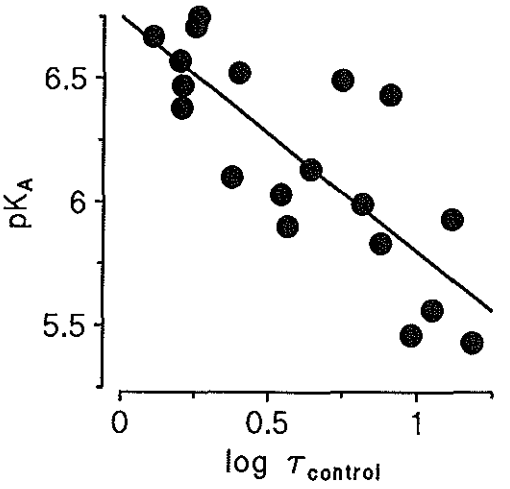

Fig. 3. Relation between individual estimates of (pK_A) and efficacy (log $\tau_{control}$) obtained from fitting pairs of control and phenoxybenzamine-treated noradrenaline concentration–effect curves obtained on rat small mesenteric arteries to the operational model of agonism.

found between the individual estimates of pK_A and log $\tau_{control}$ ($r = -0.80$, $p < 0.0001$), inconsistent with the assumption that affinity and efficacy are independent model parameters (best line fit: $pK_A = -0.96 \times \log \tau_{control} + 6.75$; Fig. 3). The pK_A and log $\tau_{control}$ estimates were not correlated with the pEC_{50} values of the NA control curve ($r = 0.31$ and 0.30, respectively, $p > 0.01$, Fig. 4). Furthermore, the outcomes of the model fitting were independent of the degree of receptor inactivation, since pK_A and log $\tau_{control}$ were not significantly correlated with the upper asymptotes of the PBZ-treated curves ($r = -0.33$ and 0.14, respectively, $p > 0.1$, Fig. 5).

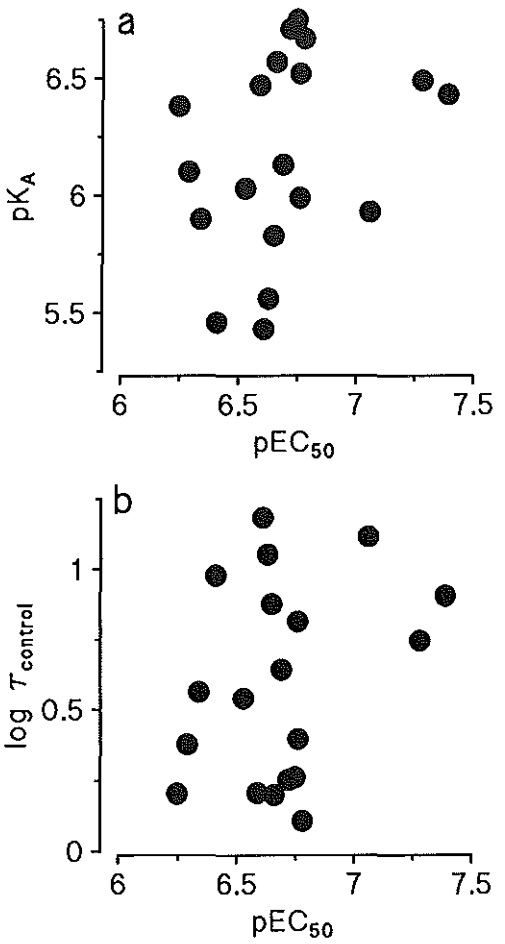

Fig. 4. Relation between individual estimates of potency (pEC_{50}) and (a) affinity (pK_A) and (b) efficacy (log $\tau_{control}$) obtained from fitting pairs of control and phenoxybenzamine-treated noradrenaline concentration–effect curves obtained on rat small mesenteric arteries to the operational model of agonism.

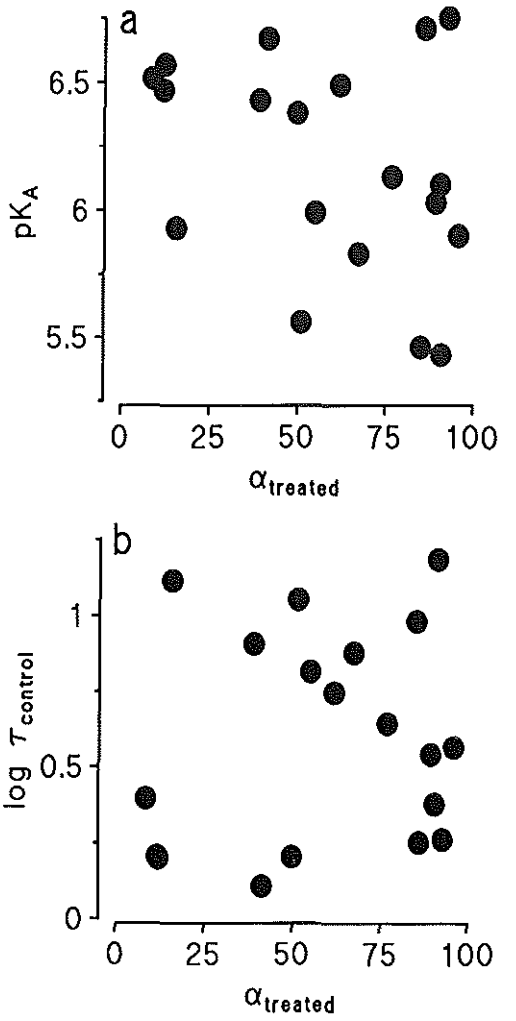

Fig. 5. Relation between individual estimates of the upper asymptote after phenoxybenzamine treatment ($\alpha_{treated}$) and (a) affinity (pK_A) and (b) efficacy (log $\tau_{control}$) values obtained from fitting pairs of control and phenoxybenzamine-treated noradrenaline concentration–effect curves obtained on rat small mesenteric arteries to the operational model of agonism.

3.3. Simulations

In an attempt to investigate whether the correlation between affinity and efficacy was due to a statistical rather than a pharmacological phenomenon, a simulation study was performed. First, "perfect" control and PBZ-treated E/[A] curves were simulated with the operational model of agonism using the average parameters estimated for NA (E_m = 120.2, log $\tau_{control}$ = 0.60, log $\tau_{treated}$ = 0.00, n = 1.88 and pK_A = 6.18; Table 2) and the same dosing scheme as em-

ployed in the experiments. The second step was to add random noise to the data points to simulate the influence of experimental error in the response measurements. For the nineteen experimental NA control curves, the coefficient of variation (CV) for each data point was found to be related to the effect level (E) as follows: log CV = $-0.02 \times E + 2.34$; $r = 1.00$). Although this empirical relationship contains both inter- and intratissue variability, for the sake of simplicity it was assumed that intertissue variability was insignificant since all responses were normalized to a calibration response. Thus, the linear relationship between log CV and E was used to add random noise to the data points as if they originated from a normal distribution and 100 pairs of curves were simulated and subsequently fitted to the operational model of agonism. Although the mean affinity and efficacy estimates were practically identical to the "true" mean values used for the simulation ($pK_A = 6.29 \pm 0.04$ and $\log \tau_{control} = 0.56 \pm 0.03$; $n = 100$), individual pK_A and $\log \tau_{control}$ estimated varied over ~2 log-unit between tissues (Fig. 6). The correlation between individual pK_A and $\log \tau_{control}$ estimates was highly significant ($r = 0.94$, $p < 0.0001$), and the best line fit was practically identical to the one obtained from the experimental data ($pK_A = -1.16 \times \log \tau_{control} + 6.94$; Fig. 6).

4. Discussion

The operational model of agonism has become a standard and widely employed tool in pharmacological research

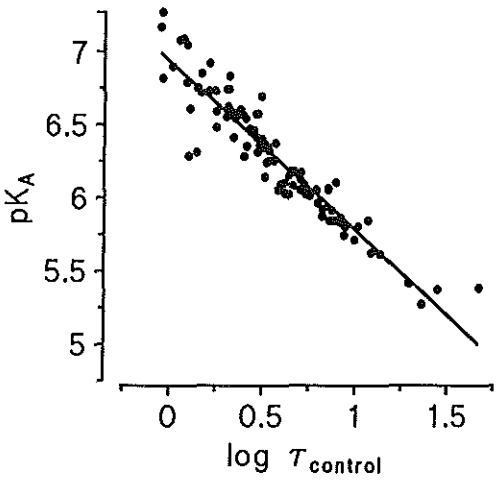

Fig. 6. Relation between individual estimates of affinity (pK_A) and efficacy ($\log \tau_{control}$) obtained from fitting 100 pairs of simulated concentration-effect curves to the operational model of agonism. In all simulations, pK_A and $\log \tau_{control}$ were fixed at 6.18 and 0.60, respectively, and the deviations from these "true" values are the result of random noise added to the data points (see text for details).

to estimate agonist affinity and efficacy from receptor inactivation experiments (see Introduction for references). Recently, we have demonstrated that the simultaneous fitting method, based on a single-curve design, only yields reliable affinity and efficacy estimates when there is practically no between-tissue variation of the upper asymptotes of the control curves, which limits to a great extent the utility of this approach for the analysis of experimental data (Van der Graaf and Danhof, 1997). In the present study, the alternative approach of a multiple-curve design was evaluated using both experimental and simulated data. The main finding was that the operational model of agonism yielded highly variable and correlated estimates of affinity and efficacy of NA at α_1-adrenoceptors in the rat SMA assay, inconsistent with the basic assumption that these are independent parameters. Remarkably, the individual pK_A estimates varied over 1.3 log units between different tissues (Table 2; Fig. 3). Previously, Bevan *et al.* (1988) have shown that the apparent affinity of NA for the α_1-adrenoceptor, determined by Furchgott's method, varied by over three orders of magnitude between 12 rabbit arteries and aortae of five species. Furthermore, it was found that NA's pK_A and potency (pEC_{50}) in these tissues were positively correlated. In contrast, there was little variation in the affinity of the α_1-adrenoceptor antagonist, prazosin, between these tissues. On the basis of these observations, the so-called *variable receptor affinity hypothesis* was proposed which suggests that variation in NA's affinity for the α_1-adrenoceptor is brought about by "local cellular influences" and does not reflect differences in the α_1-adrenoceptor subtypes between tissues. It was suggested that this mechanism can also cause large pK_A variations for NA in the same tissue (Bevan *et al.*, 1989), when it was found that during a 3-month period the pK_A for NA in rabbit thoracic aorta varied between 5.4 and 7.3 ($n = 21$) and was positively correlated with potency. The cornerstone on which Bevan and coworkers have built their hypothesis is the correlation between NA's affinity and potency. In the present case, however, there was no significant correlation between pEC_{50} values for the control curves and pK_A estimates [Fig. 4(a)], and our data are therefore not consistent with expectations of the variable receptor affinity hypothesis (Bevan *et al.*, 1988, 1989).

It is also unlikely that the complexity in the present study was due to failure to satisfy basic experimental criteria for the application of the operational model of agonism. First, following vehicle treatment, the second NA E/[A] curve was practically superimposed on the first one, confirming the validity of the paired-curve design. Second, the reversible α_1-adrenoceptor antagonist, prazosin, could protect completely against the inhibitory effect of PBZ, confirming that this was due only to inactivation of α_1-adrenoceptors. Another possibility could be the involvement of multiple receptors, since at least three α_1-adrenoceptor subtypes ($\alpha_{1A/L}$, α_{1B}, and α_{1D}) operate in vascular tissues (see Stam *et al.*, 1999). However, a 30-min pretreatment of the SMA with 10 μM CEC had no significant effect on the NA response,

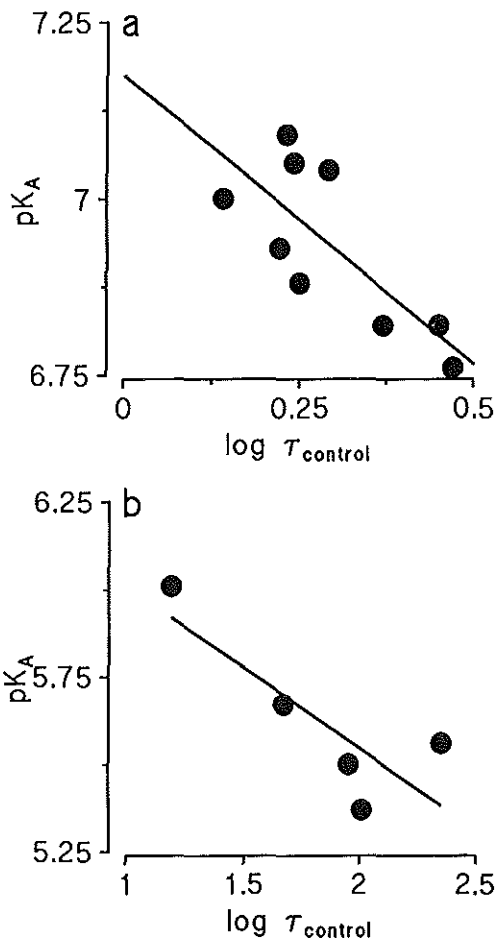

Fig. 7. Relation between previously published individual estimates of affinity (pK_A) and efficacy ($\log \tau_{control}$) obtained from operational model fitting of pairs of control and phenoxybenzamine-treated concentration-effect curves to (a) 5-hydroxytryptamine obtained on rabbit aorta (Leff *et al.*, 1990; original data from Black *et al.*, 1985b) and (b) 5-methylfurmethide obtained on guinea-pig trachea (Leff *et al.*, 1985).

while it is known to inactivate, at least partially, α_{1B}- and α_{1D}-adrenoceptors under these conditions (Michel *et al.*, 1995; Stam *et al.*, 1999). Finally, the endothelium was always removed, cocaine and timolol were present in all experiments to block $Uptake_1$ and β-adrenoceptors, respectively, and $Uptake_2$ does not play a significant role in the SMA assay (Van der Graaf *et al.*, 1996).

Overall therefore, the simulations performed in this study (Fig. 6) strongly suggest that the large variability in pK_A and $\log \tau$ estimates and the associated correlation are due to a statistical rather than a pharmacological phenomenon and call into question the validity of the widely used curve-fitting procedure for the estimation of agonist affinity and efficacy. It was shown that when random noise (at experimentally encountered levels) was added to "perfect" curves generated by the operational model of agonism, affinity estimates varied over two log units between independent experiments (Fig. 6). This implies that the failure of the operational model of agonism to estimate robust and independent values of affinity and efficacy is not limited to the present experiment but is an inherent weakness of the method. Although detailed studies in other assays are required to substantiate this conclusion further, some previously published results may be related directly to the phenomenon described in the present article. First, Henry *et al.* (1992) have reported that in guinea-pig ileum carbachol and pilocarpine affinity estimates obtained by the operational model of agonism varied by two and one orders of magnitude, respectively, and high correlations were observed between pK_A and $\log \tau$ estimates. Second, analysis of previously published pK_A and $\log \tau$ estimates for 5-hydroxytryptamine in rabbit aorta (Black *et al.*, 1985a; Leff *et al.*, 1990) and for 5-methylfurmethide in guinea-pig trachea (Leff *et al.*, 1985) also revealed notable correlations between affinity and efficacy ($r = -0.77$ and -0.83, respectively, Fig. 7), although it should be noted that the range and number of values are rather limited in these studies. Finally, Tabernero *et al.* (1996) have reported a correlation between pK_A and $\log \tau$ for the α_1-adrenoceptor agonist, phenylephrine, in the tail artery of spontaneously hypertensive rats with intact or damaged endothelium.

In conclusion, this study extends our previous (Van der Graaf and Danhof, 1997) assessment of the reliability of agonist affinity and efficacy estimation using the operational model of agonism and demonstrates that the multiple-curve design does not necessarily provide a reliable alternative for the single-curve method. Although in theory the operational model of agonism should provide independent estimates of agonist affinity and efficacy, this is unlikely to be the case with experimental data.

Acknowledgments

PHVDG was financially supported by the Academy Fellowship Program of the Royal Netherlands Academy of Arts and Sciences (KNAW).

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Chapter 4

Functional characterisation of the pharmacological profile of the putative α_{1B}-adrenoceptor antagonist, (+)-cyclazosin

Eur. J. Pharmacol. (1999) 361, 79-83

Short communication

Functional characterisation of the pharmacological profile of the putative α_{1B}-adrenoceptor antagonist, (+)-cyclazosin

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Received 18 September 1998; revised 7 October 1998; accepted 9 October 1998

Abstract

We studied the functional pharmacological profile of (+)-cyclazosin, which has been characterised as a selective, high-affinity ($pK_i = 9.68$) α_{1B}-adrenoceptor ligand in binding experiments with rat liver membranes. The pK_B/pA_2 values for antagonism of contractions mediated via $\alpha_{1A/L}$-adrenoceptors of rat small mesenteric artery, α_{1D}-adrenoceptors of rat aorta and α_{1B}-adrenoceptors of rat spleen were 7.78 ± 0.04, 6.86 ± 0.07 and 7.96 ± 0.08, respectively. Furthermore, in mouse spleen, which is also regarded as an α_{1B}-adrenoceptor preparation, (+)-cyclazosin displayed low potency and did not act as a competitive antagonist. Thus, in contrast with results obtained in radioligand binding experiments, (+)-cyclazosin does not behave as a selective α_{1B}-adrenoceptor antagonist in functional tissues. Whether this discrepancy has consequences for the classification of α_1-adrenoceptors requires further investigation.

Keywords: α_1-Adrenoceptor; Aorta, rat; (+)-Cyclazosin; Small mesenteric artery, rat; Spleen, mouse; Spleen, rat; Tamsulosin

1. Introduction

Radioligand binding studies and molecular biology experiments have demonstrated the existence of at least three α_1-adrenoceptor subtypes, now referred to as α_{1A}, α_{1B} and α_{1D} (Hieble et al., 1995). Functional studies suggest the existence of an additional α_{1L}-adrenoceptor subtype displaying low affinity for prazosin (Hieble et al., 1995). Recently, it was postulated that the α_{1L}-adrenoceptor might represent a low affinity state of the α_{1A}-adrenoceptor (Ford et al., 1997). Selective competitive antagonists for α_{1A}- and α_{1D}-adrenoceptors, have been described in detail (see Hieble et al., 1995; Stam et al., 1996; Ford et al., 1997). Although the preferential susceptibility to irreversible inactivation by chloroethylclonidine has been used to subclassify α_{1B}-adrenoceptors (Hieble et al., 1995), the lack of a selective competitive antagonist has impeded a precise quantitative characterisation of α_{1B}-adrenoceptors.

Initially, some data obtained in radioligand binding experiments suggested that spiperone and risperidone were competitive, selective α_{1B}-adrenoceptor antagonists, but functional studies were not able to confirm this (Burt et al., 1995; Eltze, 1996b). Recently, however, Giardina et al. (1996) have described a potent competitive α_{1B}-adrenoceptor antagonist, (+)-cyclazosin, which displays a 90- to 130-fold selectivity for binding to rat α_{1B}-adrenoceptors compared to α_{1A} and α_{1D} subtypes ($pK_i = 9.68$, 7.73 and 7.57 for rat liver α_{1B}, hippocampus α_{1A} and cloned α_{1d}-adrenoceptors, respectively). The selectivity of (+)-cyclazosin on functional responses mediated by the α_1-adrenoceptor subtypes has however not yet been studied. Therefore, in the present study we examined the effect of (+)-cyclazosin on the contractile responses to noradrenaline and phenylephrine in rat small mesenteric artery, rat aorta and rat and mouse spleen, responses which are believed to be mediated mainly by $\alpha_{1A/L}$- (Stam et al., 1996), α_{1D}- (Hieble et al., 1995) and α_{1B}-adrenoceptors (Burt et al., 1995; Hieble et al., 1995; Eltze, 1996a), respectively.

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2. Materials and methods

2.1. Tissue preparation

The mesentery, aorta and spleen were isolated from male Wistar rats (250–350 g) and spleen from white mice (25–30 g) which had been killed by cervical dislocation. Rats received prior anaesthesia (sodium pentobarbitone, 60 mg kg^{-1}, i.p.). Tissues were placed in ice-cold modified Krebs–Henseleit solution (KHS) of the following composition (mM): NaCl 119.0, $NaHCO_3$ 25.0, KCl 4.7, KH_2PO_3 1.2, $MgSO_4$ 1.2, glucose 5.5, $CaCl_2$ 2.5 and ethylenediaminetetraacetic acid 0.026. The Ca^{2+} concentration ($CaCl_2$ = 0.25 mM) used for rat aorta was one tenth of that of standard KHS (see Van der Graaf et al., 1996a). Tissues were mounted in thermostatically controlled (37°C) organ baths to measure isometric contractions. The bath medium was continuously gassed with 95% O_2 and 5% CO_2.

Rat small mesenteric arteries were isolated from the arterial tree and mounted as ring segments (~ 2 mm in length) in a myograph (J.P. Trading, Aarhus, Denmark), as described by us previously (Van der Graaf et al., 1996b). After a 30 min stabilization period, the preparations were challenged five times with noradrenaline (10 μM) with washouts after each challenge. The endothelium was left intact, since its removal turned out to be technically difficult and was found to be associated with a substantial decrease in the functional reactivity (unpublished observation). The integrity of the endothelium was confirmed after the first challenge with noradrenaline by using acetylcholine (10 μM), which produced at least 60% relaxation in all tissues.

After removal of the endothelium by gentle rubbing with a polyethylene tube, rat aortic ring segments (3 mm) were mounted in 15-ml organ baths and equilibrated at 20 mN for 90 min. Subsequently, a calibration contraction was obtained to 30 μM 5-hydroxytryptamine (5-HT) and the absence of the endothelium was then confirmed by the lack of relaxation in response to acetylcholine (10 μM).

Rat and mouse splenic strips, obtained after longitudinal bisection, were mounted in 15 ml organ baths and equilibrated at a tension of 15 mN for 90 min and at 8 mN for 60 min, respectively.

2.2. Experimental protocol

Tissues were incubated with desipramine (10 μM), timolol (6 μM) and corticosterone (10 μM) to block neuronal uptake, β-adrenoceptors and non-neuronal uptake, respectively. Sixty minutes later and in the presence of these substances, agonist (noradrenaline in rat small mesenteric artery and mouse spleen; phenylephrine in rat aorta and spleen) concentration-effect ($E/[A]$) curves were recorded. In the ase of rat small mesenteric artery, cocaine (30 μM) replaced desipramine, corticosterone was omitted and SCH-23390 (10 nM) was added to block dopamine D_1 receptors (Van der Graaf et al., 1996b).

A multiple-curve design was used in experiments with rat small mesenteric artery and rat and mouse spleen. After the first (rat small mesenteric artery and spleen) or third (mouse spleen) agonist $E/[A]$ curve was recorded, each tissue segment was washed (rat small mesenteric artery: 30 min, rat spleen: 120 min, mouse spleen: 60 min) and equilibrated (60 min) with vehicle or antagonist at different concentrations. Subsequently, another agonist $E/[A]$ curve was obtained and the responses were expressed as a percentage of those of the preceding agonist curve.

A single curve design was used for rat aorta. Thus, after the calibration contraction in response to 5-HT, separate segments from each vessel were incubated with either vehicle or different antagonist concentrations. Subsequently, a single $E/[A]$ curve was obtained for phenylephrine. Data are expressed as percentages of the calibration contraction.

2.3. Analysis

Individual agonist curve data were fitted to the Hill equation by using an iterative, least-squares method to calculate the midpoint location (pEC_{50}), Hill slope (n_H) and upper asymptote (α). The effect of drug treatment on these parameters was assessed by one-way analysis of variance (ANOVA) or Student's t-test, as appropriate. Values of $P < 0.05$ were considered to be significant.

When minimum criteria for competitive antagonism were satisfied, that is the antagonist produced a parallel rightward shift of the agonist $E/[A]$ curve with no change in the upper asymptote, antagonist affinity was estimated by fitting the individual pEC_{50} values obtained in the absence and presence of antagonist to the Schild equation as described previously (Van der Graaf et al., 1996a). When the Schild plot slope parameter (b) was not significantly different from unity, the data were re-fitted with b constrained to unity so that the antagonist dissociation equilibrium constant, K_B, could be estimated (Jenkinson et al., 1995). When the criteria of competitive antagonism were not completely satisfied, an empirical pA_2 value was estimated by using the Schild equation, with b constrained to unity. All data are presented as means ± S.E.M.

2.4. Compounds

Compounds were obtained from the following sources: cocaine hydrochloride, 5-hydroxytryptamine creatine sulphate (5-HT), (−)-noradrenaline hydrochloride, acetylcholine chloride, (−)-phenylephrine hydrochloride, desipramine, corticosterone were from Sigma, The Netherlands; SCH-23390 (*R*-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride) was from Research Biochemicals, USA; timolol maleate was from ICN Biomedicals, The Netherlands; tamsulosin hydrochloride was a gift from Yamanouchi

Pharmaceutical, Japan; (+)-cyclazosin (+[4-(4-amino-6,7-dimethoxyquinazolin-2-yl)-*cis*-octahydroquinoxalin-1-yl]-furan-2-ylmethanone) was a gift from Dr. A. Leonardi, Recordati, Italy. (+)-Cyclazosin was dissolved in dimethylsulfoxide to give a 0.1 M stock solution and further diluted in distilled water. Corticosterone was dissolved in ethanol to give a stock solution of 30 mM. All other drugs were dissolved in distilled water.

3. Results

3.1. Effect of (+)-cyclazosin on noradrenaline-induced contraction of rat small mesenteric artery

Noradrenaline produced concentration-dependent contractions of rat small mesenteric artery (Fig. 1A). Hill parameters of the control noradrenaline $E/[A]$ curves ($n = 7$) were: midpoint location (pEC_{50}) = 6.40 ± 0.17, Hill slope (n_H) = 3.34 ± 0.45 and upper asymptote (α) = 95 ± 2% of that of the first noradrenaline $E/[A]$ curve (20.6 ± 2.1 mN). (+)-Cyclazosin (0.1–1 μM) produced a parallel, rightward shift of the noradrenaline $E/[A]$ curve. Schild analysis (Fig. 1B) yielded a slope parameter not different from unity (1.15 ± 0.11, $df = 18$) and a pK_B of 7.78 ± 0.04 was estimated.

3.2. Effect of (+)-cyclazosin on phenylephrine-induced contraction of rat aorta

Phenylephrine produced concentration-dependent contractions of rat aortic rings: $pEC_{50} = 6.76 \pm 0.05$, $n_H = 0.62 \pm 0.04$ and $\alpha = 108 \pm 7\%$ of that of the 5-HT calibration contraction (9.3 ± 0.1 mN, $n = 5$). (+)-Cyclazosin (0.1–3 μM) concentration dependently shifted the phenylephrine $E/[A]$ curve to the right (Fig. 1C). However, the criteria for competitive antagonism were not completely satisfied, since (+)-cyclazosin produced a concentration-dependent steepening of the $E/[A]$ curves ($n_H = 0.75 \pm 0.02$, 0.82 ± 0.06, 0.89 ± 0.02 and 0.96 ± 0.06 for 0.1, 0.3, 1 and 3 μM (+)-cyclazosin, respectively ($P < 0.001$). Notwithstanding this complexity, Schild analysis was per-

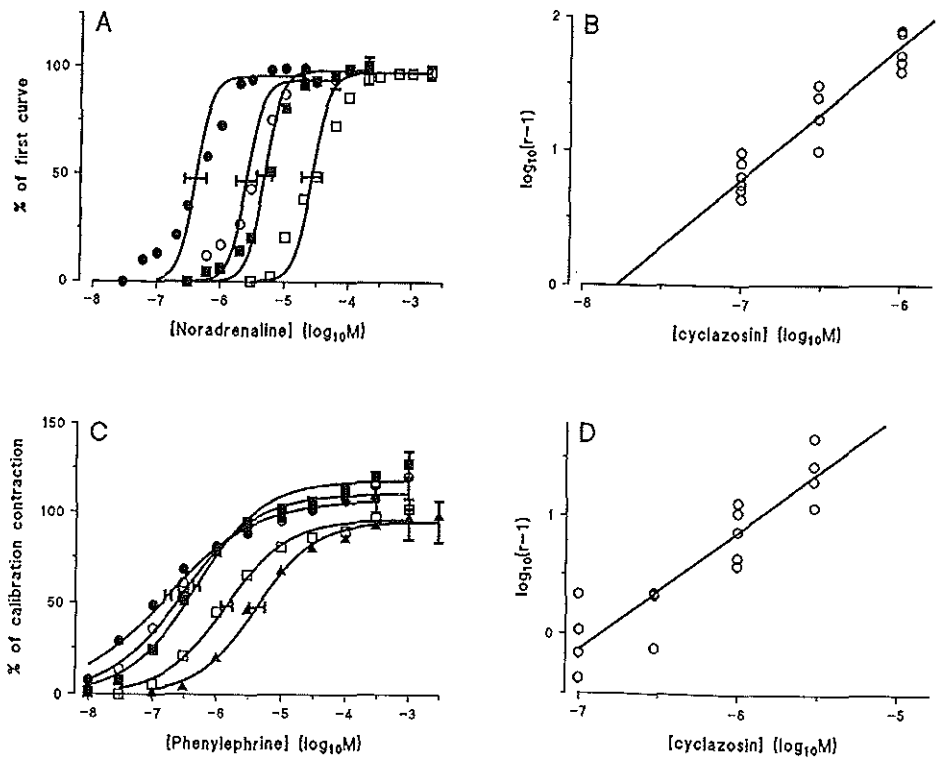

Fig. 1. $E/[A]$ curves of noradrenaline in rat small mesenteric artery (panel A) and phenylephrine in rat aorta (panel C) in the absence (●) or presence of 0.1 (○), 0.3 (■), 1 (□) and 3 (▲) μM (+)-cyclazosin. The corresponding Schild plots are shown in panels B and D. The lines superimposed on the data points were determined by using parameters obtained from the constrained model fit.

formed (Fig. 1D). The Schild slope parameter was not different from unity (1.07 ± 0.09, $df = 21$) and a pA_2 value of 6.86 ± 0.07 was estimated.

3.3. Effect of (+)-cyclazosin on phenylephrine-induced contraction of rat spleen

Hill parameters for the phenylephrine-induced contraction of vehicle-treated rat spleen ($n = 6$) were $pEC_{50} = 5.22 \pm 0.04$, $n_H = 0.75 \pm 0.05$ and $\alpha = 127 \pm 9\%$ of that of the first phenylephrine $E/[A]$ curve (3.5 ± 0.1 mN). (+)-Cyclazosin at concentrations of 0.03–0.3 μM produced a parallel, rightward displacement of the phenylephrine $E/[A]$ curve (Fig. 2A). In the presence of a higher concentration of (+)-cyclazosin (1 μM), the maximum of the agonist $E/[A]$ curve could not be attained with the highest concentration of phenylephrine (10 mM), and the phenylephrine $E/[A]$ curve flattened ($n_H = 0.45 \pm 0.01$, $P < 0.05$). Schild analysis was performed only for the concentrations of (+)-cyclazosin (0.03–0.3 μM) that met the criteria of competitive antagonism: $b = 1.02 \pm 0.20$ ($df = 11$) and $pK_B = 7.96 \pm 0.08$ (Fig. 2B).

For comparison, the affinity of the reference α_1-adrenoceptor antagonist, tamsulosin, was estimated. Tamsulosin (10 nM) produced a parallel rightward displacement of the phenylephrine $E/[A]$ curve and yielded a pA_2 of 9.16 ± 0.14 ($n = 3$), similar to that reported by Noble et al. ($pA_2 = 8.9$; Noble et al., 1997).

3.4. Effect of (+)-cyclazosin on noradrenaline-induced contraction of mouse spleen

As shown in Fig. 2C, noradrenaline produced concentration-dependent contractions of mouse spleen and the Hill parameters in the control ($n = 8$) tissue were $pEC_{50} = 6.44 \pm 0.07$, $n_H = 0.74 \pm 0.04$, $\alpha = 107.3 \pm 0.4\%$ of that of the third $E/[A]$ curve (2.7 ± 0.02 mN). (+)-Cyclazosin (0.1 μM) produced a parallel, rightward shift of the noradrenaline $E/[A]$ curve, with an associated pA_2 value of 7.38 ± 0.08. Higher concentrations of (+)-cyclazosin (0.3 and 1 μM), however, did not produce any further shift (Fig. 2C). A slight decrease in the maximal response ($\alpha = 89.4 \pm 0.6\%$, $P < 0.05$) was produced by (+)-cyclazosin (1 μM). The affinity estimate determined from

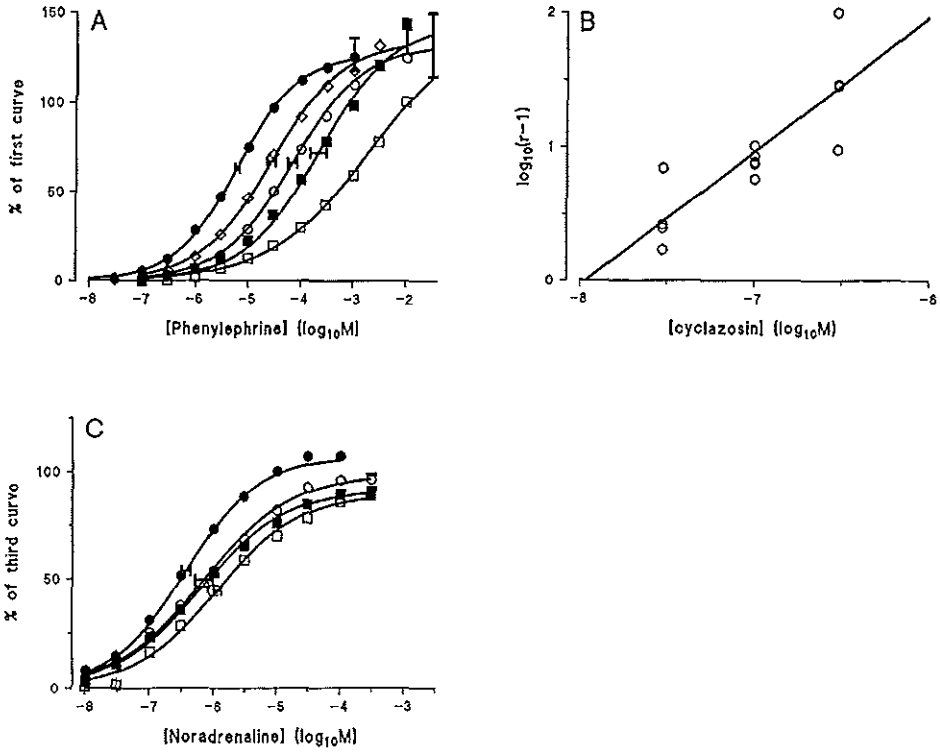

Fig. 2. $E/[A]$ curves of phenylephrine in rat spleen (panel A) and noradrenaline in mouse spleen (panel C) in the absence (●) or presence of 0.03 (◇), 0.1 (○), 0.3 (■) and 1 (□) μM (+)-cyclazosin. The corresponding Schild plot in the rat spleen is shown in panel B. The line superimposed on the data points was determined by using parameters obtained from the constrained model fit.

the rightward shift produced by 10 nM tamsulosin ($pA_2 = 8.44 \pm 0.14$, $n = 3$) was in good agreement with that of a previous report ($pK_B = 8.62$; Eltze, 1996a).

4. Discussion

In this study, we characterised the potency of the putative α_{1B}-selective antagonist (+)-cyclazosin in tissues expressing different subtypes of functional α_1-adrenoceptors. The affinity estimate of (+)-cyclazosin in rat small mesenteric artery ($pK_B = 7.78 \pm 0.04$) was in agreement with the reported binding affinity for α_{1A}-adrenoceptors in rat hippocampus, human cloned α_{1a}-adrenoceptors and α_{1L}-adrenoceptors ($pK_i/pK_B = 7.1–7.7$; Giardina et al., 1996; Kava et al., 1998).

In rat aorta, which is considered to be a functional α_{1D}-adrenoceptor correlate (Hieble et al., 1995), the rightward displacement of the phenylephrine $E/[A]$ curves by (+)-cyclazosin was accompanied by a concentration-dependent steepening of the phenylephrine $E/[A]$ curve. This phenomenon has also been reported for other antagonists and is suggested to be due to the expression of two closely related forms of the α_{1D}-adrenoceptor in rat aorta (Van der Graaf et al., 1996a). The functional potency in rat aorta of (+)-cyclazosin ($pA_2 = 6.86$), however, was within the range of its affinity for rat cloned α_{1d}-adrenoceptors ($pK_i = 7.57$; Giardina et al., 1996).

On the basis of the high sensitivity to inactivation by chloroethylclonidine, the receptors mediating contraction of rat spleen in response to phenylephrine have been classified as α_{1B}-adrenoceptors (Han et al., 1987; Burt et al., 1995). However, the pA_2 value of 7.96 estimated from the competitive antagonism displayed by (+)-cyclazosin (0.03–0.3 μM) is incompatible with its affinity for rat liver α_{1B}-adrenoceptors ($pK_i = 9.68$; Giardina et al., 1996). This discrepancy with radioligand binding data led us to study the antagonism of (+)-cyclazosin in mouse spleen, a tissue where an even better correlation of antagonist affinities with cloned α_{1b}-adrenoceptors has been observed (Burt et al., 1995; Eltze, 1996a). Surprisingly, the rightward displacement of the noradrenaline $E/[A]$ curve by (+)-cyclazosin was only small and was not concentration dependent (Fig. 2C). It should be noted that in our hands tamsulosin, the reference α_1-adrenoceptor antagonist, yielded affinity estimates in rat and mouse spleen that were in accordance with those of previous studies (Eltze, 1996a; Noble et al., 1997). Thus, in contrast with the results of radioligand binding experiments (Giardina et al., 1996), (+)-cyclazosin appears not to behave as a selective α_{1B}-adrenoceptor antagonist in functional studies. Whether this discrepancy has consequences for the classification of α_1-adrenoceptors requires further investigation.

Acknowledgements

We thank Dr. A. Leonardi and Yamanouchi for providing us with (+)-cyclazosin and tamsulosin, respectively.

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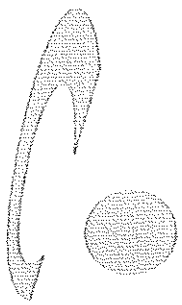
Chapter 5

Characterization of receptors mediating contraction of the rat isolated small mesenteric artery and aorta to arginine vasopressin and oxytocin

Br. J. Pharmacol. (1998) 125, 865-873



Characterization of receptors mediating contraction of the rat isolated small mesenteric artery and aorta to arginine vasopressin and oxytocin

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1 The exact nature of the receptor subtype(s) involved in the action of arg-vasopressin (AVP) on the rat aorta and small mesenteric artery (SMA) is controversial. Therefore, we have studied the effects of the selective V_{1A} receptor antagonists, OPC 21268 and SR 49059, and the oxytocin (OT) receptor antagonist, atosiban, on the AVP- and OT-induced contractions of the two vessels.

2 AVP and OT displayed similar intrinsic activities in the rat aorta and SMA, but AVP was ~130 fold and ~500 fold more potent than OT, respectively. In the rat aorta, Hill slopes (n_H) were similar for OT and AVP. However, in rat SMA, the OT concentration-effect (E/[A]) curve was significantly steeper than the AVP E/[A] curve (n_H, = 3.3±0.20, 2.3±0.15; $P<0.001$).

3 In the aorta OPC 21268, SR 49059 and atosiban competitively antagonized the AVP and OT E/[A] curves. Except for atosiban and SR 49059 against AVP, competitive antagonism was also observed in the SMA. Atosiban caused concentration-dependent steepening of the AVP E/[A] curve, whereas SR 49059 decreased the upper asymptote.

4 Schild analysis yielded affinities indicative of V_{1A} receptor involvement in both vessels: pK_B/pA_2 = 9.20–9.48, 7.56–7.71 and 6.19–6.48 for SR 49059, OPC 21268 and atosiban, respectively.

5 Neither AVP nor OT relaxed U46619 pre-contracted aorta or SMA in the presence of SR 49059, suggesting no interference of a vasodilatory component.

6 Despite predominant involvement of V_{1A} receptors in both vessels, the different Hill slopes of AVP and OT E/[A] curves as well as the steepening of the AVP E/[A] curves by atosiban are indicative of receptor heterogeneity in the rat SMA.

Keywords: Aorta; atosiban; OPC 21268; oxytocin; rat; small mesenteric artery; SR 49059; vasopressin receptors

Introduction

Arg-vasopressin (AVP) is believed to exert its action through binding to two major classes of receptors: V_1 (subdivided in V_{1A} and V_{1B} subtypes) and V_2 receptors (Manning & Sawyer, 1989). In many isolated arteries, including those from human (Lluch *et al.*, 1984; Martin De Aguilera *et al.*, 1990; Liu *et al.*, 1994; Martinez *et al.*, 1994a,b; Bax *et al.*, 1995; Jovanovic *et al.*, 1995; Medina *et al.*, 1996; Calo *et al.*, 1997), rabbit (Garcia-Villalon *et al.*, 1996), dog (Katusic *et al.*, 1984; Myers *et al.*, 1989) and the rat (Angus *et al.*, 1994), vasoconstriction is mediated by the V_{1A} receptor. However, an early study demonstrated that the potency order of vasopressin analogues on the rat mesenteric arterioles differed from that on the rat aorta, suggesting the involvement of distinct receptors (Altura, 1975). This notion seems to be substantiated by the finding that the selective peptide V_1 receptor antagonist, [d(CH_2)$_5$Tyr(Me)^2]AVP, was ten times more potent on the rat aorta (pA_2 = 10.84; Anouar *et al.*, 1996) than on the rat small mesenteric artery (SMA; pK_B = 9.76); the latter affinity value indicated the involvement of V_{1A} receptor in the rat SMA (Angus *et al.*, 1994). Although Burrell and colleagues (1994) reported that the AVP-induced contractions of the rat SMA were also potently antagonized by the non-peptide V_1 receptor antagonist OPC 21268 (Yamamura *et al.*, 1991), the displayed antagonism was non-competitive as well as too potent to account for V_1 receptor involvement. These inconsistencies concerning the action of AVP in the rat SMA and aorta might suggest interference by a vasodilator component in the rat SMA (Walker *et al.*, 1989; Matinez *et al.*, 1994a) and/or the involvement of multiple receptors (Altura, 1975; Angus *et al.*, 1994) in the two vessels. In this connection, oxytocin (OT) receptors may also be important, since OT receptors are operative in cardiovascular tissues (Yazawa *et al.*, 1996; Gutkowska *et al.*, 1997) and AVP and OT can activate each other's primary receptors (Manning & Sawyer, 1984; Jovanovic *et al.*, 1995, 1997).

In the present study we aimed to eliminate the inconsistencies concerning the receptor subtype(s) involved in the response to AVP in the rat SMA and aorta. For this purpose, we analysed the mechanisms involved in the contractile action of AVP and OT in these vessels, using the non-peptide V_1 receptor antagonists, OPC 21268 (Yamamura *et al.*, 1991) and SR 49059 (Serradeil-Le Gal *et al.*, 1993), and the peptide OT receptor antagonist, atosiban (also known as ORF22164, RWJ 22164, or 1-deamino-[D-Tyr(OEt)^{2}Thr^{4}Orn^8]OT (dETVT)) (Pettibone *et al.*, 1992). A preliminary account of part of these data was presented to the British Pharmacological Society (Stam *et al.*, 1996).

Methods

The rat small mesenteric artery preparation

Male Wistar rats (250–350 g) were anaesthetized (sodium pentobarbitone, 60 mg kg^{-1}, i.p.) and killed by cervical

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dislocation and the mesentery was removed and placed in ice-cold modified Krebs-Henseleit solution (KHS) of the following composition (mM): NaCl 119.0, $NaHCO_3$ 25.0, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, glucose 5.5, $CaCl_2$ 2.5. Arterial trees were dissected and cleared from surrounding adipose tissue. From each arterial tree, a ring segment (~2 mm in length) was mounted in a myograph (J.P. Trading, Aarhus, Denmark) with separated 6 ml organ baths (thermostatically controlled at 37 °C containing modified KHS and continuously gassed with 95% O_2 and 5% CO_2) as described previously (Mulvany & Halpern, 1977). Tissue responses were measured continuously as changes in isometric force. Following a 30 min stabilization period, the internal diameter of each vessel was set to a tension equivalent to 0.9 times the estimated diameter at 100 mmHg effective transmural pressure (I_{100} = 200–300 μm) according to the standard procedure of Mulvany & Halpern (1977). After a further 30 min stabilization period, a calibration contraction (12.5±0.5 mN, n = 61) was obtained to 100 μM phenylephrine and the presence of the endothelium confirmed. This procedure was followed by 30 min washing.

The rat isolated aortic ring preparation

The rat aorta was removed and placed in ice-cold modified KHS of the same composition as for the SMA, except for, the Ca^{2+} concentration, which was one tenth of that of standard KHS in order to eliminate the spontaneous phasic contractions seen in standard KHS (Martin, 1989). The tissue was mounted as 3 mm ring segments in 15 ml organ baths containing KHS ($CaCl_2$ = 0.25 mM) aerated with 95% O_2 and 5% CO_2 and maintained at 37 °C. The ring segments were allowed to equilibrate at a tension of 20 mN for 60 min and were washed every 15 min. After equilibration, a calibration contraction (0.90±0.02 g, n = 58) was obtained to 30 μM 5-hydroxytryptamine (5-HT) and the absence of the endothelium was confirmed. This procedure was followed by 60 min washing. Tissue responses were measured continuously as changes in isometric force with a Harvard isometric transducer.

Table 1 Estimates (means±s.e.mean) of the upper asymptote (α), midpoint location (pEC_{50}) and Hill slope (n_H) obtained after fitting the individuals AVP and OT E_i[A] curves in the rat SMA and aorta to the Hill equation

SMA	α	pEC_{50}	n_H	
AVP	118±3%	9.48±0.04	2.3±0.15	n=16
OT	126±3%	6.76±0.04*	3.3±0.20*	n=21
	P>0.05	P<0.001	P<0.001	
Aorta	α	pEC_{50}	n_H	
AVP	73±8%	9.19±0.04	1.9±0.10	n=13
OT	53±5%	7.07±0.04*	1.8±0.10	n=9
	P>0.05	P<0.001	P>0.5	

*Significantly different from AVP E_i[A] curve.

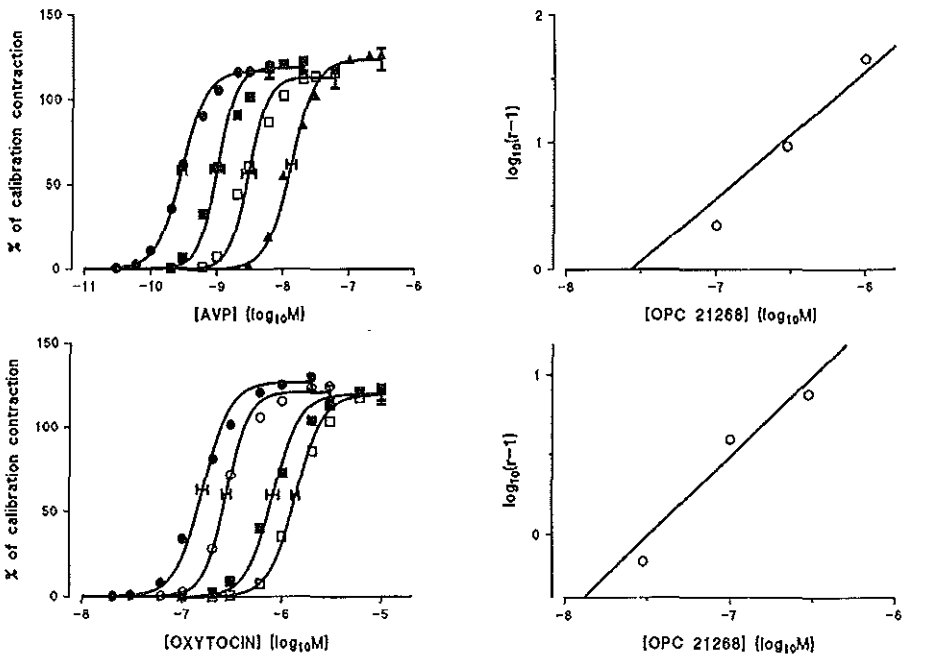

Figure 1 (Left panels) Concentration-effect curves to AVP and OT obtained on the rat SMA in the absence or presence of OPC 21268. The lines superimposed on the mean data points were simulated using the Hill equation. (Right panels) Schild plots for the interaction of OPC 21268 with AVP (upper panel) and OT (lower panel). The solid lines superimposed on mean data points were simulated using the parameters obtained from the constrained model fits.

Removal of endothelium

It is well known that contractile responses to a number of agonists can be influenced by endothelium-derived factors (Furchgott & Vanhoutte, 1989). Indeed, the contractile responses to AVP show tachyphylaxis in the rat aorta with intact endothelium (Millette & Lamontagne, 1996). Therefore, the endothelium of the aorta was denuded by gently rubbing with a poly-ethylene tube. In contrast, the endothelium of the rat SMA was left intact, since its removal turned out to be technically difficult and was found to be associated with a substantial decrease of the functional reactivity (unpublished observation). Fortunately, the necessity to remove the endothelium in the rat SMA is not that marked, since five repetitive AVP E/[A] curves could be produced without tachyphylaxis (Angus *et al.*, 1994).

The integrity of endothelium was checked with acetylcholine (10 μM), which failed to relax rat aorta segments, but produced at least 60% relaxation in all segments of the rat SMA.

Experimental protocol

Tissues were incubated for 60 min with antagonist or vehicle and single agonist concentration-effect (E/[A]) curves were then obtained by cumulative dosing at quarter- or half-log unit concentration increments.

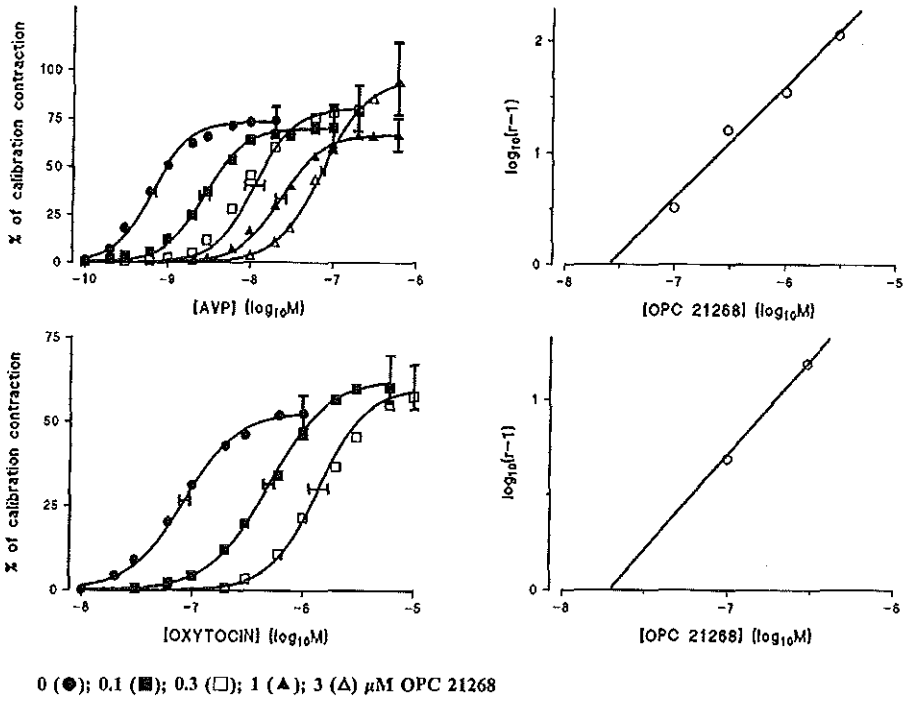

Figure 2 (Left panels) Concentration-effect curves to AVP and OT obtained on the rat aorta in the absence or presence of OPC 21268. The lines superimposed on the mean data points were simulated using the Hill equation. (Right panels) Schild plots for the interaction of OPC 21268 with AVP (upper panel) and OT (lower panel). The solid lines superimposed on mean data points were simulated using the parameters obtained from the constrained model fits.

Table 2 pK_B/pA_2 values (means ± s.e.mean) for SR 49059, OPC 21268 and Atosiban on the rat SMA and aorta against AVP and OT and reported pK_i values for rat liver V_{1A} receptors

	SMA		*Aorta*		
Antagonist	*AVP*	*OT*	*AVP*	*OT*	*pK_i for the rat liver V_{1A} receptor*
SR 49059	9.20 ± 0.13[a]	9.38 ± 0.06	9.48 ± 0.09	9.29 ± 0.12[a]	9.1[b]
OPC 21268	7.56 ± 0.11	7.49 ± 0.08	7.60 ± 0.07	7.71 ± 0.08[a]	6.5–7.6[c]
Atosiban	6.48 ± 0.11[a]	6.34 ± 0.16	6.19 ± 0.06	6.30 ± 0.04	6.7[d]

[a]pA_2. [b]Serradeil-Le Gal *et al.*, 1993. [c]Yamamura *et al.*, 1991; Pettibone *et al.*, 1992; Burrell *et al.*, 1993a,b; Serradeil-Le Gal *et al.*, 1993, 1994; Hirasawa *et al.*, 1994. [d]Pettibone *et al.*, 1992.

Analysis

Individual agonist curve data were fitted to the Hill equation using an iterative, least-squares method:

$$E = \frac{\alpha * [A]^{n_H}}{[A]_{50}^{n_H} + [A]^{n_H}}$$

to provide estimates of midpoint slope (n_H), midpoint location ($[A]_{50}$ estimated as a logarithm) and upper asymptote (α). The effect of drug treatment on these parameters was assessed by one-way analysis of variance (ANOVA) or Student's *t*-test, as appropriate. Values of $P<0.05$ were considered to be significant.

When the minimum criteria for competitive antagonism were satisfied, that is the antagonist produced parallel rightward shift of the agonist E/[A] curves with no change in upper asymptote, antagonist affinity estimates were obtained by fitting the individual midpoint location values obtained in the absence ($\log[A]_{50}$) and presence ($\log[A]_{50B}$) of antagonist (B) to the following derivation of the Schild equation as described previously (Black *et al.*, 1985a).

$$\log[A]_{50B} = \log[A]_{50} + \log(1 + [B]^b/10^{b*\log K_B})$$

When the Schild plot slope parameter (b) was not significantly different from unity, then the data were re-fitted with b constrained to unity so that the antagonist dissociation equilibrium constant, K_B, could be estimated as $\log K_B \pm$s.e. (Jenkinson *et al.*, 1995). When one concentration of antagonist was tested or the criteria of competitive antagonism were not completely satisfied, an empirical pA_2 value was estimated using the above equation, with b constrained to unity.

Compounds

Compounds were obtained from the following sources: 5-hydroxytryptamine creatine sulphate, acetylcholine chloride, (−)-phenylephrine hydrochloride, oxytocin, [Arg^8]vasopressin acetate, U46619 (9,11-dideoxy-11α,9α-epoxy-methanoprostglandin $F_{2\alpha}$): Sigma Chemical Company Ltd., The Netherlands; SR 49059 ((2*S*) 1-[(2*R* 3*S*)-5-chloro-3-(2-chlorophenyl)-1-(3,4-dimethoxybenzene-sulphonyl)-3-hydroxy-2,3-dihydro-1*H*-indole-2-carbonyl]-pyrrolidine-2-carboxamide) and OPC 21268 (1-{1-[4-(3-acetylaminopropoxy)benzoyl]-4-piperidyl}-3,4-dihydro-2(1*H*-benzazepine)): a gift from Dr D. Nisato, Sanofi Recherche, Montpellier Cedex, France; Atosiban: a gift from Dr P. Melin, Ferring Pharmaceuticals, Malmö, Sweden. U46619 was dissolved initially in 20% ethanol to give a 1 mM stock solution and further diluted in distilled water. OPC 21268 and SR 49059 were dissolved in dimethylsulphoxide to give a 1 mM stock solution and further diluted in distilled water. All other drugs were dissolved in distilled water.

Results

Contractions to AVP and OT

AVP and OT produced concentration-dependent contractions of the rat SMA and aorta. The individual curves were fitted to

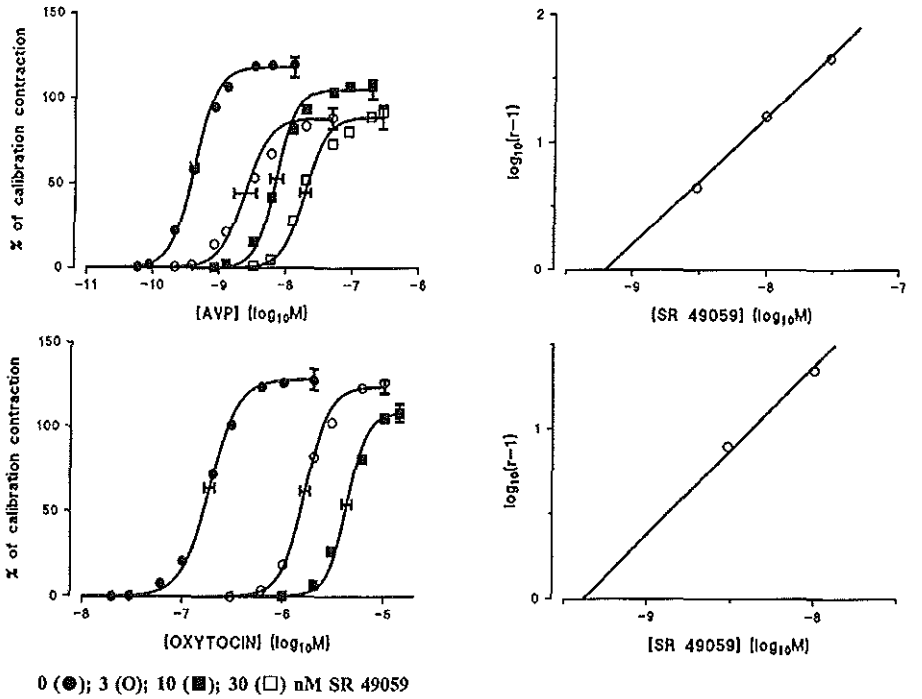

Figure 3 (Left panels) Concentration-effect curves to AVP and OT obtained on the rat SMA in the absence or presence of SR 49059. The lines superimposed on the mean data points were simulated using the Hill equation. (Right panels) Schild plots for the interaction of SR 49059 with AVP (upper panel) and OT (lower panel). The solid lines superimposed on mean data points were simulated using the parameters obtained from the constrained model fits.

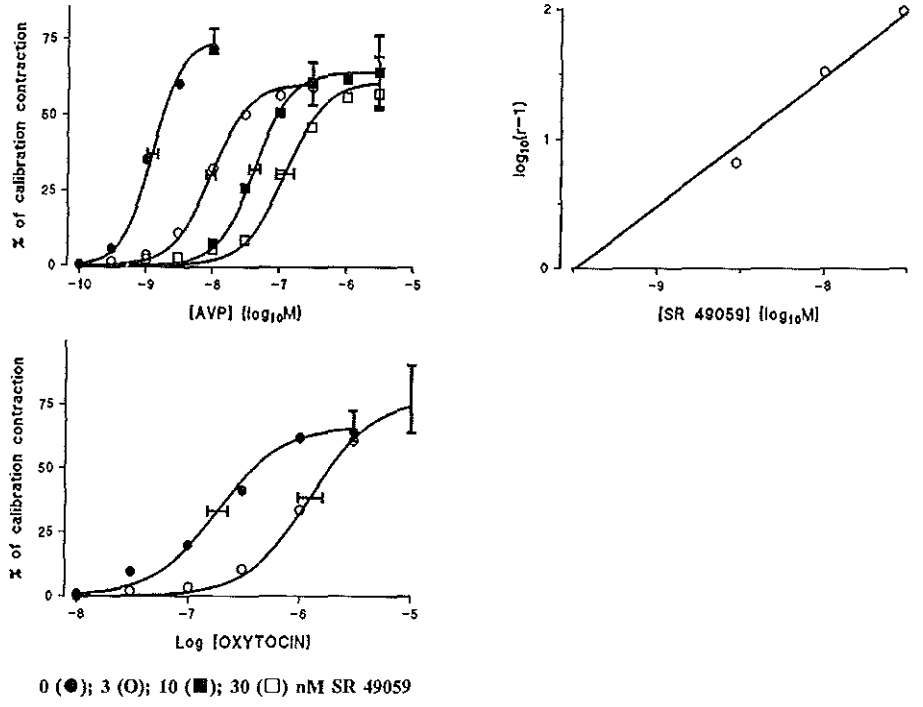

Figure 4 (Left panels) Concentration-effect curves to AVP and OT obtained on the rat aorta in the absence or presence of SR 49059. The lines superimposed on the mean data points were simulated using the Hill equation. (Right panel) Schild plot for the interaction of SR 49059 with AVP. The solid lines superimposed on mean data points was simulated using the parameters obtained from the constrained model fits.

the Hill equation to provide estimates of the midpoint location (pEC_{50}), slope (n_H) and upper asymptote (α) (Table 1). The intrinsic activities of AVP and OT were not significantly different, but AVP was ~500 and ~130 fold more potent than OT in the SMA and aorta, respectively. Interestingly, in the SMA, but not in the aorta, the OT E/[A] curve was significantly steeper than the AVP E/[A] curve.

Effect of OPC 21268 on the response to AVP and OT

The selective V_1 receptor antagonist OPC 21268 (0.1–3 μM, $n=4-11$) behaved as a competitive antagonist of AVP- and OT-induced contractions of the rat SMA (Figure 1) as well as aorta (Figure 2). The Schild slope parameters (b) for the antagonism of OPC 21268 against AVP and OT in the SMA ($b=1.27\pm0.15$ and 0.84 ± 0.14, respectively) and aorta ($b=0.82\pm0.10$ and 1.04 ± 0.48, respectively) were not significantly different from unity, allowing for the estimation of pK_B values (Table 2).

Effect of SR 49059 on the response to AVP and OT

In the rat SMA (Figure 3), the other selective V_1 receptor antagonist SR 49059 (3 and 10 nM, $n=5$) behaved as a competitive antagonist of OT ($b=0.86\pm0.15$; $pK_B=9.38\pm0.06$; Table 2). In contrast, however, SR 49059 produced a small non-concentration related depression of the maximum response to AVP. Notwithstanding this complex behaviour of SR 49059, the data were fitted to the Schild equation. The Schild slope parameter was not significantly different from unity ($b=0.97\pm0.17$) and the estimated pA_2 value was 9.20 ± 0.13 (Table 2).

In the rat aorta (Figure 4), SR 49059 (3–30 nM, $n=3-5$) produced parallel rightward shifts of the AVP and OT E/[A] curves. Schild analysis yielded a slope parameter not significantly different from unity ($b=1.15\pm0.1$) for the antagonism of the AVP response ($pK_B=9.48\pm0.09$; Table 2). pA_2 value for SR 49059 against OT, obtained after fitting the data to the Schild equation with b constrained to unity, was 9.29 ± 0.12 (Table 2).

Effect of atosiban on the response to AVP and OT

In the rat SMA (Figure 5; Table 2), atosiban (0.3–3 μM, $n=4-5$) behaved as a competitive antagonist of the OT E/[A] curves. Again, however, the AVP E/[A] curve in the rat SMA was not displaced in a parallel manner, since atosiban (1–10 μM, $n=4$) produced a significant concentration-dependent steepening (Hill slopes: 1.96 ± 0.01, 2.14 ± 0.10, 2.40 ± 0.16 and 2.70 ± 0.11 for 0, 1, 3 and 10 μM atosiban, respectively, $P<0.05$). Notwithstanding this complex behaviour, the data were fitted to the Schild equation to obtain values of b (1.06 ± 0.15) and pA_2 (6.48 ± 0.11; Table 2).

In the rat aorta (Figure 6), atosiban (0.3–10 μM, $n=5-7$) produced parallel rightward shifts of the AVP and OT E/[A] curves. Schild analysis yielded slope parameters not significantly different from unity ($b=0.82\pm0.10$ and 0.81 ± 0.14) and

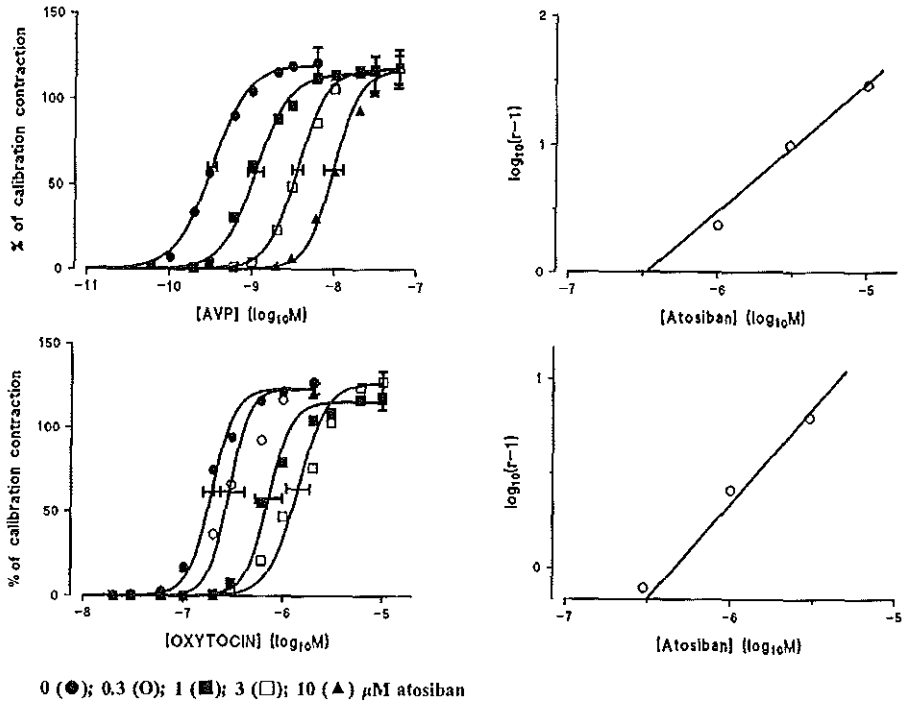

Figure 5 (Left panels) Concentration-effect curves to AVP and OT obtained on the rat SMA in the absence or presence of atosiban. The lines superimposed on mean data points were simulated using the Hill equation. (Right panels) Schild plots for the interaction of atosiban with AVP (upper panel) and OT (lower panel). The solid lines superimposed on mean data points were simulated using the parameters obtained from the constrained model fits.

pK_B values of 6.19 ± 0.06 and 6.30 ± 0.04 against AVP and OT, respectively (Table 2).

Relaxant responses to AVP and OT

In order to study whether AVP and OT displayed a non-V_{1A} receptor-mediated vasodilator response, the rat SMAs and aortae were pre-contracted with 100–200 nM and 10–30 nM U46619, respectively, after selective V_{1A} receptor blockade by 30 min pre-incubation with the SR 49059 (10 nM). After the contractile response had stabilized (78 ± 11% and 73 ± 11% of the calibration contraction, for the rat SMA and aorta, respectively) AVP or OT E/[A] curves were obtained. No relaxation to AVP and OT was observed in either tissue (n = 4–5, data not shown). In fact, a slight further contraction was seen.

Discussion

To date the receptor subtype involved in the AVP-induced contraction of the rat SMA has been controversial. The peptide V_1 receptor antagonist [d(CH_2)$_5$Tyr(Me)^2]AVP defined the receptor involved as V_1 (Angus *et al.*, 1994). However, the data obtained with OPC 21268 in the rat SMA were inconsistent with the involvement of a V_1 receptor (Burrell *et al.*, 1994). Furthermore, the potencies of AVP receptor agonist as well as antagonist peptides differed for the rat aorta and mesenteric resistance arteries (Altura, 1975; Angus *et al.*, 1994; Anouar *et al.*, 1996). This suggests regional differences in the receptor subtype(s) involved in the response to AVP.

In the present study, AVP and OT produced concentration-dependent contractions of the rat SMA and aorta, with AVP being about 500 and 130 times, respectively, more potent than OT. The estimated antagonist affinities of OPC 21268 (7.49–7.71), SR 49059 (9.2–9.5) and atosiban (6.19–6.48) were similar with respect to the agonists (AVP and OT) and vessels (SMA and aorta) studied. Since these affinity values are in accordance with the reported binding affinities for V_{1A} receptors on the rat liver membranes (Table 2) and SR 49059 displays only a 10^{-7} M affinity for the OT receptor (Serradeil-Le Gal *et al.*, 1993), it is tempting to conclude that the functional responses to both AVP and OT in the rat SMA and aorta are mediated *via* a single receptor that can be classified as V_{1A}. However, the analysis of the action of AVP suggests a more complex situation in the rat SMA. The Hill slopes of the AVP and OT E/[A] curves (n_H = 2.3, 3.3, respectively) differed significantly. In case of a homogeneous receptor population, different Hill slopes would be expected only if the intrinsic activities of the agonists were different (Black *et al.*, 1985b). This was not the case as the upper asymptotes of the AVP and OT E/[A] curves in the rat SMA were similar (see Table 1).

Studying α_1-adrenoceptor responses in the rat aorta, Van der Graaf and colleagues have modelled that the differences in Hill slope values of agonists with similar intrinsic activity are best accounted by assuming multiple receptors (Van der

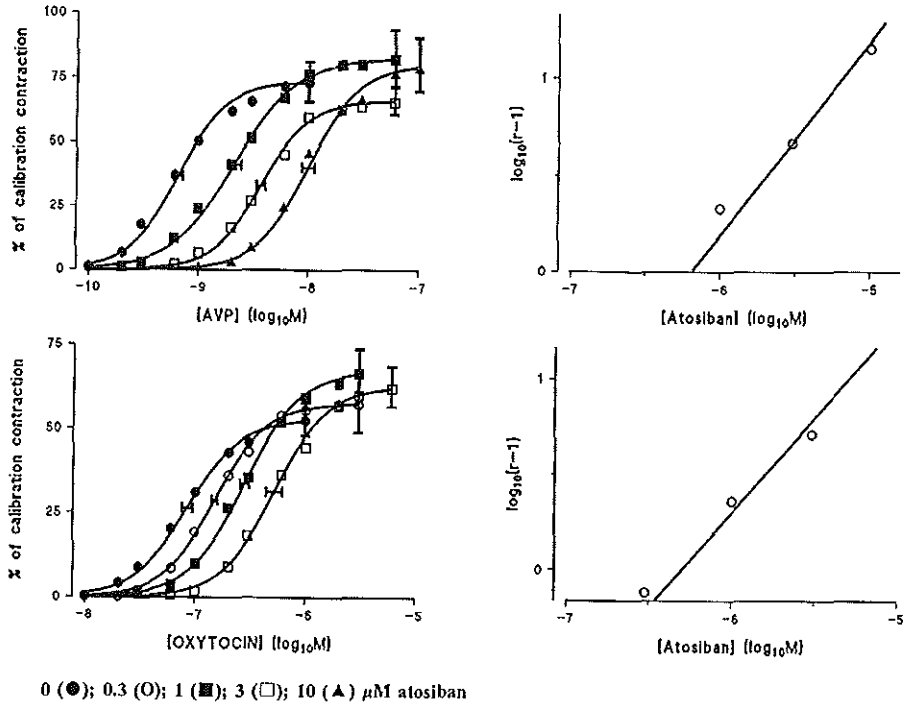

Figure 6 (Left panels) Concentration-effect curves to AVP and OT obtained on the rat aorta in the absence or presence of atosiban. The lines superimposed on the mean data points were simulated using the Hill equation. (Right panels) Schild plots for the interaction of atosiban with AVP (upper panel) and OT (lower panel). The solid lines superimposed on the mean data points were simulated using the parameters obtained from the constrained model fits.

Graaf *et al.*, 1995). The concentration-dependent steepening of the AVP E/[A] curve by atosiban in the rat SMA substantiates the significance of the difference in Hill slope parameter between OT and AVP E/[A] curves. Interestingly, atosiban caused the Hill slope parameter of the AVP E/[A] curve to shift towards that of the OT E/[A] curve (see Table 1). In other cases also, the antagonist-induced changes of the Hill slope parameter proved to be a more sensitive indicator of receptor heterogeneity than the Schild plot slope parameter (Van der Graaf *et al.*, 1996; Prentice & Hourani, 1997). Thus, in the present study, the contraction of the SMA by AVP is likely to involve a heterogeneous (V_{1A} and non-V_{1A}) receptor population. Receptor heterogeneity does not readily explain the failure of SR 49059 to satisfy the criteria for competitive antagonism of the AVP-induced contraction in the SMA. The compound exhibits slow dissociation kinetics due to its high affinity (D. Nisato, personal communication). Indeed, incubation of the rat SMAs with SR 49059 decreased the E_{max} of the AVP E/[A] curve (Figure 3). However, the decrease in E_{max} was small and independent of the concentration used. A similar small decrease in AVP E_{max} in the rat SMA has also been observed with peptide antagonists (Angus *et al.*, 1994).

Interestingly, in contrast to the non-competitive nature of SR 49059 and atosiban in the rat SMA with intact endothelium, both compounds behaved as competitive antagonists in the rat aorta, where endothelium had been removed (see Methods). Thus, it is possible that vasodilator responses elicited by AVP due to a release of endothelium-derived factors (Katusic *et al.*, 1984; Myers *et al.*, 1989; Russ & Walker, 1992; Martinez *et al.*, 1994b; Suzuki *et al.*, 1994) may interfere with its contractile responses in the rat SMA. Since, in addition, AVP can also elicit endothelium-independent vasodilatation (Martinez *et al.*, 1994a,b), we studied the effects of AVP as well as OT on both vessels after pre-contraction with the thromboxane-mimetic agent, U46619 in the presence of SR 49059. Both agonists, however, failed to relax either the rat SMA or the rat aorta. The lack of vasodilator responses with AVP and OT strengthens the notion that the AVP-induced contraction of the rat SMA seems to involve heterogeneous receptors.

We would like to point out that our results with respect to the competitive antagonism displayed by OPC 21268 in the rat SMA ($pA_2 = 7.56$) differ from those reported in an earlier study (Burrell *et al.*, 1994). Burrell and colleagues (1994) demonstrated that OPC 21268, at a concentration of only 10 nM, almost completely blocked the AVP-induced contraction of the rat SMA. Although the authors did not discuss this observation, the antagonism of OPC 21268 suggested either a non-competitive action or the co-existence of an underlying relaxant response (not observed in the present study). We cannot explain the discrepancy. However, the pA_2 values obtained by us are in agreement with the reported pK_i values in the rat liver (see Table 2). Moreover, a parallel rightward shift of the AVP-induced pressor response in the rat by OPC 21268 (Yamamura *et al.*, 1991) is also in accordance with our findings in the rat SMA, which is generally believed to represent a resistance vessel (Fenger-Gron *et al.*, 1997).

In summary, the results of the present study show that AVP and OT contract the rat aorta and SMA and, according to most criteria, the data are consistent with the response being predominantly mediated by a V_{1A} receptor. However, the non-competitive antagonism of the AVP-induced contraction of the rat SMA by atosiban and SR 49059 as well as the Hill slope difference between AVP and OT E/[A] curves indicate receptor heterogeneity in the rat SMA. In this respect, it is of interest to note that Heinemann *et al.* (1998) have suggested the involvement of a novel AVP receptor in the pressor response of the rat perfused mesentery. Overall, therefore, the existence of another atypical receptor in the rat SMA cannot be excluded.

We thank Dr D. Nisato for providing us with OPC 21268 and SR 49059 and Dr P. Melin for providing us with atosiban.

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(Received April 28, 1998
Revised July 28, 1998
Accepted August 3, 1998)

Chapter 6

Interaction of arginine vasopressin and noradrenaline in the rat isolated small mesenteric artery

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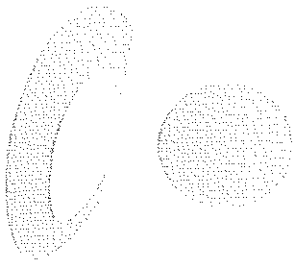


Interaction of arginine vasopressin and noradrenaline in the rat isolated small mesenteric artery

Summary

The interaction between arg-vasopressin (AVP) and noradrenaline in rat small mesenteric artery (SMA) was investigated and the data analysed according to a theoretical two-receptor:one-transducer model.

Noradrenaline produced concentration-dependent contractions of SMAs (pEC_{50}=6.50±0.08, n_H=2.62±0.23). In the presence of AVP-induced threshold contractions (5.3±0.2%, 12.4±0.7% and 28.3±1.5% of the calibration contraction), noradrenaline concentration-effect (E/[A]) curves were flattened (n_H=1.05±0.08, p<0.01; 0.86±0.03, P<0.001 and 0.78±0.03, P<0.001, respectively) and potentiated (pEC_{50}= 6.89±0.14; 7.19±0.09, P<0.0001; 7.17±0.12, P<0.0001, respectively). The maximum response to noradrenaline, however, was not affected by the presence of AVP.

The potentiation and flattening of the noradrenaline E/[A] curves by AVP was abolished by SR 49059 (2 nM), indicating involvement of the V_{1A} receptor.

AVP produced concentration-dependent contractions (pEC_{50}=9.71±0.16, n_H=2.31±0.57). In the presence of noradrenaline-induced threshold contractions (5.8±0.9%, 10.9±0.7% and 23.8±1.5% of the calibration contraction) AVP E/[A] curves were flattened (n_H=1.08±0.07; 0.86±0.09 and 0.86±0.03, respectively) and potentiated (pEC_{50}=9.95±0.09; 10.21±0.11; 9.86±0.12, respectively), but the maximum response remained unaffected.

After treatment with phenoxybenzamine, noradrenaline behaved as a weak partial agonist (pEC_{50}=4.87±0.04, n_H=1.80±0.12 and α=10.5±3.8% of the calibration contraction). Under these conditions, AVP (0.38±0.10 nM) not only produced a significant potentiation (pEC_{50}=5.59±0.11) and flattening (n_H=1.11±0.17) of the noradrenaline E/[A] curve but also significantly increased the maximum response more than 4-fold (α=43.4±6.2% of the calibration contraction).

A two-receptor:one-transducer model could satisfactory fit all experimental data and the slope of the common transducer pathway was found to be steep (n3=5.4). In conclusion, we have demonstrated that the interaction between AVP and noradrenaline on rat SMA follows the theoretical two-receptor:one-transducer model, with the slope-dependence residing in the common transducer pathway.

Introduction

Agonist-agonist interactions have been studied for many years which has resulted in a wealth of theoretical as well as experimental reports (see Scaramellini et. al., 1997). Synergistic interactions between agonists are particularly intriguing because of their clinical implications. For example, a synergistic interaction between 5-hydroxytryptamine (5-HT, serotonin) and noradrenaline has been suggested to play a role in the actiology of hypertension [1]. Moreover, the antihypertensive effects of ketanserin and captopril was suggested to be at least partially based upon reversal of synergistic interaction of 5-HT and angiotensin II, respectively, with noradrenaline [1] [2]. Additionally, MaassenVanDenBrink and

colleagues [3] suggested that a thromboxane A_2-induced enhancement of the contractile response of human coronary arteries to the anti-migraine drug, sumatriptan, may be involved in the chest symptoms observed with the drug.

Several studies have reported amplification (the response to the combination of two agonists exceeding the sum of their individual effects) and/or potentiation (increase in pEC_{50}) of responses elicited by agonists acting at two different receptors, including 5-HT and noradrenaline [4-6], angiotensin II and noradrenaline [7, 8], melatonin and noradrenaline [9], thromboxane A_2 and 5-HT [3, 10, 11] as well as arg-vasopressin (AVP) and noradrenaline [12, 13]. Although most studies on the interaction between two agonists are merely of a descriptive nature, for one type of agonist-agonist interaction, namely two receptors connected with one transducer pathway, a theoretical model has been developed [14]. According to the geometry of the agonist E/[A] curve, this two-receptor:one-transducer model accounted for phenomena like threshold amplification and potentiation, and predicted the conditions under which they will occur [15] [10] [5]. Recently, this model was extended to allow for the interacting agonists to have E/[A] curves with different slopes [16]. Interestingly, this model provides a framework by which agonist-agonist interactions can be interpreted and predicted. Accordingly, in the present investigation in rat small mesenteric arteries (SMA), we studied the interaction between AVP and noradrenaline, which cause contractions via predominantly V_{1A} [17] and α_{1L} adrenergic receptors, respectively [18] [19].

Methods

The rat small mesenteric artery preparation

Male Wistar rats (250-350 g) were anaesthetised (sodium pentobarbitone, 60 mg kg^{-1}, i.p.) and killed by cervical dislocation. The mesentery was removed and placed in ice-cold modified Krebs-Henseleit solution (KHS) of the following composition (mM): NaCl 119.0, $NaHCO_3$ 25.0, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, glucose 5.5, $CaCl_2$ 2.5 and EDTA 0.026. Arterial trees were dissected and cleared from surrounding adipose tissue. From each arterial tree, a ring segment (~2 mm in length) was mounted in a myograph (J.P. Trading, Aarhus, Denmark) with separated 6 ml organ baths (thermostatically controlled at 37°C) containing modified KHS and continuously gassed with 95% O_2 and 5% CO_2), as described previously [20]. Tissue responses were measured continuously as changes in isometric force. Following a 30 min stabilisation period, the internal diameter of each vessel was set to a tension equivalent to 0.9 times the estimated diameter at 100 mm Hg effective transmural pressure (I_{100}=200 - 300 μm) according to the standard procedure of Mulvany & Halpern (1977). After a further 30-min stabilisation period, the preparations were challenged five times with noradrenaline (10 μM) with washouts after each challenge. The integrity of the endothelium was confirmed after the first challenge with 10 μM of methacholine, which produced at least 60% relaxation in all vessel segments. After a first noradrenaline E/[A] curve, each vessel segment was washed for 30 min and equilibrated for 45 min. Subsequently, we aimed to induce a threshold contraction by either AVP or noradrenaline that amounted about 5%, 10% or 25% of the maximal contraction of the first noradrenaline E/[A] curve. After the threshold contraction to AVP or noradrenaline had stabilised, a second noradrenaline or AVP E/[A] curve, respectively, was produced. Responses were expressed as percentage of fifth noradrenaline challenge, which served as calibration contraction (13.4 ± 0.4 mN, n=31).

In one set of experiments, it was investigated whether the potentiation of the noradrenaline E/[A] curve was mediated via the V_{1A} receptor. Therefore, after the AVP-induced threshold contraction reached its maximum, SR 49059 (2 nM), a selective V_{1A} receptor antagonist [21], was added followed by a second noradrenaline E/[A] curve.

In another set of experiments, the interaction between AVP and noradrenaline on the contraction of rat SMA was assessed after partial inactivation of a-adrenoceptors by phenoxybenzamine. After the 5 challenges with noradrenaline (10 μM), phenoxybenzamine (3 nM)

was added to vessel segments for 5 min. Subsequently, the segments were washed 8 times during a 30-min period and equilibrated for 45 min. A control noradrenaline E/[A] curve was obtained and, after washing and equilibration, a threshold contraction to AVP (~10 %) was induced. Upon stabilisation of the threshold contraction to AVP, a second noradrenaline E/[A] curve was produced. In one set of experiments BHT-933 was used as agonist to observe possible involvement of α_2-adrenoceptors. After washing and equilibration, a third noradrenaline E/[A] curve was produced.

In all experiments, a mixture of cocaine (30 μM), timolol (6 μM) and SCH-23390 (R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride; 10 nM) was added during the equilibration period to block neuronal uptake, β_1/β_2-adrenoceptors and D_1 receptors, respectively [22].

Analysis

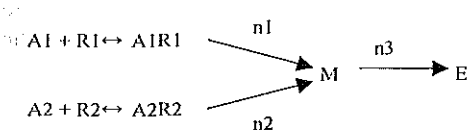
Individual agonist curve data were fitted to the Hill equation using an iterative, least-squares method

$$E = \frac{\alpha * [A]^{n_H}}{[A]_{50}^{n_H} + [A]^{n_H}} \quad (1)$$

to provide estimates of midpoint slope (n_H), midpoint location ($[A]_{50}$ estimated as a logarithm) and upper asymptote (α). The effect of drug treatment on these parameters was assessed by one-way analysis of variance (ANOVA) or Student's t-test, as appropriate. Values of $P<0.05$ were considered to be significant.

Application of the two-receptor:one-transducer model

Experimental data were fitted to the two-receptor:one-transducer model derived by Scaramellini and colleagues (1997).



The model describes the interaction between two interacting agonists, A1 and A2, which occupy different receptors (R1 and R2), to produce a common intracellular mediator, M, leading to a pharmacological effect, E. The separate and common elements of the transduction pathway have the algebraic form of the Hill equation with n1 and n2 as slope factors for the separate parts and n3 for the common part. The production of M by A1 and A2 is described by the following equations:

$$[M]_{A1} = \frac{m_1 * [A_1]^{n1}}{K_1^{n1} + [A_1]^{n1}} \quad (2)$$

$$[M]_{A2} = \frac{m_2 * [A_2]^{n2}}{K_2^{n2} + [A_2]^{n2}} \quad (3)$$

$$[M]_{tot} = [M]_{A1} + [M]_{A2} \quad (4)$$

In which m1 and m2 are the maximal concentrations of M that A1 and A2 can produce, respectively,, and K1 and K2 are the midpoint location parameters of the functions. The total concentration of M is given by

and the pharmacological effect is related to $[M]_{tot}$ as follows:

$$E = \frac{E_m * [M]_{tot}^{n3}}{K^{n3} + [M]_{tot}^{n3}} \quad (5)$$

where E_M is the maximum effect in the system and K is the value of $[M]_{tot}$ for half E_M.

The AR module (derivative-free, non-linear regression) of the BMDP statistical software package [23] was used for the fitting procedures. At first instance we applied a graphical method (see Results section) to deduce the slope parameter, n3, corresponding to the common part of the transducer pathway. Subsequently, the noradrenaline control E/[A] curves (n=19) and the phenoxybenzamine treated E/[A] curves (n=7), both produced in the absence of AVP, were simultaneously fitted to equation 5 to obtain estimates of Em, pK1 (that is -log K1) and n1 and individual estimates of m1. Subsequently, the noradrenaline E/[A] curves obtained in the presence of an AVP threshold contraction (n=26), either with or without previous

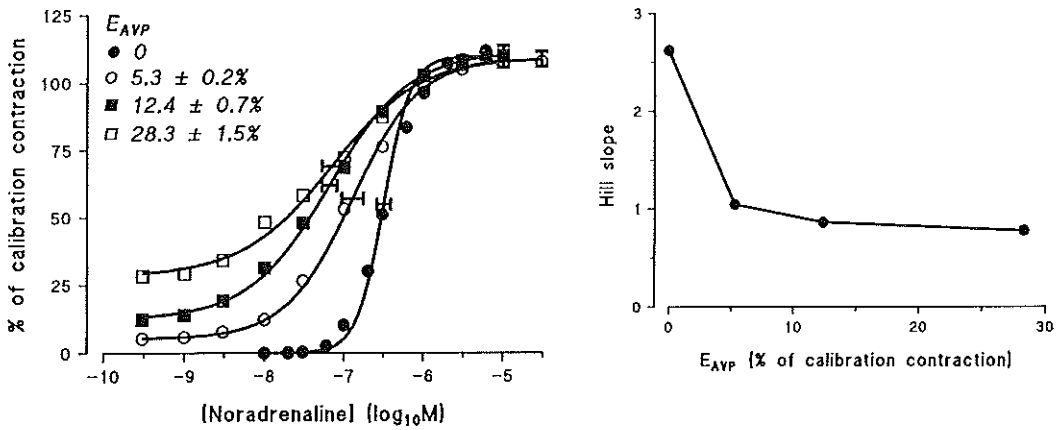


Figure 1. Left panel: *E/[A] curves of noradrenaline in rat SMA in the absence or presence of threshold contractions of AVP (E_{AVP}). The lines superimposed on the mean data points were simulated using the Hill equation.* Right panel: *The relationship between the Hill slope parameter and threshold contraction of AVP (E_{AVP}).*

inactivation by phenoxybenzamine, and AVP E/[A] curves (n=17) in the absence or presence of a noradrenaline-induced threshold contraction, were simultaneously fitted to equation 5, to obtain estimates of m1, m2, n2 and pK2. For the sake of simplicity, the midpoint location (K) of the $E/[M]_{tot}$ relation was constrained to unity in the present analysis.

Compounds

[Arg^8]vasopressin, methacholine bromide, l-noradrenaline hydrochloride, phenoxybenzamine hydrochloride and timolol maleate (purchased from Sigma, The Netherlands); BHT-933 (azepexole,2-amino-6-ethyl-4,5,6,7-tetrahydro-6H-oxozalo-(5,4-d)-azepindihydrochloride), SCH-23390 and (purchased from Research Biochemicals Incorporated, U.S.A); SR 49059 ((2S) 1-[(2R 3S)-5-chloro-3-(2-chlorophenyl)-1-(3,4-dimethoxybenzene-sulfonyl)-3-hydroxy-2,3-dihydro-1H-indole-2-carbonyl]-pyrrolidine-2-carboxamide; a gift from Dr. D. Nisato, Sanofi Recherche, Montpellier Cedex, France). Noradrenaline was dissolved in stoichiometric ascorbic acid solution. Methacholine and phenoxybenzamine were dissolved in ethanol. SR 49059 was dissolved in dimethylsulfoxide to give a 1 mM stock solution and further diluted in distilled water. All other drugs were dissolved in distilled water.

Results

Effect of AVP threshold contractions on noradrenaline-induced contraction of rat SMA

Noradrenaline produced concentration-dependent contractions of SMAs (Fig. 1) and the individual E/[A] curves (n=8) were fitted to the Hill equation to provide estimates of midpoint location (pEC_{50}=6.50±0.08), Hill slope (n_H=2.62±0.23) and upper asymptote (α=110±1% of the fifth noradrenaline calibration contraction). Threshold contractions that amounted 5.3±0.2%, 12.4±0.7% and 28.3±1.5% of the calibration contraction were induced by 0.13±0.05, 0.16±0.09 and 0.17±0.08 nM AVP (n=4-8), respectively. The three threshold contractions to AVP caused a flattening of the noradrenaline E/[A] curves (n_H=1.05±0.08, p<0.01; 0.86±0.03, P<0.001 and 0.78±0.03, P<0.001, respectively; see Fig. 1) together with a leftward shift which became significant at a threshold of 12.4% (pEC_{50}= 6.89±0.14; 7.19±0.09, P<0.0001; 7.17±0.12, P<0.0001, respectively). The maximum response

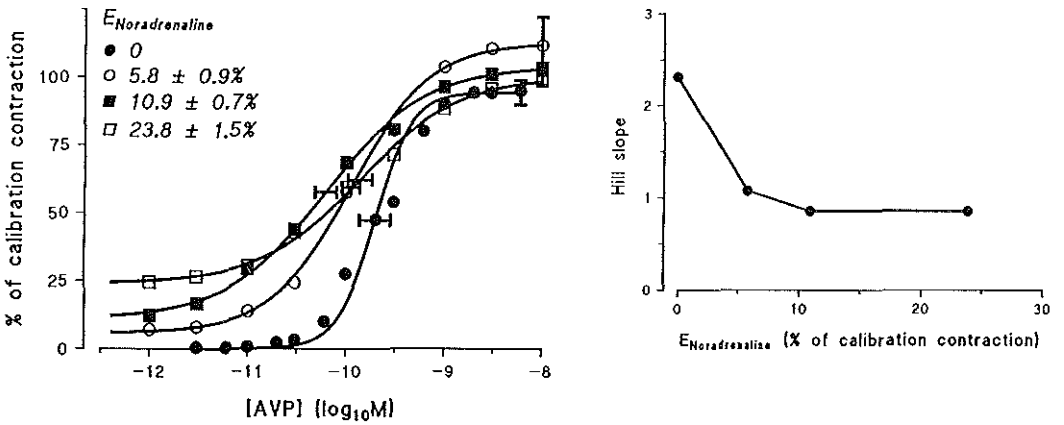


Figure 2. Left panel: *E/[A] curves of AVP in rat SMA in the absence or presence of threshold contractions of noradrenaline. The lines superimposed on the mean data points were simulated using the Hill equation.* Right panel: *The relationship between the Hill slope parameter and threshold contraction of noradrenaline ($E_{Noradrenaline}$).*

obtained with noradrenaline was not affected.

Effect of noradrenaline threshold contractions on AVP-induced contraction of rat SMA

Subsequently, we investigated the reciprocal interaction between a fixed concentration of noradrenaline and variable concentrations of AVP. AVP produced concentration-dependent contractions of rat SMA (Fig. 2). Hill parameters of the control AVP E/[A] curves (n=4) were: pEC_{50}=9.71±0.16, n_H 2.31±0.57 and α=94±5% of the fifth noradrenaline (10 μM) calibration contraction. Threshold contractions amounting 5.8±0.9%, 10.9±0.7% and 23.8±1.5% of the noradrenaline calibration contraction were induced by 0.08±0.02, 0.08±0.02 and 0.21±0.03 μM noradrenaline (n=4-5), respectively. The three threshold contractions to noradrenaline caused a flattening of the AVP E/[A] curves (n_H=1.08±0.07; 0.86±0.09 and 0.86±0.03, P<0.001, respectively; see Fig. 2 insert). There was also a leftward shift (pEC_{50}=9.95±0.09; 10.21±0.11; 9.86±0.12, respectively), which was significant at a threshold of ~10.9% (p<0.05). The maximum response obtained with AVP was not affected.

Effect of SR 49059 on potentiation of noradrenaline response by AVP

Since the AVP-induced potentiation of α_1-adrenergic pressor responses in the perfused mesentery was reported not to be mediated via a vasopressin V_1 receptor [24], we investigated whether this was also the case in the SMA. After a control noradrenaline E/[A] curve (pEC_{50}=6.31±0.08, n_H=2.38±0.36 and α=111±2% of the calibration contraction, n=4), a threshold contraction was induced by AVP (0.02-0.06 nM) that resulted in 41±7% of the calibration contraction. Addition of a V_{1A}-selective concentration (2 nM) of SR 49059 produced a complete reversal of the AVP contraction within seconds. Furthermore, the subsequent noradrenaline E/[A] curve was identical to the first control noradrenaline E/[A] curve (pEC_{50}=6.45±0.10, n_H=2.04±0.32 and α=107±1% of the calibration contraction), indicating that V_{1A} receptors mediated the potentiation of the noradrenaline E/[A] curve.

Effect of partial inactivation of α-adrenoceptors with phenoxybenzamine on potentiation of the noradrenaline response by AVP

After pre-treatment with phenoxybenzamine (3 nM) for 5 min, noradrenaline behaved as a weak partial agonist (pEC_{50}=4.87±0.04, n_H=1.80±0.12 and α=10.5±3.8% of the calibration contraction). Under these conditions, a threshold contraction (10.6±0.5%) with AVP (0.38±0.10 nM) not only produced a significant potentiation (pEC_{50}=5.59±0.11) and flattening (n_H=1.11±0.17) of the noradrenaline E/[A] curve but also significantly increased the maximum response more than 4-fold (α=43.4±6.2% of the calibration contraction).

This increase in α proved to be highly significant after subtraction of the AVP threshold contraction (P<0.002). Following washout, a third noradrenaline E/[A] curve did not differ from the first (pEC_{50}=4.75±0.06, n_H=1.62±0.14 and α=13.1±5.4% of the noradrenaline calibration contraction).

In the presence of a threshold contraction

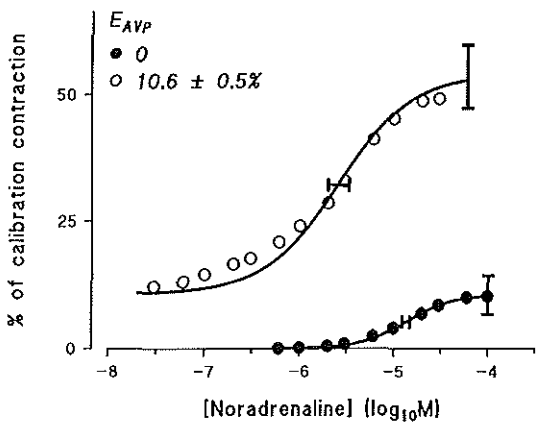


Figure 3. *E/[A] curves of noradrenaline in rat SMA after receptor alkylation with phenoxybenzamine (3 nM for 5 min) in the absence or presence of a threshold contraction of AVP. The lines superimposed on the mean data points were simulated using the Hill equation.*

to AVP the selective α_2-adrenoceptor agonist BHT-933 (0.01-100 μM) did not induce any contraction (data not shown).

Application of the two-receptor:one-transducer model

To further analyse the data in a quantitative manner we applied the two-receptor:one-transducer model as developed by Scaramellini et al. (1997). As described in detail by these authors the slope ratios (the quotient of the E/[A1] curve obtained in the presence and absence of A2) depend purely on the slope of the common transducer pathway, n3. Interestingly, the slope ratios of AVP and noradrenaline (assigned to represent A1 and A2 in the model described in the Methods section, respectively) E/[A] curves plotted against the contractile effect of the corresponding agonist overlap (Figure 4), consistent with expectations for a reciprocal interaction via a common transducer pathway [16]. Since an algebraic relationship between slope ratio and n3 was found to be intractable [16], a set of standard curves was produced by Scaramellini et al. (1997) that displayed the relationship between slope ratios <1 and n3 (Figure 5, left panel). We employed this data set (kindly provided by Clare Scaramellini, AstraZeneca, Loughborough) to estimate n3 for noradrenaline and AVP in rat SMA via a graphical method. We found that linear regression of the semi-logarithmically plotted data yielded lines that displayed a strong (r>0.98) and highly significant (P<0.001) correlation (Figure 5, middle panel). Interestingly, a subsequently performed linear regression of the intercepts of these lines (Figure 5, right panel) yielded another line (intercept = -0.112808*n3 + 1.1579) that showed an almost perfect correlation (r=0.99) with the corresponding slope parameter, n3. Additionally, the line obtained by linear regression of the noradrenaline and AVP slope ratios from our experimental data (Figure 5, middle panel; Slope ratio = -0.1560705 Log [A2] + 0.546693) also displayed a significant correlation (r=0.49, p=0.0036). Accordingly, the intercept of this line corresponded with an estimate for n3 of 5.4 (Figure 5, right panel).

In order to obtain the model parameters for

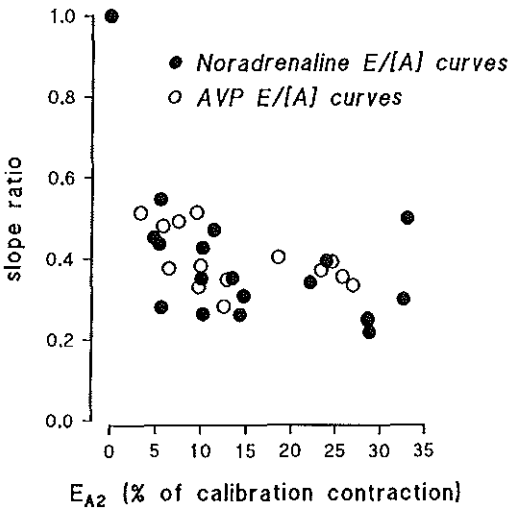


Figure 4. Plot of the slope ratios of noradrenaline and AVP E/[A] curves (see Figures 1 and 2, respectively) versus the threshold contraction of the interacting agonist (E_{A2}).

noradrenaline in the SMA, the control noradrenaline E/[A] curves (n=19) and the noradrenaline E/[A] curves produced after phenoxybenzamine treatment (n=7), both obtained in the absence of [AVP], were simultaneously fitted to the two-receptor:one-transducer model (equation 2-5 with $[M]_{tot}=[M]_{A1}$) to estimate the parameters of the noradrenaline E/[A] curve: EM (108.48), pK1 (5.58), n1 (0.59). The individual estimates of m1 were averaged to obtain M1 (5.06 ± 0.30). It should be noted that in this fit the converge criteria were not met (standard errors are lacking). However, since the parameters that were estimated with the smallest sum of squares appeared realistic, we used these for further analysis. Subsequently, the noradrenaline E/[A] curves (either with or without previous inactivation by phenoxybenzamine), obtained in the presence of an AVP-induced threshold contraction (n=26) and AVP E/[A] curves (n=17) in the absence or presence of a noradrenaline-induced threshold contraction, were simultaneously fitted to two-receptor: one-transducer model (equation 2-5) to obtain estimates of m1 (5.04 ± 0.32), m2 (1.88 ± 0.14), n2 (0.48 ± 0.03) and pK2 (9.25 ± 0.08). Some examples of the two-receptor:one-transducer model fit are shown in Figure 6, where parameter estimates were used to simulate the curves shown superimposed on the experimental data.

Discussion

Recently, Scaramellini and co-workers (1997) presented a theoretical model which considers the interaction between two agonists occupying two different receptors to produce a common intracellular mediator leading to a pharmacological effect. This theoretical model, which extended an earlier version [14] by taking into account a possible role of the separate parts of the pathway, predicts a wide variety of possible location and slope changes of E/[A] curves upon interaction of two agonists [16]. Briefly, the location of the slope-dependence of the agonist E/[A] curve, in either the separate (n1) or common pathway (n3), determines the geometry and location of the agonist E/[A1] curve interacting with a fixed concentration of the second agonist [A2]. When n3>1, E/[A1] curves are potentiated and flatten with increasing [A2] and, if A1 is a partial agonist, the E/[A1] curve will also be amplified (the response to the combination exceeds the sum of the individual effects of A1 and A2). When n3<1, E/[A1] curves are right shifted and steepen with increasing [A2]. Although n1 contributes to the location and shape of the control E/[A1] curve, its impact on agonist interaction, other than quantitative changes, is rather insignificant [16]. Thus, the [A2]-induced relative changes in the slope of the E/[A1] curve depend totally on n3. Although there have been many reports considering agonist-agonist interactions, only few studies have examined their observations according to this model or to its earlier version [14] [25] [15] [5]. As a consequence, the applicability of this model to predict agonist-agonist interactions lacks thorough experimental backup.

In the present study, we interpreted the interaction between AVP and noradrenaline in rat SMA using this model as a framework. Noradrenaline and AVP mutually potentiated the

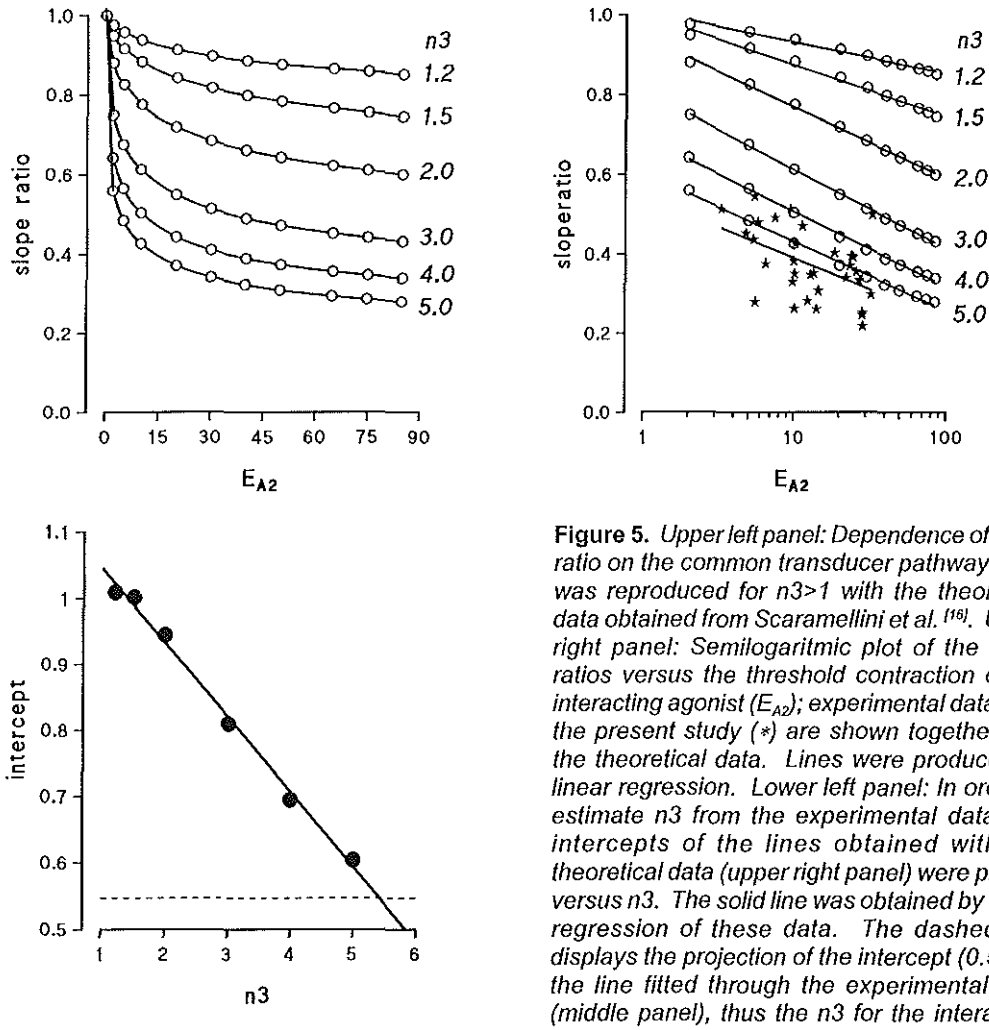


Figure 5. *Upper left panel: Dependence of slope ratio on the common transducer pathway. Plot was reproduced for n3>1 with the theoretical data obtained from Scaramellini et al. [16]. Upper right panel: Semilogaritmic plot of the slope ratios versus the threshold contraction of the interacting agonist (E_{A2}); experimental data from the present study (*) are shown together with the theoretical data. Lines were produced by linear regression. Lower left panel: In order to estimate n3 from the experimental data, the intercepts of the lines obtained with the theoretical data (upper right panel) were plotted versus n3. The solid line was obtained by linear regression of these data. The dashed line displays the projection of the intercept (0.55) of the line fitted through the experimental data (middle panel), thus the n3 for the interaction between AVP and noradrenaline was found to be 5.4.*

contraction elicited by the other (Figures 1 and 2). The initial leftward (potentiation) and subsequent rightward shift with higher threshold contraction was in accordance with the theoretical model, assuming that the slope dependence of the observed agonist E/[A] curve lies in the common transducer pathway (n3) [16]. The dependence of curve shape on the common transducer pathway was further strengthened by the predicted and the observed flattening of the noradrenaline and AVP E/[A] curves caused by the threshold contractions induced by the interacting agonist. Thus, the steep slopes of both AVP and noradrenaline E/[A] curves (n_H=2.31 and 2.62, respectively) depend on a commonly shared transducer pathway for which we also estimated a steep slope (n3=5.4, Figure 5, right panel). Moreover, the dependence of the steep slopes of the AVP and noradrenaline E/[A] curves on the shared transducer pathway was confirmed

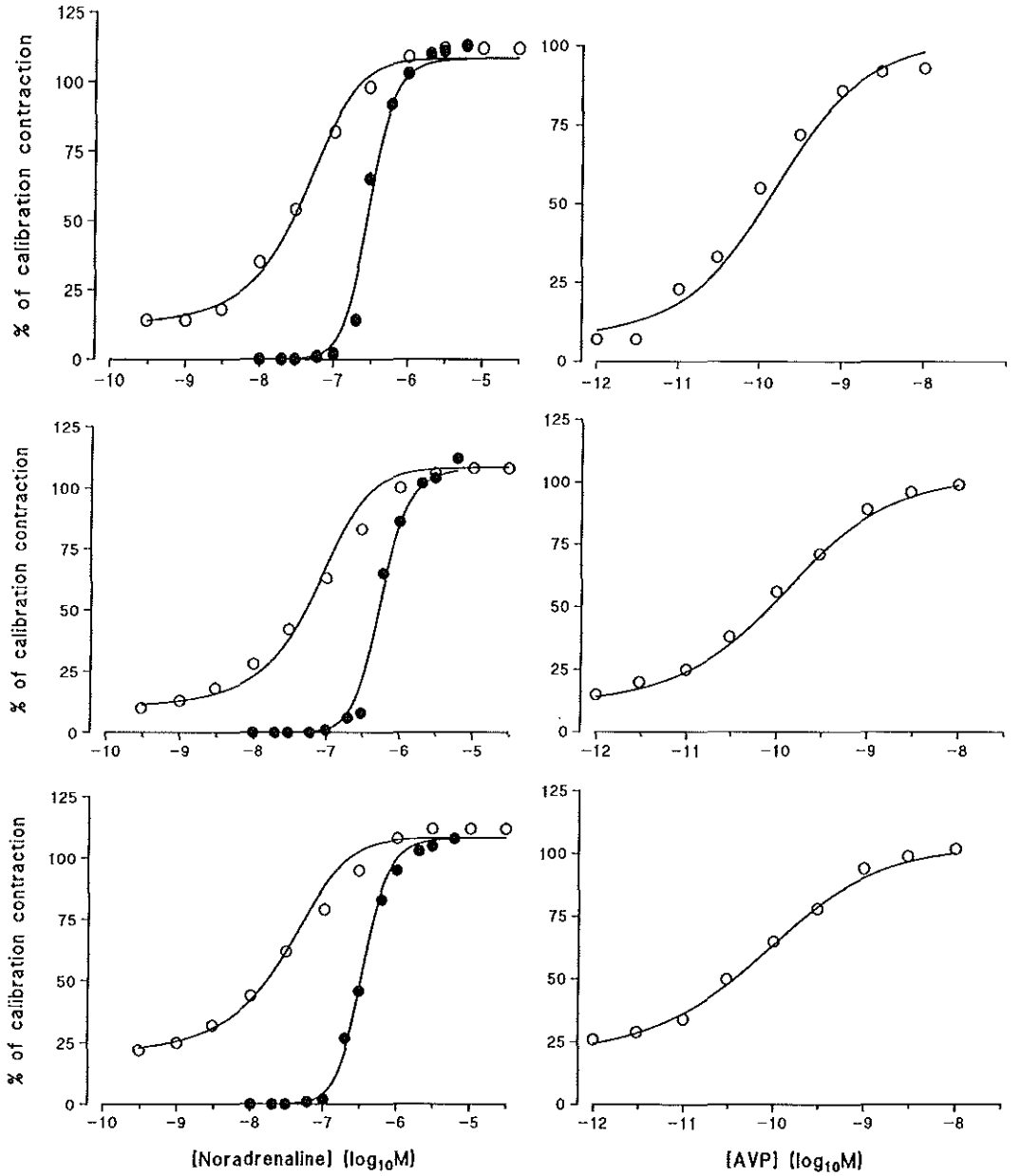


Figure 6. *Six individual examples of the two-receptor:one-transducer model fit the E/[A] curves obtained in the absence (solid circles) or presence of a thrshol contraction (open circles). The lines superimposed on the data points represent the fit of the two-receptor:one-transducer model.*

by the interaction of noradrenaline and AVP after partial inactivation of α-adrenoceptors by phenoxybenzamine. The E/[A] curve to noradrenaline, now behaving as a less efficacious partial agonist, was potentiated, flattened and amplified (see Figure 3), again fully consistent with the model predictions for n3>1 [16]. Interestingly, a similar interaction as described in the present study was observed previously in the rabbit femoral artery for noradrenaline and angiotensin II, which mutually potentiated and amplified each other's effect obtained after partial receptor inactivation via either an irreversible or a non-competitive antagonist [7, 8].

From the interaction experiments, model parameters could be derived that could satisfactory describe all data (see Figure 6). It should be noted that this good fit was obtained with a K_1value for noradrenaline (5.58#) that were very similar to previously reported functional agonist affinity constants in rat SMA (pK_A = 5.64-6.18; [26] Furthermore, , the K_2 model parameter for AVP (9.25) was in good agreement with the binding affinity reported for the cloned rat V_{1A} receptor (pK_D and pKi = 9.17 and 8.73; [27]). This substantiates the validity and physiological relevance of the theoretical two-receptor-one transducer model to describe the interaction between AVP and noradrenaline in rat SMA. Interestingly, in branches of the superior mesenteric artery, the contraction induced by endogenous (nerve released) as well as exogenous noradrenaline was also potentiated by low concentrations of AVP [12]. Though the interaction was neither analysed nor interpreted according to the two-receptor-one-transducer model, the figures in the original paper clearly show that, in accordance with our study, the AVP-induced potentiation accompanies a flattening of the noradrenaline E/[A] curve.

Previously, we have demonstrated the AVP-induced contraction of rat SMA is predominantly mediated by the V_{1A} receptor, although there was some indication for the co-involvement of an atypical receptor [17]. The SMA is generally considered as model of a resistance vessel [28]. Nevertheless, the AVP-induced potentiation of methoxamine responses in the perfused rat mesentery was reported to be mediated via an atypical vasopressin receptor, not antagonised by SR 49059 [24]. However, in contrast to this perfused assay system, the V_{1A} receptor antagonist SR 49059, at a concentration (2 nM) selective for the V_{1A} receptor [21], blocked the AVP-induced potentiation of noradrenaline in the SMA. In previous studies, we demonstrated that the noradrenaline-induced contraction of rat SMA is mediated via α_{1L}-adrenoceptors [18, 19], without involvement of α_2-adrenoceptors [29]. Other studies demonstrated that α_2-adrenoceptor-mediated contractions could be uncovered in the presence of a threshold contraction to U46619 [30, 31]. However, AVP did not uncover any contractile response to the α_2-adrenoceptor agonist BHT-933 in the SMA. Therefore, we feel confident that the interaction between AVP and noradrenaline is mediated by the V_{1A} receptor and α_{1L} adrenoceptor.

The theoretical model used to analyse our data does not consider the molecular entities involved in the transducer pathway. However, it is likely that the common transducer pathway involves the activation of phospholipase C, followed by the production of inositol-1,4,5-triphosphate (IP_3) and diacylglycerol, release of Ca^{2+} and activation of protein kinase C [32]. The contraction of vascular smooth muscle via α_1-adrenoceptors is mediated via the second messengers IP_3 and DAG [33, 34]. Although the exact nature of the adrenoceptors in rat SMA is not clear [18], the $\alpha_{1A/L}$-adrenoceptors in rat vas deferens as well as the cloned α_{1A}-adrenoceptor expressed in cell-lines display a similar pharmacological profile [18] [35, 36] and are coupled to IP_3 [35, 36]. Similarly, the V_{1A} receptor has been shown to couple to this second messenger system [37]. Therefore, the hydrolysis of inositol phospholipids might be the physical representative of the common transducer pathway. Since the steepness of the AVP and noradrenaline E/[A] curve resides in the transducer pathway, a similar interaction profile is predicted between all agonists which couple to the same transducer pathway in the SMA. Interestingly, 5-HT as well as U46619

both produced steep E/[A] curves in rat SMA via 5-HT_2 and TP receptors, respectively [38, 39]. Since, both these receptors couple to the inositol phosphate pathway [40, 41], a similar pattern of interaction can be expected for the interaction between these agonists and with noradrenaline and AVP. Although not studied intensely enough for definite conclusions, the potentiation and flattening of the noradrenaline E/[A] curve by U46619 (pEC_{50}=5.92 and 6.65; n_H=3.1 and 1.4 in the absence or presence of U46619) is in accordance with the interaction pattern between AVP and noradrenaline [18].

Another type of interaction in isolated vessels has been described for 5-HT, acting via the 5-HT_{1B} receptor negatively coupled to adenylyl cyclase, and U46619, acting via TxA_2 receptor coupled to inositol phospholipids [10, 31, 42]. A threshold contraction by U46619 uncovered 5-HT-induced contractile responses, which were either minimal or even absent in quiescent vessel segments. This type of interaction was not mutual. Accordingly, different transducer pathways were implied to account for this different type of interaction [10] [42].

In conclusion, we have demonstrated that the contractions of rat SMA induced by AVP and noradrenaline are mutually potentiated. This interaction between AVP and noradrenaline, which involves the V_{1A} receptor and α_{1L}-adrenoceptor, respectively, follows the theoretical two-receptor:one-transducer model, with the slope-dependence residing in the common transducer pathway.

We thank Dr. Clare Scaramellini for providing us with the data of figure 5 (upper left panel)

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General discussion

Functional affinity states: a general feature of α_1-adrenoceptors ?

In the classical receptor concept an antagonist is devoid of efficacy and its interaction with a distinct receptor subtype is governed solely by its binding affinity. This binding affinity is considered to be system and agonist independent. These characteristics have made antagonists very valuable tools for the classification of receptor subtypes. Throughout history novel receptor subtypes were initially defined by the construction of a 'fingerprint' of antagonist affinities. Via this method the existence of an α_{1L}-adrenoceptor subtype was proposed in functional studies. Initially, through the displayed low affinity values for prazosin and subsequently by its low affinity for the selective α_{1A}-adrenoceptor antagonist, RS-17053 (see Chapter 1). Despite its recognised potential as a drug target in a cloning era, intensive cloning has thus far failed to identify a gene coding for this 'novel' subtype. This led several investigators to the belief that the α_{1L}-adrenoceptor might not exist as a separate entity. It was postulated that the α_{1L}-adrenoceptor might be a low affinity state of the α_{1A}-subtype [1]. Evidence for this belief has been provided in pharmacological experiments with cells expressing α_{1A}-adrenoceptors. α_{1A}-Adrenoceptors, displayed high binding affinities for prazosin, RS-17053, WB 4101 and 5-Mu (pKi = 9.9, 9.3, 9.8 and 9.2, respectively), yet the functional estimated affinities were about one log unit lower, whereas that of others (tamsulosin, indoramin and Rec 15/2739) remained unchanged [1]. Furthermore, in radioligand binding studies the expressed α_{1a}-adrenoceptor gene product could display, governed by environmental factors, either an α_{1A}- or α_{1L}-adrenoceptor profile [2]. However, the estimated affinities for RS-17053 in those cellular experiments could not cover the range of functional affinities that have been estimated in isolated 'α_{1A}-tissues' (see Chapter 1).

In the last two decades, molecular biology, in particular, has put severe pressure on the classical receptor concept (see paragraph *inverse agonism* below). Thus far, however, the classical theory has proven to be adequate for explaining the displayed pharmacology of most receptors in 'non-genetically modified' systems. We envisaged the $\alpha_{1A/L}$-adrenoceptor controversy as a possible challenge to the classical receptor concept (see Chapter 1). It is particularly interesting this challenge originates in native tissue. Because of its possible impact, it was our ambition to gain a better insight in this controversy. As a model we used rat SMA.

In Chapter 2 we demonstrated that the α_{1L}-adrenoceptor subtype was involved in noradrenaline-induced contraction of rat SMA, without any co-involvement of an α_{1A}-adrenoceptor subtype. The displayed α_{1L}-profile proved to be relatively stable, since we were unable to identify environmental factors that could induce an affinity switch for RS-17053 (Chapter 2). Furthermore, the α_{1L}-adrenoceptor did not display any variable affinity for an agonist (noradrenaline, Chapter 3). Apparently, in a tissue system (SMA) the α_{1L}-conformation is more stable than in a cellular assay [2]. As a consequence we were unable to prove the hypothesis that the α_{1A}-adrenoceptor could present itself functionally as different affinity states. However, the possibility of affinity states is still a valid hypothesis. Moreover, from the data in this thesis and those reported elsewhere we belief that affinity states might be a general feature among the class of α_1-adrenoceptor instead of unique for α_{1A}-adrenoceptors. I believe that for all three α_1-adrenoceptor subtypes the existence of 'receptor affinity states' should be considered as a serious possibility. For α_{1B}- and α_{1D}-adrenoceptors the reasoning for this stand can be summarised as follows.

α_{1B}-adrenoceptors: A discordance between radioligand binding and functional studies was noted for the interaction of (+)-cyclazosin with α_{1B}-adrenoceptors, similar to that of

α_{1A}-adrenoceptors (Chapter 4). The high binding affinity in rat liver (pK_i=9.68) initially designated (+)-cyclazosin as a selective α_{1B}-adrenoceptor antagonist [3]. However, the functional pA_2 value (7.96) for (+)-cyclazosin estimated in rat spleen was clearly incompatible with this binding affinity (Chapter 4). Furthermore, in the mouse spleen, another 'α_{1B}-tissue' [4, 5], (+)-cyclazosin did not behave as a competitive antagonist. Thus, apart from the discordance between binding and functional assays (+)-cyclazosin does not behave homogeneously in different functional α_{1B}-assays. This substantiates earlier reports with spiperone and risperidone. Initially, radioligand binding studies identified spiperone and risperidone as selective α_{1B}-adrenoceptor antagonists that display a 13- and 120-fold higher affinity, respectively, for binding to rat α_{1B}-adrenoceptors than for α_{1A}adrenoceptors [6],[7], [8, 9]. However, the functional selectivity of spiperone was only 2-5 fold and even more remarkable risperidone functionally behaved as moderate selective (10-fold) antagonist of α_{1A}-adrenoceptors [5, 10].
Therefore, as proposed for the α_{1A}-adrenoceptors [1] it is tempting to speculate that the α_{1B}-adrenoceptor can present itself as different affinity states.
α_{1D}-adrenoceptors: In the rat aorta, which is considered to be a functional α_{1D}-adrenoceptor correlate [11], the rightward displacement of the phenylephrine E/[A] curves by (+)-cyclazosin was accompanied by a concentration-dependent steepening of the phenylephrine E/[A] curve (Chapter 5). This phenomenon was also reported for other antagonists, and has been suggested to be due to the expression of two closely-related forms of the α_{1D}-adrenoceptor in rat aorta [12]. Analysis of agonism provided more evidence that α_1-adrenoceptors in rat aorta do not operate as a homogenous one-receptor-one-transducer system [13].

In the absence of molecular evidence in support of additional α_1-adrenoceptor subtypes, I believe that multiple receptor states should be considered for all α_1-adrenoceptor subtypes as an explanation for these observations. It should be noted that evidence provided from molecular pharmacology in recent years (as discussed below) supports such a stand.

Functional receptor affinity states: what is the mechanism?

Splice variants
Splice variants of the α_{1A}-adrenoceptor have been considered as a possible explanation for affinity states of α_{1A}-adrenoceptors. However, although four functionally active splice variants of α_{1A}-adrenoceptors were defined, their pharmacological profiles were similar [14] [15]. Interestingly, a recent study identified additional splice variants that led to truncated receptors lacking a transmembrane domain [16]. Though these truncated isoforms were incapable of ligand binding and signal transduction co-expression with functional isoforms diminished the number of prazosin binding sites. Prazosin affinity was not affected by the interaction with truncated isoforms, but the cell surface trafficking of the co-expressed original seven transmembrane α_{1A}-adrenoceptor was inhibited. At present splice variants do not offer a direct explanation for low affinity subtypes, but should be considered as a regulatory pathway for α_{1A}-adrenoceptors.

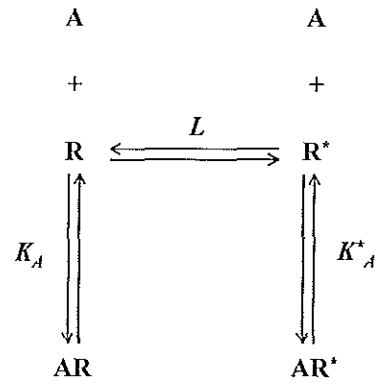
Localisation
It was demonstrated that α_{1A}-adrenoceptors localise predominantly intracellular, whereas most of the α_{1B}-adrenoceptors localise on the cell surface. This subtype-specific cellular distribution rather than the receptor structure determined the sensitivity for CEC inactivation of α_1-adrenoceptors [17, 18]. Recent studies reported that all α_1-adrenoceptor subtypes display a degree of cellular distribution and are localised at the plasma membrane as well as intracellularly [19, 20]. Interestingly, α_{1A}-adrenoceptors display a different, more α_{1L}-adrenoceptor-like, pharmacological profile when treated as whole cells in contrast to an α_{1A}-adrenoceptor profile in membrane preparations [1]. Possibly, differences in subcellular localisation of α_1-adrenoceptors among tissues may explain the different affinity states that are observed. Indeed, MacKenzie and colleagues speculated on the basis of their observations that the ability to penetrate

cells might influence ligand affinity for α_1-adrenoceptors [20]. However, this hypothesis is only conceivable on the condition that these intracellular receptors do actually transduce signals. Signal transduction by intracellular receptors has not been demonstrated yet. Lipophilicity of ligands would be the most likely physicochemical property that enables or disables cellular penetration. Interestingly, RS-17053 is a highly lipophilic ligand. In contrast, however, Rec 15/2739 which is another example a highly lipophilic ligand did not display a discriminatory potency for α_{1A}- or α_{1L}-adrenoceptors similar to that of RS-17053 [1]. Apparently, lipophilicity is not the crucial factor that explains the α_{1L}-profile. Furthermore, it should be noted that the two subtypes could not be discriminated by their agonist profiles [1]. Some variation between agonist profiles would have been expected in case physiochemical properties of ligands would be of crucial importance.

Another interesting feature that should be considered is the ligand-induced redistribution of α_1-adrenoceptors. Mc Cune *et al.* [19] demonstrated that prazosin caused a redistribution of α_{1D}-adrenoceptors from an intracellular localisation to the cell membrane. Consistent with other reports (see [21]), the authors suggest that this receptor redistribution is associated with ligands that displayed negative intrinsic activity at α_{1D}-adrenoceptors. However, redistribution of receptors is normally studied, and likely requires, a presence of the inverse agonists over a longer time period (>24h) than in our experiments [19, 21]. Nevertheless, the concept of inverse agonism offers other directions for explaining affinity states.

Inverse agonism

In the classical concept the only relevant parameter which accounts for an antagonist's capability to recognise a receptor and form a complex with it is its binding affinity. This affinity is agonist and system independent. Consequently, affinity values for antagonists are not expected to differ between functional assays and binding studies and this makes antagonists suitable tools for receptor characterisation. However, in recent years a concept developed which redefines agonism and antagonism and introduced terms like constitutive activity, neutral antagonism and inverse agonism (see [22, 23]). The concept postulates that receptors exist in a variety of conformational states, some of which are spontaneously active. These spontaneously active conformations can interact with the effector mechanisms in the absence of a ligand and explain constitutive activity. In the simplest model, the two-state model (see Figure), receptors are proposed to exist in equilibrium between two conformations, an active form (R*) and an inactive form (R). Agonists act by preferentially binding to and enriching the active conformation, thereby increasing effector activity, whereas inverse agonists bind preferentially to the inactive (R) conformational state, leading to a reduction of constitutive activity. Neutral antagonists bind equally well to R and R*, and therefore do not alter the equilibrium and constitutive receptor activity. Thus antagonists do not simply block the action of an agonist but can also posses efficacy, ranging from negative antagonism to neutral antagonism.



The equilibrium between the two states is controlled by the equilibrium constant L in the absence of a ligand. The interaction of an agonist (A) with the receptor alters the equilibrium between the two states according to its dissociation equilibrium constants at the two receptor states, namely K_A and K'_A. In this concept the action of an antagonist in a given tissue depends first on its

negative or neutral efficacy but also on the basal R:R* ratio which is determined by the constant L. It is not unlikely that L and consequently the basal R:R* ratio might vary among tissues and thereby introducing a system dependency which will influence the affinities of non-neutral antagonists estimated in functional studies. Thus for negative antagonists the affinity values estimated in functional assays may not be comparable with those obtained in binding experiments. Furthermore, the functional affinities of negative antagonists may differ amongst tissues, according to the allosteric parameter L.

In Chapter 3 we have demonstrated a highly significant correlation between the affinity and efficacy for noradrenaline in the SMA, which traditional pharmacology views as independent parameters. We offered the plausible explanation that this correlation is a statistical phenomenon. However, alternatively this correlation could be interpreted to support the existence of multiple receptor states in rat SMA. In the multiple state receptor model efficacy is the consequence of affinity [14, 23, 24]. Since the relative affinity for either R or R* determines the efficacy of the system. This concept of inverse agonism and system dependent equilibria between R:R* offers a framework for speculation about the observed discrepancies in antagonist affinities among functional assays and between radioligand binding studies and functional assays. Recently it became clear that prazosin, 5-Mu, WB- 4101, indoramin, but not Rec15/2739 displayed negative intrinsic activity at wild type α_{1A}-adrenoceptors [25]. Furthermore, all of the aforementioned antagonists were inverse agonists at wild type α_{1B}-adrenoceptors. Additionally, RS-17053, tamsulosin as well as (+)-cyclazosin behaved as inverse agonists at constitutively active mutant α_{1A}- and α_{1B}-adrenoceptors. Similarly, for α_{1D}-adrenoceptors; BMY 7378, phentolamine, 5-Mu and prazosin have been identified as inverse agonists [19, 26]. Moreover, soon a report will be published which suggests the presence of a constitutively active α_{1D}-adrenoceptor population in rat aorta *(Gisbert R. et al. J.Pharmacol. Exp. Ther. 295(2) (2000))*. Clearly the ingredients for multiple affinity states are present within the class of α_1-adrenoceptors. However, a straightforward quantitative and qualitative account for our observations cannot be provided yet. Particularly, since antagonists like (+)-cyclazosin and indoramin were characterised as inverse agonists at α_{1A}-adrenoceptors [25] but their functional α_{1L}-adrenoceptor profile is similar to the binding affinities at α_{1A}-adrenoceptors. Furthermore, many antagonists display negative efficacy at α_{1B}-adrenoceptors though functionally only for (+)-cyclazosin a discrepancy with binding affinities was reported (Chapter 4). One of the problems is that a two state model is clearly too simple to explain the experimental data. In a two-state model differences amongst tissues between the R:R* ratio should have been expressed as levels of constitutive activity. However, constitutive tissue activity or loss of it by RS-17053 or (+)-cyclazosin was neither observed in our experiments, nor has it been reported elsewhere. Obviously, a two-state model is a too simplistic model to offer a completely satisfactory explanation for 'antagonist affinity states'. Interestingly, a three state model containing two active receptor states has been proposed in order to explain the phenomenon that the same receptor, when coupled to different G protein effector pathways, can display different affinity/ efficacy patterns also designated as 'agonist trafficking'[23]. In fact it was suggested that this feature might partially account for the incompatibility of α_1-adrenoceptors in rat aorta with a one-receptor-one-transducer system [13]. Though the validity of the above-described mechanisms is well established in genetically engineered systems, they do not offer clear-cut explanations for our observations in native tissue. Nevertheless, molecular pharmacology has identified concepts that offer useful directions for further thinking and studying the inconsistencies of α_1-adrenoceptors with traditional receptor theory.

Does the rat small mesenteric artery represent a resistance vessel?

Isolated SMA assays have been used widely as models of resistance vessels [27]. Since small

mesenteric arteries (SMAs; internal diameter 100-300 μm) contribute substantially to vascular resistance in rat [27-29], these vessels are believed to posses regulatory potential and consequently have predictive value for the blood-pressure response [30]. However, this belief was challenged by the lack of effect of idazoxan in SMA, a partial α_1-adrenoceptor agonist that increases blood pressure in pithed rat [31].

Our data question the predictive value of the SMA for pressor responses within the mesenteric circulation. In the perfused mesentery the selective α_{1A}-adrenoceptor antagonist, RS-17053, antagonised the noradrenaline induced pressor response displaying high affinity ($pA_2 = 9.9$; [32]). This pharmacological profile confirmed the resistance in the perfused mesentery is determined by α_{1A}-adrenoceptors [32]. However, the affinity of RS-17053 in the rat SMA was 35-fold lower ($pK_B = 8.3$-8.4 ; Chapter 2). Therefore, it appears that α_{1A}-adrenoceptors mediate the pressor response in rat perfused mesentery, whereas the contraction in rat SMA is mediated by a α_{1L}-adrenoceptor.

Also comparison of our AVP experimental data in the SMA (Chapter 5) with reported data in the perfused mesentery argues against the SMA being a resistance vessel. The AVP-induced potentiation of methoxamine responses in the perfused rat mesentery was reported to be mediated via an atypical vasopressin receptor, not antagonised by SR 49059 [33]. However, in contrast to this perfused assay system, the V_{1A} receptor antagonist SR 49059, at a concentration (2 nM) selective for the V_{1A} receptor [34], blocked the AVP-induced potentiation of noradrenaline in the SMA. Apparently, discrepancies between the SMA and the perfused assay are not confined to α_1-adrenoceptors.

These discrepancies seem to contrast with the general view that SMA's with internal diameters of 100-300 μm represent resistance vessels [27-30]. However, at least for α_1-adrenoceptors the possibility of affinity states should be considered. Therefore, the SMA might represent a resistance vessel on the basis of its diameter, but clearly it lacks predictive value for pressor responses. Alternatively, the pressor response in the perfused mesentery might reflect resistance changes in the more distal arteriolar circulation that co-determine resistance rather than in the SMAs [30]. In summary our findings cast doubt on the validity of the SMA as a resistance-vessel model for predicting the pharmacology of pressor responses.

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Summary and conclusions

In **Chapter 1** we provided an overview of the historical aspects of the classification of α_1-adrenoceptors. The official nomenclature now recognises α_{1A}-, α_{1B}- and α_{1D}-adrenoceptor subtypes which all display high affinity for prazosin. However, functional experiments have suggested the existence of another α_{1L}-adrenoceptor subtype that displays a low affinity for prazosin. The controversy over the existence of an α_{1L}-adrenoceptor subtype appears to focus on tissues that were initially characterised as functional α_{1A}-tissues like, for example, rat mesenteric resistance vasculature. The range of estimated affinities of the selective α_{1A}-adrenoceptor antagonist, RS-17053, in functional "α_{1A}-tissues" substantiates the discordance with the official α_1-adrenoceptor nomenclature. Also in Chapter 1, the nomenclature of vasopressin and oxytocin receptors was reviewed more briefly. Subsequently, the aims and outline of the thesis are described. In the absence of a molecular correlate, we envisaged that the α_{1L}-adrenoceptor could bear a challenge to the traditional receptor concept. Given the possible impact of this subject it was our primary ambition to clarify the nature of the α_{1L}-adrenoceptor subtype.

In **Chapter 2** we characterised the α_1-adrenoceptor subtype involved in noradrenaline-induced contractions of rat SMA. This adrenoceptor subtype was designated α_{1L}, because it displays a low affinity for prazosin and RS-17053. Since, it has been suggested that the α_{1L}-adrenoceptor represents a conformational affinity state of the α_{1A}-adrenoceptor we elaborated on the nature of the α_{1L}-adrenoceptor pharmacology. In brief, the pharmacological α_{1L}-adrenoceptor profile was agonist independent and we were unable to define environmental factors that influenced this profile. Therefore we did not obtain evidence for the hypothesis that the α_{1L}-adrenoceptor represents an affinity state of the α_{1A}-adrenoceptor. Furthermore, a co-existing α_{1A}-adrenoceptor in rat SMA is unlikely.

Variable receptor affinity, rather than different receptor subtypes, has been suggested to account for the variation in estimated agonist affinities in functional studies on α_1-adrenoceptors. In case of a variable affinity for agonists, it is not unreasonable to assume that this phenomenon would also manifests itself for other ligands like antagonists and could account for the α_{1L}-adrenoceptor profile in rat SMA. In **Chapter 3** we have studied the agonism of noradrenaline in rat SMA by analysis of receptor inactivation experiments using the operational model of agonism. The main finding of the study was that the operational model of agonism yielded highly variable and correlated estimates of affinity and efficacy. The correlation of affinity and efficacy is inconsistent with the basic assumption that these parameters are independent. Interestingly, introducing random noise on simulated 'perfect' control and PBZ treated noradrenaline E/[A] curves yielded affinity and efficacy estimates that displayed not only a similar degree of variation as experimentally obtained estimates, but also a highly significant correlation. This suggests that a statistical rather than a pharmacological phenomenon accounts for the large variability and correlation of affinity and efficacy estimates. Therefore, the α_{1L}-adrenoceptor in rat SMA does not display variable affinity for noradrenaline.

Although the preferential susceptibility to irreversible inactivation by CEC has been used to subclassify α_{1B}-adrenoceptors, an α_{1B}-adrenoceptor selective competitive antagonist has not been available for quantitative characterisation of functional α_{1B}-adrenoceptors. In **Chapter 4** we characterised the functional pharmacological profile of (+)-cyclazosin, a selective competitive α_{1B}-adrenoceptor antagonist as defined in radioligand binding studies. We used rat SMA, rat aorta and rat and mouse spleen as representants of functional $\alpha_{1A/L}$-, α_{1D}- and α_{1B}-adrenoceptors

respectively. The functional potency of (+)-cyclazosin for $\alpha_{1A/L}$-and α_{1D}-adrenoceptors was in accordance with radioligand binding affinity for these subtypes. However, the functional antagonising potency of (+)-cyclazosin in rat and mouse spleen was much lower than its affinity for α_{1B}-adrenoceptors in radioligand binding studies. Furthermore, (+)-cyclazosin displayed a different pharmacology at mouse and rat α_{1B}-adrenoceptors. Thus, a discordance between radioligand binding and functional studies was noted for the interaction of (+)-cyclazosin with α_{1B}-adrenoceptors, similar to that of α_{1A}-adrenoceptors.

In **Chapter 5** we addressed the inconsistencies between various reports concerning the functional pharmacology of vasopressin in rat SMA and aorta. In order to illuminate these discrepancies we studied the effects of the selective V_{1A} receptor antagonists, OPC 21268 and SR 49059, and the oxytocin receptor antagonist, atosiban, on the AVP- and oxytocin-induced contractions of the two vessels. AVP and oxytocin contracted rat SMA and aorta without any vasodilatory component. The antagonist affinities indicated predominant involvement of V_{1A} receptors in both vessels. However, the concentration-dependent steepening of AVP E/[A] curves by atosiban and the Hill slope difference between oxytocin- and AVP E/[A] curves could indicate receptor heterogeneity. Therefore, despite predominant involvement of V_{1A} receptors, receptor heterogeneity should be considered in the SMA.

Because interactions between agonists are the *in vivo* reality, we aimed to analyse the interaction between α_{1L}-adrenoceptors and vasopressin receptors in rat SMA (**Chapter 6**). We used the previously described theoretical two-receptor:one-transducer model as a framework for the design and analysis of the experiments. This model predicts, by taking into account separate and common parts of the transducer pathway, a wide variety of possible location and slope changes of E/[A] curves upon interaction of two agonists. Threshold contractions by either AVP or noradrenaline potentiated and flattened the E/[A] curves of the interacting agonist without affecting maximum responses. Also the response to noradrenaline, which behaved as a partial agonist after phenoxybenzamine treatment was potentiated, flattened and the maximum response was increased by an AVP-induced threshold contraction. All observed characteristics (potentiation, flattening and the increased maximum) of agonist E/[A] curves by threshold contractions of the interacting agonists are predicted by the two-receptor:one-transducer model, assuming a steep common transducer pathway. Indeed a steep slope (n=5.4) was estimated for the common part of the transducer pathway and together with the other derived parameters the theoretical two-receptor:one-transducer model could satisfactory fit all experimental data. In conclusion, we have demonstrated that interaction between AVP and noradrenaline, which involves the V_{1A} receptor and α_{1L}-adrenoceptor, respectively, follows the theoretical two-receptor:one-transducer model, with the slope-dependence residing in the common transducer pathway.

In **Chapter 7** the nature of the α_{1L}-adrenoceptor is further discussed. From the data in this thesis and those reported elsewhere it is proposed that affinity states might not be unique for α_{1A}-adrenoceptors, but are a common feature of the α_1-adrenoceptor class. Though the nature of the affinity states in native tissue is not established, molecular pharmacology has identified concepts like inverse agonism that offer useful directions for further thinking and studying the inconsistencies of α_1-adrenoceptors with traditional receptor theory. Finally, we questioned the validity of the rat SMA as a resistance vessel, which has a predictive value for pressor responses, since our findings with α_1-adrenoceptors and vasopressin in the SMA differ from those in the perfused mesentery.

Conclusions with reference to our predefined aims

- The α_1-adrenoceptor mediating noradrenaline-induced contractions in rat SMA displays a distinct α_{1L}-adrenoceptor pharmacology.
- There is no co-existing α_{1A}-adrenoceptor in

the rat SMA.

- Environmental factors that might affect the pharmacological profile for antagonists of the α_{1L}-adrenoceptor could not be defined. Furthermore, the considerable variation of functionally estimated agonist affinities within the SMA was not based on variable affinity of the α_{1L}-adrenoceptor. Therefore, we did not obtain evidence for the hypothesis the α_{1L}-adrenoceptor represents an affinity state of the α_{1A}-adrenoceptor.
- (+)-Cyclazosin does not behave as a selective α_{1B}-adrenoceptor antagonist in functional tissues. This could suggest that the α_{1A}-adrenoceptor is not the only subtype within the α_1-adrenoceptor class that displays a heterogeneous pharmacological profile.
- The interaction between noradrenaline and AVP follows the theoretical two-receptor:one-transducer model assuming a steep common transducer pathway.

Samenvatting

Binnen de klasse van α_1-adrenerge receptoren onderscheidt men nu officieel 3 subtypen: α_{1A}, α_{1B} en α_{1D}. Voor deze 3 subtypen zijn moleculaire en farmacologische bindings- en functionele data met elkaar in overeenstemming. Functionele studies hebben echter aanwijzingen geleverd voor het bestaan van een vierde subtype. Deze α_{1L}-adrenerge receptor, kenmerkte zich in eerste instantie door een lage affiniteit voor prazosine. De affiniteit voor prazosine was gemiddeld een log-eenheid lager dan die voor de andere subtypen, welke prazosine zonder onderscheid met hoge, subnanomolaire, affiniteit herkent in zowel functionele als bindingsstudies. Later is gerapporteerd dat RS-17053, een selectieve α_{1A}-receptor antagonist, een groter onderscheidend vermogen heeft en dat het α_{1L}-subtype in verschillende weefsels een range van affiniteiten lijkt te hebben voor RS-17053. Echter, ondanks het huidige moleculair biologische tijdperk is een coderend gen voor dit α_{1L}-subtype 'nog' niet geïdentificeerd. Dit is opmerkelijk omdat de α_{1L}-adrenerge receptor toch als een potentiële "drug-target" gezien wordt. Om deze reden wordt aan het bestaan van de α_{1L}-adrenerge receptor als separate identiteit getwijfeld. Als alternatieve verklaring is de hypothese geopperd dat de α_{1L}-adrenerge receptor een affiniteitsvorm is van de α_{1A}-adrenerge receptor. De affiniteitsvorm waarin de α_{1A}-adrenerge receptor zich presenteert wordt volgens deze hypothese bepaald worden door omgevingsfactoren. Dit zou ook verklaren waarom de affiniteiten voor prazosine en RS-17053 bepaald in bindingsstudies verschillend zijn van die in functionele studies, terwijl die van andere antagonisten niet verschilden.

Volgens de traditionele receptor theorie is de bindingsaffiniteit de enige parameter van belang voor de antagonist-receptor interactie. Bovendien is deze bindingsaffiniteit onafhankelijk van omgevingsinvloeden. Duidelijk is dat de traditionele receptor theorie geen ruimte laat voor affiniteitsvormen van receptoren. Ook zijn grote en selectieve verschillen in affiniteiten voor antagonisten tussen radioligand bindingsdata en functioneel verkregen data niet te verwachten. Moleculaire biologische bevindingen hebben al behoorlijke druk is uitgeoefend op de traditionele receptor theorie en zelfs aanleiding gegeven tot de formulering van nieuwe concepten. Echter, dergelijke druk vanuit genetisch ongemodificeerde systemen is veel zeldzamer. Om inzicht te krijgen in de mogelijke implicaties was het primaire doel van dit onderzoek om de aard van de α_{1L}-adrenerge receptor op te helderen. In hoofdstuk 2 hebben we de α_1-adrenerge receptor in de kleine mesenteriale vaten gekarakteriseerd als een α_{1L}-subtype, met een lage affiniteit voor de antagonisten, prazosine en RS-17053. Er konden geen omgevingsfactoren worden geïdentificeerd die van invloed waren op de affiniteit van de receptor voor RS-17053. Bovendien was co-aanwezigheid van een α_{1A}-subtype onwaarschijnlijk. In Hoofdstuk 3 hebben we het agonisme van noradrenaline bestudeerd in de kleine mesenteriale vaten. Middels het operationele model voor agonisme hebben we de parameters affiniteit en intrinsieke activiteit van noradrenaline geschat. De correlatie van deze parameters, die volgens de theorie onafhankelijk zijn, leek geen mechanistisch, maar eerder een statistisch fenomeen te zijn. De belangrijkste uitkomst met betrekking tot onze doelstelling was, dat hoewel de geschatte affiniteit voor noradrenaline in de verschillende experimenten met dit preparaat sterk varieerde, deze variabiliteit niet berustte op een variatie van de affiniteit van de receptor. Samenvattend blijkt uit hoofdstuk 2 en 3 dat het affiniteitsprofiel van de α_{1L}-adrenerge receptor stabiel is voor zowel een antagonist als een agonist. Hoewel beide studies geen bewijs leverden voor het bestaan van fenotypische affiniteitsvormen van de α_{1A}-adrenerge receptor is dit nog steeds een valide hypothese. Op basis van de resultaten voor α_{1B}-adrenerge receptoren (hoofdstuk 4) en gegevens uit de literatuur dient men het bestaan van affiniteitsvormen mijns inziens voor de gehele klasse van α_1-adrenerge receptoren als serieuze

mogelijkheid te beschouwen (algemene discussie). In hoofdstuk 4, karakteriseren we het functionele farmacologische profiel van (+)-cyclazosine. In radioligand bindingsstudies werd (+)-Cyclazosine gedefinieerd als een selectieve α_{1B}-adrenerge receptor antagonist. Echter, wanneer de functionele respons van α_{1B}-receptoren (contractie van de milt van een rat en muis) bestudeerd wordt komt er een totaal ander farmacologisch profiel naar voren. In de rattenmilt gedraagt (+)-cyclazosine zich weliswaar als een competitieve antagonist, maar met een bijna 100* lagere affiniteit dan in bindingsstudies. In de muizenmilt gedraagt (+)-cyclazosine zich zelfs niet als een competitieve antagonist. De functionele gemeten affiniteit voor $\alpha_{1A/L}$- en α_{1D}-adrenerge receptoren kwam wel overeen met die gemeten in radioligand bindingsstudies. Evenals voor de α_{1A}-adrenerge receptor lijkt er nu ook voor de α_{1B}-adrenerge receptor een discrepantie te bestaan voor de affiniteit gemeten in radioligand binding studies en in functionele studies.

In aanwezigheid van (+)-Cyclazosine nam de helling van de phenylefrine curve in de rattenaorta (α_{1D}-adrenerge receptoren) op een concentratie-afhankelijke manier toe. Deze bevinding bevestigt eerdere resultaten van een uitgebreidere analyse in rattenaorta, welke de aanwezigheid van 2 nauwverwante receptor subtypen suggereerde. Met de huidige resultaten voor de interactie van (+)-cyclazosine en α_{1B}-en α_{1D}-adrenerge receptoren en eerder verschenen studies betoogt algemene discussie onder andere dat het bestaan van affiniteitsvormen voor de totale klasse van α_1-receptoren een serieus te nemen optie is. Met name omdat er genetisch géén aanwijzingen zijn voor extra subtypen. Vanuit de moleculaire biologie zijn er wel concepten als affiniteitsvormen en invers agonisme gedefinieerd. Hoewel deze concepten niet direct vertaald kunnen worden om onze waarnemingen te verklaren, kunnen zij wel richting geven aan verder filosoferen over en onderzoek naar een verklaring.

In hoofdstuk 2 tot en met 4 hebben we ons onderzoek gericht op de farmacologische analyse van één receptor subtype. In de *in vivo* situatie zijn er verschillende vaatverwijdende en vaatvernauwende stoffen aanwezig die allen verschillende receptoren stimuleren. De uiteindelijke respons van een bloedvat vormt de resultante van alle receptorinteracties. Synergistische interacties zijn met name interessant vanwege de klinische implicaties. Om deze reden wilden we de interactie tussen vasopressine receptoren en α_{1L}-adrenerge receptoren in de kleine mesenteriale vaten onderzoeken. Om drie redenen werd gekozen voor vasopressine: (1) vasopressine is een zeer potente vasoconstrictor en is daardoor waarschijnlijk al in lage concentraties betrokken bij interacties, (2) vasopressine is mogelijk belangrijk in pathologische situaties zoals hartfalen en (3) de interactie tussen vasopressine receptoren en α_{1L}-adrenerge receptoren is nog niet uitgebreid onderzocht. Alvorens we deze interactie daadwerkelijk onderzochten was het noodzakelijk om voorafgaand de receptor(en) betrokken bij de vasopressine respons te karakteriseren (hoofdstuk 5). Met name omdat de literatuur hierover in de mesenteriale vaten niet eenduidig was. Vasopressine en oxytocine (beiden zijn agonist voor zowel vasopressine als oxytocine receptoren) gaven een contractie van de mesenteriale vaten en rattenaorta zonder aanwezigheid van een vasodilatoire component. De gemeten affiniteiten voor antagonisten suggereerden dat met name V_{1A} receptor de contractie medieerde. Echter, in de kleine mesenteriale vaten was er enige indicatie voor betrokkenheid van meer receptor typen. Vervolgens hebben we in hoofdstuk 6 de agonist-agonist interactie van noradrenaline met vasopressine onderzocht. In de literatuur zijn diverse interacties tussen agonisten beschreven. De verschillende onderzoeken hebben echter niet gestreefd naar het classificeren van het type van interactie. Daartegenover staat dat er één type van interactie met name als theoretisch model beschreven. Dit twee-receptor:één-transducer model beschrijft de interactie tussen twee receptor typen die uiteindelijk koppelen aan 1 transductie systeem en voorspelt een variëteit aan vorm en locatie van agonist curves bij een interactie. Lage contracties van vasopressine of noradrenaline gaven een potentiering en afvlakking van de concentratie respons curve van de interacterende

agonist (noradrenaline of vasopressine respectievelijk). De maximale respons van de agonisten wijzigde niet door de interactie. Echter, na inactivatie van α_1-adrenerge receptoren nam de maximale respons van noradrenaline, welke zich nu gedroeg als een partiële agonist, wel toe door een drempelcontractie van vasopressine. Al deze observaties zijn verklaarbaar door het twee-receptor:één-transducer model mits de hellingsparameter van het gedeelde transductiesysteem groter is dan 1. Via een grafische methode werd deze hellingshoek inderdaad geschat op 5.4. Vervolgens bleek dat met deze hellingshoek en andere berekende parameters de totale dataset goed te simuleren was met het twee-receptor:één-transducer model. Concluderend, kan gesteld worden dat de interactie tussen α_1-adrenerge en vasopressine receptoren in kleine mesenteriale vaten verloopt volgens het twee-receptor:één-transducer model.

Dankwoord

Bij het nadenken over het te schrijven dankwoord realiseer ik me al snel dat eigenlijk alle mensen die je een warm hart toedraagt (en omgekeerd) belangrijk zijn voor alles wat je onderneemt. Dit geldt ook voor het uitvoeren van een promotieonderzoek. In dat opzicht is dit dankwoord uiterst onvolledig. Desondanks wil ik een aantal mensen direct noemen.

Als eerste wil ik Prof. dr. Pramod Saxena bedanken voor de begeleiding en de mogelijkheid die hij mij geboden heeft om te promoveren. Pramod, hoewel je er op menig moment weleens een zwaar hoofd in zult hebben gehad was dat nooit merkbaar. Ik heb dan ook het vertrouwen dat je in mij stelde zeer gewaardeerd. De rust en het geduld waarmee je samen met mij aan een artikel kon werken verbaasde me altijd weer, maar was erg prettig. Bovendien waren deze sessies erg vruchtbaar. Als géén ander ben jij in staat om de boodschap op papier te zetten.

Piet Hein jouw betrokkenheid aan het totale project is van cruciaal belang geweest voor het slagen ervan. Hoewel ik voor de start van dit onderzoek nogal neerkeek op de wetenschappelijkheid van de klassieke farmacologie ben ik nu totaal om. Met name jouw enthousiasme en gedrevenheid hebben mij overtuigd dat deze tak van wetenschap dé manier is om nieuw bedachte concepten te toetsen en te vergelijken. Jij was ook de persoon waarmee je farmacologisch kon 'freaken' wat erg motiverend werkte. Met name in jouw Rotterdamtijd leidde dit tot veel gedachten-experimenten. Gelukkig had jij ook altijd oog voor de realiteit en heb jij als zodanig een belangrijke 'brugfunctie' vervuld waardoor er nu gepromoveerd wordt.

Prof. dr. J.F. Koster, Prof. dr. P.D. Verdouw en Prof. dr. J. Zaagsma dank ik voor de snelle beoordeling van mijn proefschrift.

Alle mensen van de afdeling Farmacologie wil ik danken voor hun bijdrage aan de gezelligheid en goede sfeer op de afdeling. Speciaal wil ik Magda en Antoinette bedanken voor alle ditjes en datjes, Ed, *de widowmaker*, Kalkman en Marcel van Gelderen voor alle lol.
Richard van Wezel en Pieter Mies dank ik voor de paranymfelarij. Jerome Baidjoe en zijn bedrijf JLB automatisering dank ik voor de altijd parate en goede hard- en software ondersteuning wat me met name in de laatste fase enorm geholpen heeft. Ook Mebiklote bedankt voor de ontspanning.

Tot slot bedank ik Virginia die er altijd in is blijven geloven en in tijden van paniek rustig bleef. Isa en Inez jullie aanwezigheid tijdens het promoveren was verfrissend en stimuleerden de afronding. Bovendien maken jullie het post-promotie tijdperk tot een heerlijk vooruitzicht.

Curriculum vitae

Wiro Stam werd op 2 juni 1965 geboren in Goirle. Na het behaalde VWO-diploma in 1984 (Sint-Paulusiyceum, Tilburg) startte hij met de opleiding Fysiotherapie in Breda. Na 2 jaar besloot hij in 1986 Medische Biologie te gaan studeren aan de Rijksuniversiteit Utrecht. Zijn eerste onderzoeksstage deed hij bij de vakgroep Farmacologie (faculteit farmacie), waar hij de rol van imuunmediatoren op de β-adrenerge receptor functie onderzocht. Een tweede onderzoeksstage werd uitgevoerd bij de vakgroep Immunologie en Infectieziekten (faculteit Diergeneeskunde). Hier werd de mogelijkheid onderzocht om middels synthetische peptiden die competeren voor antigeenpresentatie het verloop van auto-immuunziekten te beïnvloeden. In 1992 werd het doctoraal examen met succes afgelegd.

Na hier en daar wat gewerkt te hebben begon hij in september 1993 als assistent in opleiding bij het instituut Farmacologie van de Erasmus Universiteit Rotterdam. Het resultaat vindt u in dit proefschrift. Sinds 1998 is hij werkzaam als medisch informatiespecialist bij Novartis Pharma te Arnhem.

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Abbreviations

AR:	Adrenoceptor
AVP:	Arg-vasopressin
CEC:	Chloroethylclonidine
E/[A]:	Concentration-effect
KHS:	Krebs Henselheit solution
NA:	Noradrenaline
OT:	Oxytocin
PBZ:	Phenoxybenzamine
SMA:	Small mesenteric artery
5-HT:	5-hydroxytryptamine