SIGNAL TRANSDUCTION PATHWAYS INVOLVED IN INTESTINAL SALT AND WATER SECRETION

Signaal overdracht routes betrokken bij zout en water secretie in de darm

PROEFSCHRIFT

Ter verkrijging van de graad van doctor

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Ik geloof dat er maar één weg naar wetenschap leidt; een probleem tegenkomen, de schoonheid ervan zien en er verliefd op worden; ermee trouwen en er nog lang en gelukkig mee leven, tot de dood je scheidt. Tenzij je een ander, nog boeiender probleem tegenkomt, of eventueel een oplossing vindt. Maar zelfs als je een oplossing vindt, ontdek je soms tot je vreugde het bestaan van een hele schare bekoorlijke, zij het wellicht moeilijke probleemkinderen voor wier welzijn je doelgericht kunt zorgen tot het einde van je dagen.

Marc Popper, uit " Realism and the aim of science".

Aan mijn ouders

Voor Michael en Ronnie

Ontwerp voorkant: Pieter van Gageldonk.

CONTENTS

LIST OF ABBREVIATIONS

AC	adenylate cyclase
ATP	adenosine 5'-triphosphate
BBM	brush border membrane
BBMV	brush border membrane vesicle
BLM	basolateral membrane
CaCaMK	calcium/calmodulin-dependent protein kinase
cAMP	adenosine cyclic 3',5'-monophosphate
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
cGMP	guanosine cyclic 3',5'-monophosphate
CT	cholera toxin
DAG	1,2-diacylglycerol
I_{sc}	short circuit current
GC	guanylate cyclase
Gpp(NH)p	guanyl-5'-yl ßγ-imidodiphosphate
G-protein	guanine nucleotide binding protein
GTP	guanosine 5'-triphosphate
GTPγS	guanosine 5'-(3-O-thio)triphosphate
InsPn	inositol phosphates
PDB	4ß phorbol 12,13-dibutyrate
PI	phosphatidylinositol
PIP	phosphatidylinositol monophosphate
PIP2	phosphatidylinositol bisphosphate
PKA	cAMP-dependent protein kinase
PKC	calcium/phospholipid-dependent protein kinase
PKG	cGMP-dependent protein kinase
PLA2	phospholipase A2
PLC	phospholipase C
PLD	phospholipase D
PMA	4ß phorbol 12-myristate 13-acetate
PT	pertussis toxin
SD	secretory diarrhoea
ST_A	heat stable toxin of E.coli

CHAPTER 1

GENERAL INTRODUCTION

This thesis describes some novel aspects of the regulation of salt and water secretion in the intestinal epithelium. This process is not unique for the intestine, but a common and necessary function of many other organs, including the stomach (gastric juice), kidney (urine), sweatglands (sweat), gallbladder (bile), testis, some endocrine glands and the lungs. Also most of the transport systems involved are not uniquely expressed in the intestine but shared by a variety of other salt transporting epithelia; this is exemplified by the generalized exocrinopathy seen in cystic fibrosis (CF), a genetic disease characterized by a mutation in a Cl^- channel protein (designated CFTR) commonly expressed in epithelial tissues [Riordan et al., 1990; Kartner et al., 1991; Anderson et al, 1991a; Bear et al., 1992].

Some characteristics of ion transport regulation, however, are exclusively found in intestinal epithelium. These include: i) the pseudohormonal action of microbial enterotoxins (e.g. cholera toxin, heat labile and heat stable Escherichia coli toxin) on intestinal salt and water transport, resulting in secretory diarrhoea (SD) and dehydration: SD is still a major cause of morbidity and mortality among children in the developing countries [Guerrant, 1985]; ii) the role of cGMP and a cGMP-dependent protein kinase as intracellular mediators of intestinal ion secretion [De Jonge, 1981; De Jonge and Lohman, 1985; De Jonge and Rao, 1990]; iii) the presence of G-protein-activated phospholipase C at the brush border membrane, despite the absence of G-protein-coupled hormone receptors at this subcellular region [Vaandrager et al., 1990]; iv) the CF defect in cholinergic/Ca^{2+}-mediated activation of intestinal Cl^- secretion [De Jonge et al., 1987; Berschneider et al., 1988; De Jonge et al., 1989], in sharp contrast to the normal Ca^{2+}-linked Cl^- channel activation observed in most other CF-affected epithelia (airway [Boucher et al., 1989; Wagner et al., 1991], sweat gland [Sato and Sato, 1984]). This and some other unique aspects of intestinal signal transduction described in this thesis, render the study of ion transport regulation in the intestine especially rewarding.

In this first chapter a brief overview is presented of current concepts

about the molecular basis of transmembrane and intracellular signal transduction mechanisms in mammalian cell types in general and in intestinal epithelium in particular. The last paragraph summarizes the specific aspects of intestinal ion transport regulation that were examined in the studies described in Chapter 2-6.

1.0 Salt and water transport in the intestine

The intestinal tract consists of the small intestine, divided into duodenum, jejunum and ileum and the large intestine or colon. Absorption of salt, water and nutrients is performed by the villous epithelial cells lining the lumen of the small intestine. In each villus a network of blood and lymph vessels serves to transport the nutrients taken up by the epithelial cells. Each absorptive cell bears a striated structure at its apical membrane, the so-called brush border equipped with numerous microvilli, facilitating the uptake of nutrients by enlargement of the membrane surface. The villous cells are surrounded by a circular depression known as the crypts of Lieberkuhn, responsible for salt and water secretion

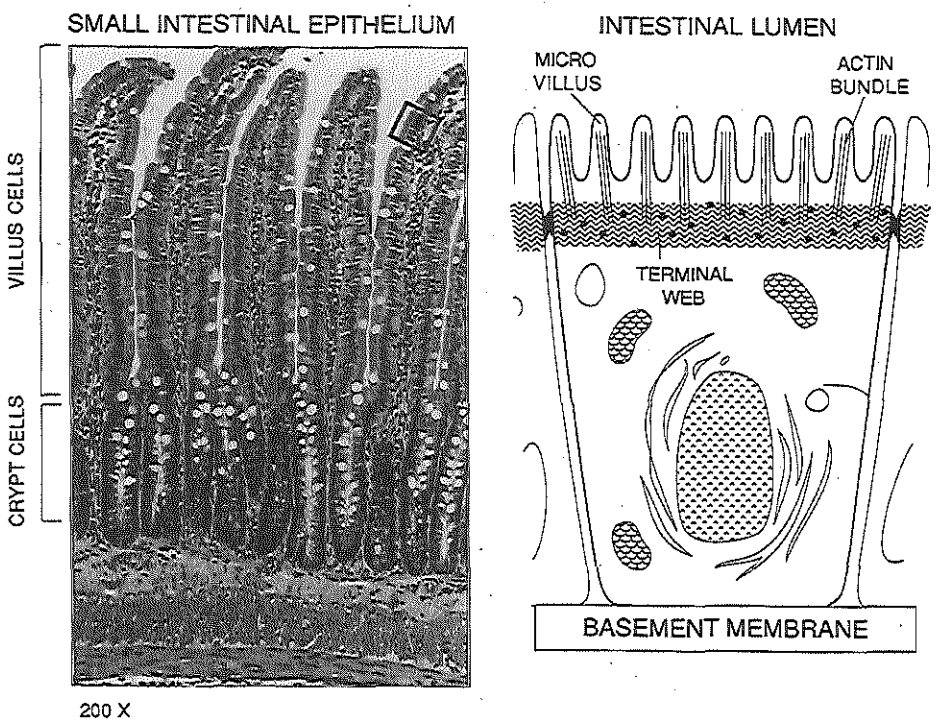

FIGURE 1. Morphology of the small intestine. Left: light microscopic view. Right: schematic drawing of an epithelial cell.

(Fig.1). Some absorption and secretion of salt and water is also performed by the epithelial cells lining the large intestine. All intestinal epithelial cell types, including the mucus-secreting Goblet cells, originate from progenitor cells (stem cells) located at the basis of the crypt [Ponder et al., 1985]. Most epithelial cells, with the striking exception of Paneth cells, subsequently migrate along the crypt/villus axis and are finally extruded into the lumen, resulting in the loss of 2.10^{10} villous cells each day and renewal of the epithelial cell layer every 4 days [Madara, 1991]. The regenerative capacity of the intestinal epithelium facilitates a fast recovery from irreversible damage or biochemical modifications brought about by toxic substances in the intestinal lumen, including cholera toxin [De Jonge, 1975a].

A considerable amount of fluid and enzymes is secreted and added to the lumen of the gastro-intestinal tract to facilitate mixing and digestion of nutrients each day: orally 1.5 liters of fluid is consumed, opposed to 8.5 l secreted by the gastro-intestinal tract (salivary glands 1.5 l, stomach 2.5 l, bile and pancreas 2 l, small intestine 1.5 l and large intestine 1 l). To prevent dehydration, water must be reabsorbed, which is carried out by the villous cells of the small intestine (80-90%) and the colonic epithelial cells. This reabsorption is very effective, causing loss of only 150 ml fluid in the faeces [Powell, 1987]. Since enterocytes do not have an active transport system for water, its transport is driven by an osmotic gradient across the epithelium. This gradient is built up by transcellular Na^+ absorption and Cl^- secretion, coupled to Cl^- absorption and paracellular Na^+ secretion, respectively [Field et al., 1989].

1.1 Ion transport systems involved in intestinal chloride secretion

The vectorial transport of electrolytes across epithelial barriers depends on the asymmetry of permeability and pumping activities of mucosal and serosal portions of the epithelial cell membranes. For chloride secretion, this is provided by targeting of Cl^- channels to the apical membrane and of K^+ channels, Na^+/K^+-ATPase and the $NaKCl_2$-cotransporter to the basolateral membrane (Fig.2). In intestinal epithelial cells the inwardly directed electrochemical gradient for Na^+, maintained by the Na^+/K^+-pump, provides the energy for secondary active ("uphill") transport of K^+ and Cl^- through the basolateral $NaKCl_2$-cotransporter into the Cl^- secreting cell types. Na^+ and K^+ leave the cell basolaterally by the Na^+/K^+-ATPase and K^+ channels, respectively. Cl^-, however, is secreted apically via Cl^- channels. The resulting transepithelial electrical potential difference drives Na^+ from the interstitium to the luminal solution via the paracellular pathway, leading

to net NaCl and water secretion.

It should be noted that all transport mechanisms indicated in Fig.2 are needed to provoke net chloride secretion. Therefore, inhibition of any of these systems will also inhibit chloride secretion. Thusfar, none of these intestinal ion transport systems has been characterized at the molecular level, i.e. by protein purification or molecular cloning. In view of this lack of information, current estimates of the number of different subspecies of K^+ channels (at least two [McRoberts et al., 1985]) and Cl^- channels (at least three [Worrell and Frizzell, 1991]) expressed in mammalian intestine are based only on pharmacological and biophysical evidence rather than on differences in genes or splice variants. A clear exception is the molecular cloning of the cystic fibrosis-gene encoded protein (CFTR) which, according to very recent information [Anderson et al., 1991a; Bear et al., 1992], serves as the major Cl^- channel in the apical membrane of enterocytes and colonocytes. The molecular properties and function of CFTR will be considered later following the discussion of signal transduction pathways involved in CFTR regulation.

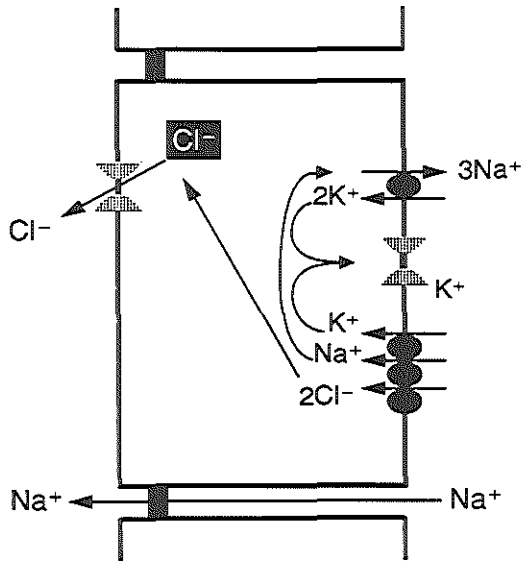

FIGURE 2. General cell model of transport pathways in transepithelial salt secretion. The transporters involved are: Cl^- channels, K^+ channels, the NaKCl-cotransporter and the Na^+/K^+-ATPase.

2.0 Regulation of chloride secretion

The net flow of water and salt in the intestine is determined by a balance between absorption and secretion. Both processes are under strict neurohormonal control. In general, the transduction of extracellular signals (hormones, neurotransmitters) to a biological response, occurs through a cascade of reactions, starting with the binding of an agonist to a membrane receptor. In many cases, the resulting conformational change in the receptor activates a guanine nucleotide binding protein (G-protein), which transduces the extracellular signal either by activating or inhibiting membranal signalling enzymes (e.g. adenylate cyclase, phospholipases) or ion transporters (e.g. K^+ or Ca^{2+} channels) [Gilman, 1987; Brown and Birnbaumer, 1990]). The cytosolic second messengers generated by these enzymes (e.g. cAMP, inositol phosphates) can transfer the signal to multiple intracellular locations. The final messenger is often a protein kinase. Protein kinases constitute a class of enzymes that catalyzes the transfer of the terminal phosphate group of ATP to the hydroxyl group on a serine, threonine or tyrosine residue of a protein. This addition of negative charges to the protein generally results in conformational changes, responsible for altered function and/or interaction with other proteins, thereby initiating a variety of biological responses.

Occasionally, the receptor protein is bifunctional and acts itself as a transmembrane signalling enzyme, e.g. a guanylate cyclase (generating cGMP) or a tyrosine kinase (cf. the insulin and EGF receptors [Ullrich and Schlessinger, 1990]). Examples of each of these signal transduction pathways have been described in intestinal epithelium [Pillion et al., 1985; De Jonge and Rao, 1990; Rao and De Jonge, 1990]. In this tissue, the most prominent intracellular signalling molecules include cAMP, cGMP and the phosphatidyl-inositol-derived messengers, Ca^{2+} and diacylglycerol. An increase in intracellular concentrations of any of these mediators results in a stimulation of chloride secretion, the net effect being fluid accumulation in the lumen. A schematic drawing of these three signal transduction pathways is given in Fig.3. The properties and characteristics of the individual components of these pathways are summarized in the following paragraphs.

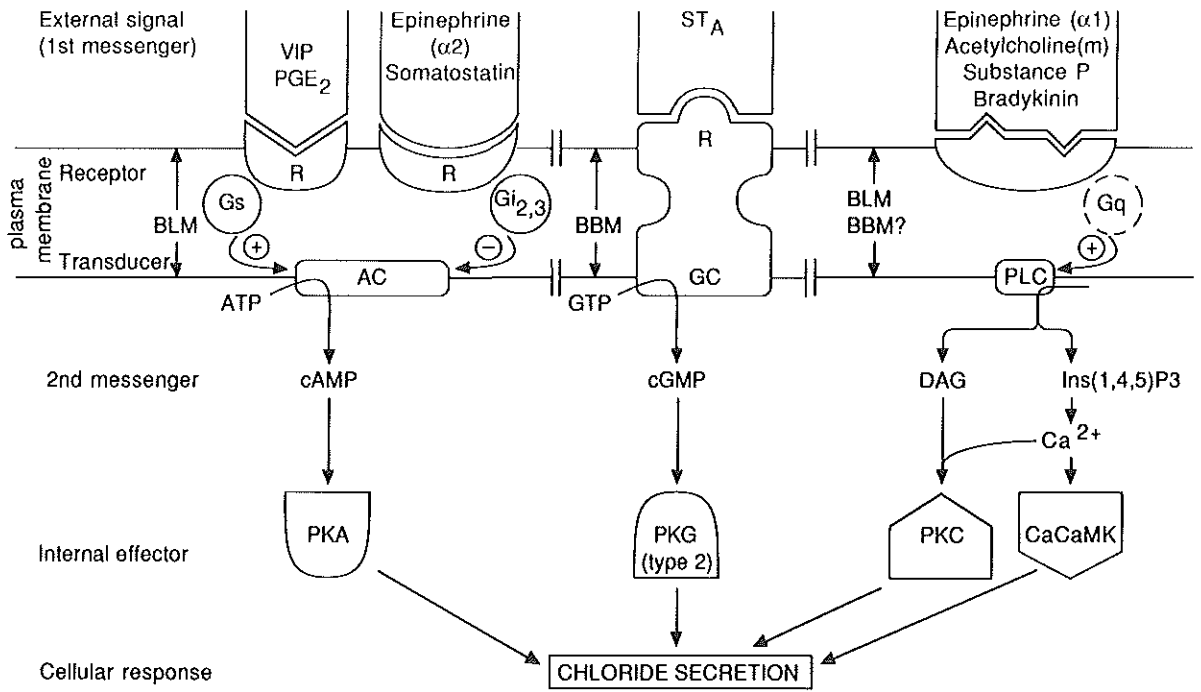

FIGURE 3. Signal transduction pathways leading to chloride secretion in the intestine. R, receptor; G, G-protein, AC, adenylate cyclase; GC, guanylate cyclase; PLC, phospholipase C, PT, pertussis toxin; DAG, diacylglycerol; Ins(1,4,5)P3, inositol (1,4,5) trisphosphate; A-kinase, cAMP dependent protein kinase; G-kinase, cGMP dependent protein kinase, C-kinase, calcium/phospholipid dependent protein kinase; Ca/CaM kinase, calcium/calmodulin dependent protein kinase; BLM, basolateral membrane; BBM, brush border membrane.

2.1 G-protein-coupled receptors

The receptors that regulate G-proteins are structurally and functionally homologous despite the variety of stimuli, which range from light and smell to neurotransmitters, hormones and inflammatory agents. Each receptor is about the same size (40-50 kDa) and forms seven membrane spanning regions linked by three cytoplasmic and three extracellular loops [Taylor, 1990]. The third intracellular loop and the cytoplasmic C-terminal tail have several serine and threonine residues that are likely or demonstrated sites of phosphorylation [Taylor, 1990]. Interaction among the second and the third intracellular loop is important to determine G-protein selectivity [Van Koppen and Nathanson, 1990]. The C-terminal tail is also involved in G-protein recognition [Taylor, 1990]. In intestinal epithelial cells, receptors of this type have sofar been localized exclusively to the basolateral membrane ([Vaandrager et al., 1990], see Fig.3 for typical examples).

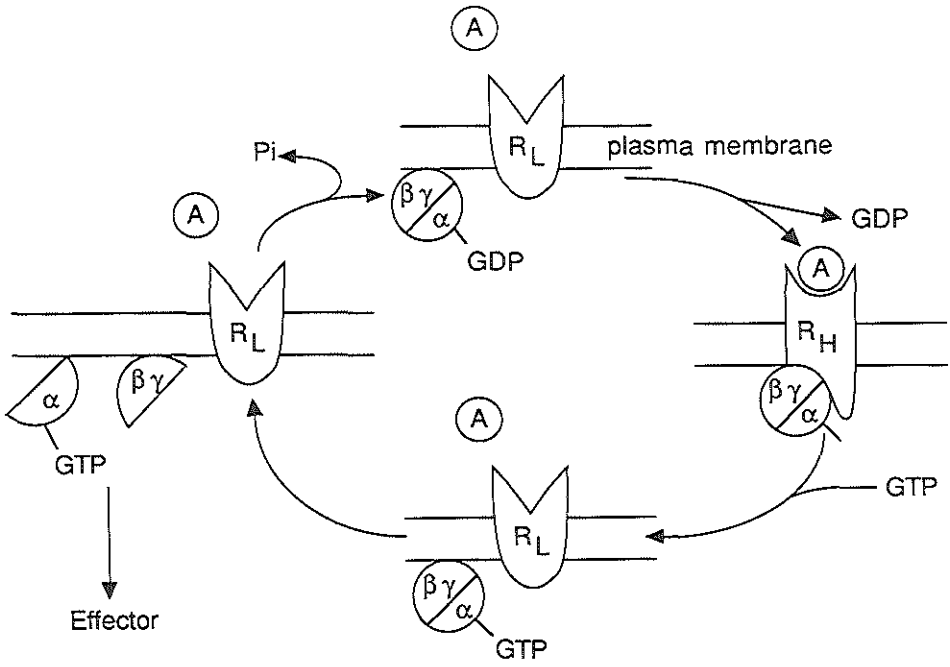

FIGURE 4. (In)activation cycle of G-proteins. The interactions between agonist (A), receptor (R) and G-protein ($\alpha\beta\gamma$) are described in the text. Binding of the agonist to its receptor activates the G-protein by increasing the rate of guanine nucleotide exchange. Activation of the G-protein is coupled to the receptor cycle, in which the receptor switches between conformations with high (R_H) and low (R_L) affinity for its agonist.

2.2 G-proteins

G-proteins are grouped into at least two classes: the hormone-linked trimeric G-proteins and the single (α) subunit G-protein typified by the ras family. As the (hormonal) regulation of chloride secretion is the main topic of this thesis, I will limit the discussion to the first of these two G-protein classes. The structural and functional properties of G-proteins have already been described in a variety of reviews [Gilman, 1987; Birnbaumer et al., 1987; Milligan, 1988; Taylor, 1990; Brown and Birnbaumer, 1990; Brown, 1991 and Boege et al., 1991]. Hereafter, the essential information from these reviews together with recent additional data will be discussed. Only the references not provided in the reviews above will be quoted separately in this section.

GTP-binding proteins participating in hormonal signal transduction are a closely related family of heterotrimers, consisting of an α-subunit (39-52 kDa), a ß-subunit (35-37 kDa) and a γ-subunit (8-11 kDa). Only the α-subunit can bind and hydrolyse GTP to GDP and P_i. The ß and γ subunits are always tightly associated. In the basal state, the G-protein exists as an $\alpha ß \gamma$ heterotrimer with GDP bound to the α-subunit. Upon activation of the receptor by the appropriate agonist, the receptor binds to the G-protein and promotes the release of GDP. The resulting agonist-receptor-G-protein complex binds the agonist with markedly higher affinity than it is to the receptor alone. At physiological concentrations, GTP binds instantaneously to the α-subunit. This leads to dissociation of the agonist and the G-protein from the receptor as well as to the formation of the activated GTP-bound α-subunit which dissociates from the $ß\gamma$-complex. The α-subunit is biologically active, although some responses are also mediated by the $ß\gamma$-subunits [Lotersztajn et al, 1992]. G-protein activation is terminated by an intrinsic GTPase activity followed by the reassociation of the GDP-bound α-subunit and the $ß\gamma$ subunits. A schematic drawing of this G-protein cycle is given in Fig.4. The α subunit of G-proteins have been implicated in a variety of biological responses (Table 1): regulation of adenylate cyclase (AC), cGMP phosphodiesterase (cGMP-PDE), phospholipase C (PLC), phospholipase A2 (PLA2) and a variety of ion channels. The effectors that are activated by the different G-proteins have little in common in contrast to the G-protein-linked receptors and the G-proteins themselves.

AC is dually regulated by Gs (stimulation) and Gi (inhibition). Gs is expressed as two long (± 52 kDa) and two short (± 45 kDa) forms, which are all splice variants of the same gene. Apart from the activation of AC, Gs is also involved in the regulation of multiple ion channels: the dihydropyridine-sensitive

Ca^{2+} channel (stimulation), cardiac I_f channels (stimulation) and neuronal Na^+ channels (inhibition). All forms can activate AC and the Ca^{2+} channel and can be expressed together in one cell type [Mattera et al., 1989]. At present it is therefore not clear why different forms of Gs exist. Inhibition of AC is mediated by Gi, a family of proteins encoded by three separate genes: Gi1, Gi2 and Gi3. Gi's are not only implicated in the inhibition of AC, but also in the activation of the muscarinic and ATP-sensitive K^+ channel, activation of an epithelial Cl^- channel and a Na^+ channel, and the activation of PLC and PLA2. Although some responses can be induced by all three Gi forms, e.g. activation of the [Ach]K^+ channel, other responses are more selective, e.g Gi2 and not Gi1 or Gi3 selectively couple to the δ subclass of the opioid receptor and some other hormone receptors [McKenzie and Milligan, 1990; Strussheim et al., 1990].

Activation of PLC is mediated both through pertussis toxin (PT) sensitive and PT insensitive pathways, suggesting the involvement of at least two different G-proteins. The identity of the G-proteins involved in the PT-sensitive pathway has only recently been clarified. In some systems Gi2 was implicated. In contrast, Go and not Gi1-3, was found to stimulate PLC in Xenopus oocytes [Moriarty et al., 1990]. Go has not only been implicated in the activation of PLC, but also in the regulation of Ca^{2+} and K^+ channels. Go is the most abundant G-protein in brain and constitutes a family of four proteins encoded by at least two genes [Inanobe 1990]. Whether these different effector systems are regulated by different Go's is not known yet.

Candidate G-proteins for the PT-insensitive activation of PLC are Gz and Gq, which both lack the PT site. However, the distribution of Gz is restricted to neural tissues and platelets and it does not appear to be present in all tissues that show PT insensitive activation of PLC, arguing against a role for Gz in PLC coupling. In contrast, Gq was found to be ubiquitous distributed and to activate one of the isoforms of PLC [PLC ß1, Taylor et al., 1991]. Finally, the hormonal activation of PLC by a number of agonists was inhibited by addition of α_q-specific antibodies in a variety of cell types [Gutowski et al., 1991]. These data indicate that Gq couples several hormone receptors to PLC. Gq is a family of five proteins: Gαq, Gα11, Gα12, Gα13 and Gα14. Whether the PLC coupling is mediated by one of these Gq-subtypes or that each member of the Gq family can mediate this response is presently unknown.

In vitro experiments suggest that a single G-protein can interact with multiple receptors (convergence). Interaction of a single receptor with multiple G-proteins (divergence) is also found. The G-protein in turn can interact with multiple

effectors (divergence) or multiple G-proteins can interact with one effector (convergence). However, molecular cloning techniques have identified a still growing family of multiple closely related isoforms of intracellular effectors such as phospholipase C [Majerus et al., 1990] and K^+ channels [Brown and Birnbaumer, 1990]. The apparent convergence of multiple G-proteins to the same effector may therefore be no more than a reflection of our present inability to detect a more specific interaction between distinct G-proteins and particular effector isoforms. Alternatively, the existence of multiple pathways resulting in the same response could be used as a fine-regulatory mechanism.

TABLE 1. Function of G-proteins in signal transduction.

G-protein subunit	Effector	Response
αs	Adenylate cyclase	stimulation, cAMP ↑
	DHP-sensitive Ca^{2+} channel	opening
	TTX-sensitive Na^+ channel	closing
	Cardiac pacemaker I_f channels	opening
αi1-3	Adenylate cyclase (αi2)	inhibition, cAMP ↓
	Phospholipase A2 (αi3)	stimulation, AA ↑
	Phospholipase C (αi2)	stimulation, InsPn ↑
	Muscarinic K^+ channel (αi1-3)	opening
	ATP-sensitive K^+ channel (αi3)	opening
	Epithelial Cl^- channel (αi3)	opening
αo	Phospholipase C	stimulation, InsPn ↑
	Neuronal K^+ channels	opening
	Ca^{2+} channels	closing
	Cardiac pacemaker I_f channels	closing
αq	Phospholipase C	stimulation, InsPn ↑
αt (transducin)	cGMP phosphodiesterase	stimulation, cGMP ↓
βγ	Plasma membrane Ca^{2+} pump	inhibition of basal activity and stimulation of agonist-sensitivity
	K^+[Ach]	closing (in absence of agonist)
	?	pheromone response in yeast

2.3 Techniques used to study G-protein involvement

To study whether a G-protein is involved in a particular biological response and which G-protein is involved, a variety of techniques can be used. First, G-proteins can be activated independently of their receptor by using i) stable analogues of GTP, e.g. GTPγS or Gpp(NH)p; ii) AlF_4^-, which is thought to mimic the structure of the third phosphate group of GTP or iii) a wasp venom, mastoparan, which is thought to mimic the cytoplasmic part of the receptor. The elicited response should be inhibited by addition of a stable analogue of GDP, GDPβS, when a G-protein is involved.

Secondly, the α subunit of some G-proteins can be covalently modified by cholera toxin (CT) and/or pertussis toxin (PT) catalyzed ADP-ribosylation. Based on this property, the G-proteins can be divided in four groups: a substrate for PT (Gi, Go), a substrate for CT (Gs), a substrate for PT and CT (transducin) or not a substrate for these toxins (Gz, Gq). CT dependent ADP-ribosylation requires lipid, NAD, GTP and ARF (ADP-ribosylation factor, a member of the small G-protein family). The small G-proteins differ from the hormone-activated heterotrimeric G-proteins in that, i) they have no intrinsic GTPase activity, ii) they are not activated by AlF_4^- and iii) Mg^{2+} is needed for nucleotide binding to the small G-proteins. CT-catalyzed ADP-ribosylation induces a constitutively active αs subunit, leading to elevated cAMP levels (Fig.3). The PT-dependent ADP-ribosylation also requires lipid and NAD, but ARF is not needed and GTP inhibits rather than stimulates the PT action as PT can only recognize the heterotrimer which dissociates upon addition of GTP. The PT-induced ADP-ribosylation is not activating the G-protein, but uncouples it from its receptor, which in the case of Gi, also leads to a (small) increase in cAMP levels. Thus, the sensitivity of a response to PT or CT provides information on which G-protein(s) could be involved. Furthermore, by using ^{32}P-NAD^+ in the ADP-ribosylation assay, G-proteins are covalently labeled and their localization and behavior can be studied. Alternatively, the involvement of Gi can also be studied by using CT, since guanine nucleotide free Gi can be recognized and ADP-ribosylated by CT. In vivo, this can be used to identify which hormone receptor couples to Gi: hormonal activation of Gi induces the formation of a G-protein without bound guanine nucleotide (Fig.4). Labeling of Gi by CT will thus only occur when Gi has been activated by the agonist [Milligan and MacKenzie, 1988].

Third, G-protein subunit-specific antibodies have been commonly used for purifying G-proteins and for studying the localization and phosphorylation of known G-proteins and has led together with molecular cloning techniques to the

identification of families of G-proteins and of novel G-protein-like proteins. By microinjection of specific antibodies, the contribution of a specific G-protein to a particular biological response can be studied. The purified G-protein subunits can be used in reconstitution assays to obtain information on their selectivity in coupling to different receptors and/or effectors.

In Chapter 2, several of the techniques mentioned above have been used to study G-protein expression and distribution in intestinal epithelial cells.

3.0 The phosphoinositide cycle

Many hormones, neurotransmitters and mediators of inflammatory reactions (e.g. epinephrine (α1), acetylcholine, substance P, histamine and bradykinin) act by activating the phosphoinositide cycle [reviewed by Berridge and Irvine, 1989 and Shears, 1989]. Coupling of the agonist to its receptor is thought to induce a conformational change such that a G-protein is activated. This G-protein, called Gp and in some cells identified as Gq, Gi2 or Go (see earlier), activates a phospholipase C (PLC). This enzyme cleaves phosphoinositide (4,5) bisphosphate (PIP2) in inositol (1,4,5) trisphosphate (IP3) and diacylglycerol (DAG). IP3 induces calcium release from intracellular IP3-sensitive stores, followed by calcium entry via Ca^{2+} channels in the plasma membrane. Calcium can act together with DAG to activate protein kinase C (PKC), a phospholipid and calcium dependent serine/threonine kinase. Calcium can also activate a calcium/calmodulin dependent serine/threonine kinase by binding to calmodulin (Fig.3). The activated kinases phosphorylate their specific substrates, thereby eliciting a variety of biological responses, e.g. chloride secretion in epithelial cells [Rao and De Jonge, 1990; Fig.3].

3.1 Phospholipase C

Phospholipase C (PLC) is the enzyme that cleaves polyphosphoinositides to yield diacylglycerol and inositol phosphates (InsPn). PLC consists of a family of cytosolic proteins of which 9 isoforms are yet identified: 1 PLC α, 3 PLC β, 2 PLC γ and 3 PLC δ [reviewed by Majerus et al., 1990]. All isoforms were shown to cleave PI, PI(4)P as well as PI(4,5)P2. However, PI(4,5)P2 is the preferred substrate at physiological calcium levels. The tissue distribution differs somewhat in that PLC α is most abundant in liver, PLC β and δ most abundant in brain, whereas PLC γ is more ubiquitously distributed over many tissues. The differences in tissue distribution can however not explain the existence of so many isoforms, since more than one isoform can be expressed in one cell.

PLC γ1 has been implicated in tyrosine kinase receptors mediated signal transduction pathways: PLC γ1 was selectively phosphorylated by the epidermal growth factor (EGF) receptor and the platelet-derived growth factor (PDGF) receptor [Majerus et al., 1990; Rhee, 1991], leading to activation of PLC [Nishibe et al., 1990]. Protein kinase A-induced phosphorylation of PLC γ in C6Bu1 cells correlated with inhibition of the norepinephrine-stimulated InsPn formation, suggesting a role for PLC γ also in the action of G-protein coupled hormone receptors [Kim et al., 1989]. In contrast, overexpression of PLC γ in NIH 3T3 cells led to an increase in InsPn release by PDGF, but not by bombesin, suggesting that G-protein coupled hormone receptors in these cells are not coupled to PLC γ [Cuadrado and Molloy, 1990]. This was confirmed by the finding that EGF, but not G-protein-coupled agonists induced the translocation of PLC γ1 from the cytosol to the membrane [Todderud et al., 1990]. Recently, PLC ß1 and not PLC γ1 or δ1, has been shown to be activated by α subunits of Gq, a new class of G-proteins [Taylor et al., 1991], suggesting a role for this isoform in signal transduction by G-protein-coupled hormone receptors. In conclusion, dependent on the cell type the different PLC isoforms may be involved in different signal transduction pathways and therefore couple to different types of cell surface receptors. With regard to the intestine, no specific information is yet available about the PLC isoforms expressed in enterocytes and colonocytes, their intracellular distribution and their contribution to PI-cycling in the basolateral and the apical membrane.

3.2 Inositol phosphates and calcium mobilization

Calcium-mobilizing agonists stimulate the production of inositol (1,4,5) triphosphate (IP3), which is rapidly metabolized to a number of other inositol phosphates by a series of kinases and phosphatases, including inositol (1,3,4,5) tetrakisphosphate (IP4), which is postulated to act together with IP3 to regulate calcium entry. The metabolism of the various inositol phosphates, shown in Fig.5, terminates the signalling activities of IP3 and IP4. The resulting formation of inositol is used for lipid resynthesis. To enable the measurement of inositol phosphate production, Li^+ is used, which inhibits the degradation of InsP to inositol and causes the accumulation of inositol phosphates [see also Chapter 4].

Recently, the receptor for IP3 has been cloned and characterized [Furuichi et al., 1989; Mignery and Sudhof, 1990]. The receptor has a transmembrane domain that forms a Ca^{2+} channel and a large cytoplasmic domain that contains the IP3 binding site. Binding of IP3 induces a conformational

change leading to opening of the Ca^{2+} channel and a rise in cytosolic calcium. Often, the calcium signal initiates in one region and is than distributed over the cell in the form of a wave. In hepatocytes, the site of origin is shown to be independent of the type of PI-coupled receptor, suggesting that the IP3-sensitive calcium pool has a restricted localization in the cell [Berridge, 1990]. The propagation of the calcium signal is now thought to occur through calcium-induced-calcium release and diffusion of IP3 deeper within the cell [Berridge, 1990; Irvine, 1990]. The resulting IP3-induced calcium oscillations can be explained in two ways, which have been shown to occur both: first, the receptor controlled model: IP3 itself oscillates presumably caused by negative feedback of protein kinase C on phospholipase C; secondly, the second messenger controlled model: IP3 itself is at a constant level, but calcium oscillates presumably as a

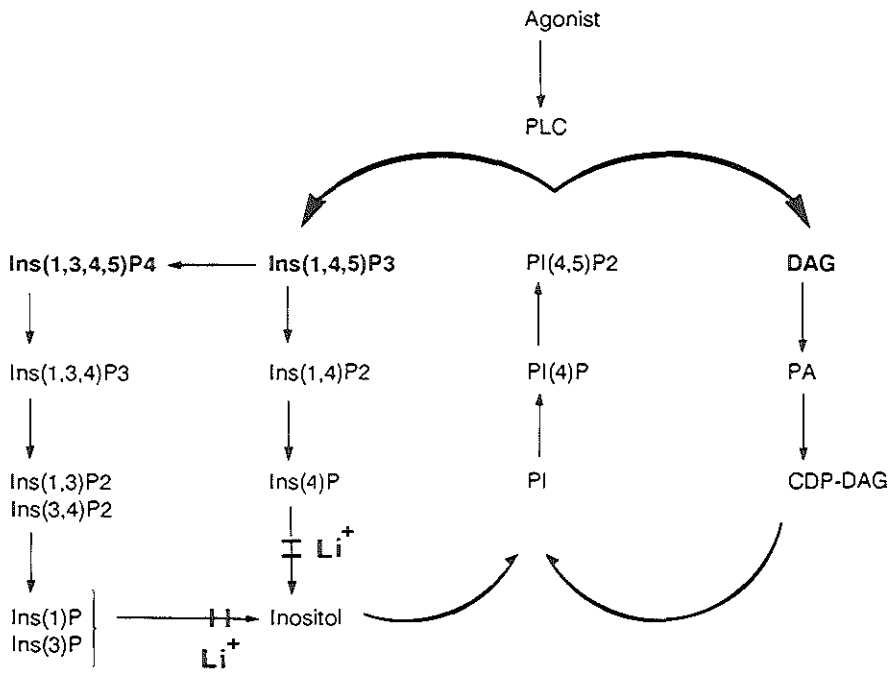

FIGURE 5. The phosphoinositide cycle. Binding of an agonist to its receptor activates phospholipase C, which catalyzes the hydrolysis of phosphatidylinositol (4,5) bisphosphate (PI(4,5)P2). The products diacylglycerol (DAG) and inositol (1,4,5) trisphosphate (Ins(1,4,5)P3) are further metabolized and finally combined to form phosphatidylinositol (PI). The biological active compounds are indicated by the fat appearance. The formation of inositol and thus the resynthesis of PI can be blocked by Li^+.

result of calcium-induced-calcium release [Berridge, 1990]. The frequency of the spikes in the IP3-induced calcium oscillations varies with agonist concentration, suggesting that calcium signalling might be frequency-encoded [Berridge, 1990].

The mechanism by which Ca^{2+} and IP3 interact to control calcium release is still unknown. Irvine has hypothized that the IP3 receptor contains both a IP3 binding site (cytosolic) and a calcium binding site (intraluminal) [Irvine, 1990]. Binding of Ca^{2+} to the receptor will change the affinity for IP3 and vice versa. A build up of cisternal calcium sensitizes the IP3 receptor to the endogenous level of IP3, leading to potentiation of calcium release in filled calcium pools. The intraluminal calcium concentration thus plays a major role in controlling the cytosolic calcium levels.

The mechanism of calcium entry is still controversial, but it is partly regulated by the intracellular calcium pools and by IP4. The IP3 receptor is proposed, in analogy with the ryanodine receptor, to play a role in the communication between the endoplasmic reticulum and the plasma membrane [Irvine, 1990]. The calcium binding site of the IP3 receptor could be important in transducing information about the filling of the intracellular calcium pool. Interaction of the IP3 receptor with the plasma membrane could occur by interaction with the IP4 receptor. The IP3 receptor interacts with the IP4 receptor when calcium and/or IP3 are bound. Binding of IP4 to its receptor causes dissociation from the IP3 receptor and induces calcium entry. Low intraluminal calcium will also result in dissociation of the IP3 from the IP4 receptor and thus stimulate calcium entry. This model [Irvine, 1990] is based on available knowledge and needs to be substantiated by further experimentation.

It should be noted that in spite of the known occurrence of IP3-provoked Ca^{2+} release [Van Corven et al., 1987] and secretagogue-operated Ca^{2+} channels [MacLeod et al., 1991] in intestinal epithelial cells (cf. Chapter 4), no detailed studies have been undertaken to define the role of IP4 in Ca^{2+} entry in enterocytes. Another intriguiing question pertinent to the enterocyte is the mechanism by which the vitamin D-dependent Ca^{2+}-uptake pathway in the duodenum [Nemere and Norman, 1991] is kept separate from the Ca^{2+} pool triggered by intestinal secretagogues.

3.3 Diacylglycerol

A well-defined pathway for generation of diacylglycerol (DAG) is initiated by the receptor-mediated stimulation of inositol phospholipid (PI) breakdown by a specific phospholipase C (PLC). In response to various calcium mobilizing

agonists, many cells produce DAG in a biphasic manner. The early transient phase peaks within 30 sec., whereas the delayed and quantitatively larger second phase reaches a maximum within 2-15 min. of stimulation and is long lasting [Billah and Anthes, 1990]. The initial rise in DAG temporally correlates with IP3 generation and its fatty acid composition (mainly 1-stearoyl-2-arachidonoyl-sn-glycerol) closely resembles that of PI. These observations indicate that the early DAG is formed by PLC action on PIP2. Considerable evidence now emerges that the sustained DAG formation is derived from phosphatidylcholine (PC) and not from PI: (i) the DAG mass is kinetically distinct from and exceeds the mass of InsPn; (ii) the fatty acid composition is similar to PC (rich in myristic acid) and (iii) certain agonist (e.g. EGF, interleukin 1 and 3 and p21 ras) induce a sustained increase in DAG without provoking PI hydrolysis [reviewed by Billah and Anthes, 1990; and by Exton, 1990].

The second messengers derived from PIP2 hydrolysis, calcium and DAG, act synergistically to stimulate PC breakdown, suggesting that initial increases in calcium and DAG by PLC action on PIP2 may be necessary for subsequent PC hydrolysis. However, sometimes PC hydrolysis preceded PI breakdown, suggesting that PC hydrolysis can couple to its own membrane receptor, independently from PIP2 hydrolysis. Several reports suggest the involvement of a G-protein. However, unequivocal proof for G-protein regulation of receptor-mediated PC hydrolysis must await appropriate reconstitution studies.

Other pathways to raise DAG levels are also described, such as de novo synthesis from glucose [Farese et al., 1987; Rossi et al., 1991], PDGF-stimulated acylation of monoacylglycerol [Hata et al., 1989]) and IGF-1-stimulated hydrolysis of glycosyl-PI [Kojima et al., 1990]. Often more than one DAG generating pathway is activated by an agonist, e.g. IGF-I stimulates PC hydrolysis, de novo synthesis of DAG and glycosyl-PI hydrolysis [Kojima et al., 1990].

The physiological relevance of these different DAG species is unclear. The distribution of PI and PC differs within the membrane, which could lead to compartimentalization of different DAG species within the cell and in that way elicit specific responses [Martinson et al., 1989]. Alternatively, the different species of DAG could be a mechanism to specifically activate one of the PKC isoforms. At present, it is not known whether these different DAG species are all capable of activating protein kinase C (PKC) in the intact cell. In vitro studies indicate that most classes of DAG are capable of activating purified forms of PKC [Go et al., 1987]. The sustained DAG generated by PC hydrolysis could be involved in cellular control mechanisms that require prolonged activation of PKC. These may

include the regulation of transcription and other events related to cell growth and differentiation. In contrast, PI-derived DAG could be responsible for mediating short-term effects of PKC, e.g. secretory processes.

In intestinal epithelial cells, large quantities of DAG are formed from monoglycerides and fatty acids during triglyceride resynthesis and chylomicron formation in duodenal and jejunal villous cells [Shiau, 1987]. Although it is likely that this pool of DAG is kept separate from the DAG involved in PKC activation, the mechanism underlying such a compartimentalization is still unknown.

3.4 Protein kinase C

Protein kinase C (PKC) is a calcium/phospholipid dependent serine/threonine kinase that is activated by diacylglycerol or phorbol esters. Its properties and characteristics are reviewed in Nishizuka, 1988; Parker et al., 1989; Ohno et al., 1990; Bell and Burns, 1991 and are discussed in the paragraph below. Phorbol esters can be used to activate PKC independent of receptor-mediated processes. PKC exists as a family of at least 8 genes, encoding 9 PKC isoforms: α, ß1, ß2, γ, δ, ϵ, ζ, η and θ, which can be subdivided in calcium dependent (α, ß1, ß2, γ) and calcium independent (δ, ϵ, ζ, η, θ) PKC's. All PKC isoforms have been cloned and characterized, except PKC θ which has not been fully sequenced yet. PKC consists of 4 conserved and 5 variable regions which form a regulatory (V1-C2) and a catalytic (C3-V5) domain. PKC ß1 and ß2 are splice variants of one gene and differ in their V5 region. The regulatory domain of PKC contains 1) the pseudosubstrate (V1/C1), an amino acid sequence that closely resembles PKC substrates; 2) one (PKC ζ) or two (all other PKC's) cysteine rich regions (C1) probably involved in DAG and phorbol ester binding (PKC ζ has lost its phorbol ester binding site and its response to DAG) and 3) the putative calcium binding domain (C2), which is only found in the calcium dependent PKC isozymes. The C-terminal catalytic domain of PKC contains the ATP binding site and the protein substrate binding sites. The catalytic domains can be separated from the regulatory domain by proteolysis, resulting in a constitutive active PKC fragment, PKM.

Activation of PKC is believed to occur through binding of diacylglycerol or other lipid activators (e.g. arachidonic acid), which causes dislocation of the pseudosubstrate from the active site by conformational changes. The binding of DAG greatly increases the affinity of PKC for calcium and phosphatidylserine, causing enzyme activation at resting calcium levels. Upon activation PKC shows a redistribution from the cytosol to the membrane and sometimes to the

cytoskeleton [Kiley and Jaken, 1990] or nucleus [Leach et al., 1989]. Calcium is thought to increase the potency and rate of action of phorbol ester by eliminating any lag-time required for recruitment of PKC to the membrane [May et al., 1985]. Sometimes, one or more PKC isotypes were already found in the particulate fraction of unstimulated cells, suggesting that PKC translocation is not a prerequisite for activation of the enzyme [Isakov et al., 1990; Chapter 5].

In general, PKC α, $\beta 1/\beta 2$ and γ have similar properties with respect to effector dependence and substrate specificity, although there are relative differences that are likely to affect cellular responses. In contrast, PKC ϵ has a clearly different substrate specificity than PKC α, β, γ: Histone IIIS a common in vitro substrate for PKC α, β, and γ was barely phosphorylated by PKC ϵ [Schaap et al., 1989]. The substrate specificity seems to be defined by the regulatory domain, since 1) the proteolytic fragment, which has lost the regulatory domain, can use Histone IIIS as a substrate [Schaap et al., 1990] and 2) a fusion protein containing the regulatory domain of PKC γ and the catalytic domain of PKC ϵ shows PKC γ defined substrate specificity [Parker, personal communication]. The properties of PKC δ, ζ and η are not defined yet.

Long-term treatment of cells with phorbol ester causes down-regulation of PKC. The mechanism is most likely proteolytic breakdown of PKC, since antibodies that recognize the cleavage site for the protease can prevent down-regulation [Young et al., 1988]. The sensitivity for down-regulation differs per isozyme and per cell type [Huang et al., 1989; Adams and Gullick, 1989]. Down-regulation of PKC is not only triggered by phorbol esters but is also observed in response to physiological stimuli. In GH4C1 cells, thyrotropin releasing hormone (TRH) induced translocation of PKC α, β and ϵ, but only PKC ϵ was down-regulated [Kiley et al., 1990]. The reason for this PKC ϵ specific down-regulation could be the prolonged activation of the calcium independent PKC ϵ by the sustained increase in PC-derived DAG which is formed without a rise in calcium. The physiological importance of PKC down-regulation is unknown.

Several inhibitors of PKC are known to exist: staurosporin, H7, sphingosine and pseudosubstrate peptides. However, these inhibitors cannot distinguish between the different PKC isotypes and the catalytic domain targeted inhibitors are not highly specific for PKC, but can also inhibit protein kinase A and G or CaCaM-kinase at somewhat higher concentration [staurosporin: Yanagihara et al., 1991]. Results obtained with these inhibitors in *in vivo* studies are therefore difficult to interpret. Furthermore, the effectiveness of the inhibitors in preventing PKC-mediated phosphorylation of intracellular substrates may depend on both the

nature of the substrates and, in the case of regulatory domain targeted inhibitors, on the relative participation of PKC versus its constitutively active proteolytic fragment PKM in the phosphorylation process [Junco et al., 1990].

3.5 Methods to study PKC isozyme specific effects

Several approaches are being used to establish the role of the individual PKC isoforms in the variety of PKC-mediated responses. PKC isotype specific differences in sensitivity to down-regulation can be used to study PKC isozyme specific effects. Using this approach, PKC α has been implicated in negative feedback of phosphoinositide hydrolysis [Huwiler et al., 1991; Chapter 5] and in the initial events of differentiation of a promonocytic cell [Strulovici et al., 1989], whereas PKC ε may play a role in the stimulation of prostaglandin synthesis [Huwiler et al., 1991]. However, the outcome of these studies based on correlations need to be confirmed by more definitive studies, such as reconstitution assays.

Another approach to study isozyme-specific effects is the generation of cell lines that stably overproduce one of the PKC isoforms. Until now, these studies were not very informative with regard to the specificity of the different isozymes of PKC. For example, overexpression of PKC β1 was found to enhance tumorigenicity [Housey et al., 1988], to have no effect on the cell morphology [Eldar et al., 1990] or to inhibit growth [Choi et al., 1990]. Although these studies strongly indicate a role for PKC β1 in the control of cellular growth mechanisms, the effect of PKC β1 on the cell morphology seemed to be dependent on the cell line used, even between different fibroblast cell lines. Therefore, a comparison within one cell line overexpressing each of the different PKC isotypes is necessary to establish whether the various isotypes have specialized functions. Such an approach showed a functional difference *in vivo* between PKC α, γ and ε in a rat fibroblast cell line: cells overexpressing PKC γ failed to enhance the expression of c-jun in contrast to cells overproducing PKC α or ε [Ohno et al., 1990]. Nevertheless, PKC γ was able to mediate gene expression in a rat neuroblastoma cell line [Bernards, 1991], indicating that PKC isoforms can exhibit different functions probably dependent on the cell line in which they are expressed.

Functional differences between PKC isoforms can also be studied by using permeabilized cells. In that way, exocytosis in pituitary cells was found to be induced by introduction of PKC α or PKC β, but not by PKC γ [Naor et al., 1989], again indicating that some specificity exists. An alternative approach will be microinjection of (not yet available) inhibitory PKC isozyme specific antibodies,

which will interfere with those physiological responses in which a particular PKC subtype is involved. Interestingly, transfection of a rat neuroblastoma cell line with the oncogene N-myc induced a shift in PKC isozyme expression from PKC δ to PKC ζ and resistence to phorbol ester [Bernards, 1991]. An alteration of PKC isoform expression was also found in H-ras transfected fibroblasts [Borner et al., 1990]. These results suggest that alterations in PKC isoform expression may be a common event in cell transformation, and could provide new information on the role of the individual PKC isoforms.

In summary, it should be clear that much further work is needed to define the exact physiological role of the individual PKC family members in cellular regulation. However, the differential expression of PKC isozymes in various tissues, the expression of more PKC isotypes in one cell and the occurrence of many DAG subtypes together with the results of the studies described in the previous paragraphs are suggestive for the existence of specific roles for PKC isozymes in eliciting a biological response.

4.0 The cAMP dependent pathway

Many hormones and neurotransmitters, e.g. vasoactive intestinal polypeptide (VIP) and prostaglandin E2 (PGE2) in the intestine act by raising cAMP levels (Fig.3). Activation of the receptor by binding of the agonist stimulates a G-protein, Gs, which activates adenylate cyclase. The resulting increase in cAMP activates a cAMP dependent serine/threonine protein kinase: protein kinase A (PKA).

The structure and properties of both adenylate cyclase and protein kinase A has been extensively reviewed of which a short overview is given below [Edelman et al., 1987; Taylor, 1989; De Jonge and Rao, 1990].

Cholera toxin is (mis)using this pathway by irreversibly activating Gs. ADP-ribosylation of its α subunit is keeping it in the active GTP bound form, resulting in permanently high cAMP levels and thus excessive salt and water secretion in the intestine.

4.1 Adenylate cyclase

Adenylate cyclase (AC) is a transmembrane glycoprotein, that catalyzes the synthesis of cAMP from adenosine triphosphate (ATP) on the cytoplasmic side of the plasma membrane. Genetic and biochemical evidence indicates the existence of multiple forms of AC. The amino-acid sequence of a hormone sensitive AC predicted two alternating sets of hydrophobic and hydrophylic

domains [Krupinski et al., 1989]. The hydrophobic domains each contain six transmembrane spans. The hydrophylic domains contain a sequence homologous to a single cytoplasmic domain of several guanylate cyclases, which are proposed nucleotide binding sites. AC was found to resemble a transporter or ion channel, related to the G-protein regulated dihydropyridine (DHP)-sensitive Ca^{2+} channels and K^+ channels. This relationship, although not based on primary amino-acid sequence, together with the possible multiple nucleotide binding sites in AC, suggest an additional role for the enzyme as a transporter. The most intriguing hypothesis is that AC can synthesize and transport cAMP. Such a function, although not yet observed in mammalian cells, has been described to occur in the slime mold Dictyostelium discoideum [Gerisch, 1987]. AC can be activated independently of the receptor by forskolin. Another tool to study effects of an increase in cAMP levels, without activation of receptor-mediated responses, is the use of cell-permeable analogues of cAMP, e.g. 8-Br-cAMP or 8-pCPT-cAMP [Sandberg et., 1991]. A characteristic feature of AC in intestinal epithelium is its distinct localization in the basolateral membrane together with the AC-linked hormone receptors [De Jonge and Hülsmann, 1974; Murer et al, 1976]. In contrast, the receptors exposed to cholera toxin *in vivo* are localized at the luminal membrane. In addition, the *in vitro* AC response to Ca^{2+} is biphasic, being activated in a calmodulin dependent manner by submicromolar Ca^{2+} [Lazo et al., 1984] and inhibited at higher concentrations. However, this potentially important link between Ca^{2+}-mobilizing secretagogues and those that regulate cAMP has been questioned more recently considering the lack of compelling evidence for calmodulin regulation of AC in non-neuronal tissues [Cooper and Caldwell, 1988]. Moreover, the inability of Ca^{2+}-ionophore, in contrast to forskolin or 8-Br-cAMP, to provoke Cl^- secretion in some human colonic cell lines (HT29cl.19A, Chapter 5) also argues against cross-talk between Ca^{2+} and cAMP in this cell model.

4.2 Protein kinase A

Protein kinase A (PKA) is composed of two regulatory (R) and two catalytic (C) subunits, R2C2. Binding of cAMP to the R subunits of PKA results in the dissociation of the R and C subunits to form R2cAMP4 and two active C subunits. The C subunit is responsible for the phosphorylation of its specific target proteins. Mg-ATP is the preferred substrate of PKA. The catalytic domain is comprised of a nucleotide and a peptide binding region. The most highly conserved regions in the protein kinases are the two nucleotide binding domains, containing the consensus sequence Gly-X-Gly-X-X-Gly. The C subunit is

myristylated. However, the role of this modification is not clear, as it is not likely to be involved in membrane association of PKA.

There are two major forms of mammalian PKA, type I and type II, which differ in their R subunits. RI and RII are products of separate genes, which differ in their tissue distribution and in their specificity for various cAMP analogues. The role of these isoforms of PKA in the biological function is still not known. Each subunit of PKA is known to be phosphorylated at multiple sites. Autophosphorylation of the RII subunit on Ser-95 was found to influence the functioning of PKA by reducing the rate of reassociation of the RII and the C subunits. The importance of autophosphorylation on the other sites is not elucidated yet.

A naturally occurring inhibitor of PKA has been detected, called PK-I or Walsh inhibitor. Inhibition of PKA is mediated by the pseudosubstrate sequence present on PK-I. Other inhibitors of PKA are derivatives of isoquinoline sulfonamide, H7 and H8. However, H7 and H8 are not specific for PKA, but are also inhibiting PKG and H7 is also inhibiting PKC. PK-I is relatively specific for PKA, but at concentrations higher than 100 μM, PKG is also inhibited.

The expression of both the type I and type II isoform of PKA in rat enterocytes have been demonstrated by photoaffinity labeling and immunological experiments [De Jonge and Lohman, 1985]. The preferential localization of type II PKA in the apical membrane suggests a major role of this isozyme in cAMP regulation of intestinal Cl^- secretion.

5.0 The cGMP dependent pathway

Agonists raising cGMP (e.g. nitroprusside and atrial natriuretic peptide, ANP) do not seem to activate guanylate cyclase via a G-protein, but through direct interaction with a receptor domain of cytosolic or membranal forms of guanylate cyclase, respectively [reviewed by Garbers, 1991]. The resulting rise in cGMP activates a cGMP dependent serine/threonine protein kinase (PKG) or interacts with a cGMP-inhibited or cGMP-stimulated-phosphodiesterase [Nicholson et al., 1991].

5.1 Guanylate cyclase

Guanylate cyclase (GC) catalyzes the synthesis of cGMP from GTP at the intracellular side of the plasma membrane. Two types of GC can be found, a soluble and a particulate form. The soluble enzyme is a heterodimer composed of an α and ß subunit. Both subunits contain a cyclase-like catalytic region, but

only the αß subunit complex has GC activity. The soluble GC is activated by nitric oxide or EDRF. This isoenzyme of GC is ubiquitously expressed in mammalian tissues. However, the soluble GC is absent in intestinal epithelium [De Jonge, 1975], excluding a direct role for EDRF/NO-compounds as regulators of intestinal Cl^- secretion.

The particulate GC contains one transmembrane domain. The extracellular region serves as the ligand binding domain. The intracellular region contains a protein kinase like domain and a cyclase catalytic domain. Three isoforms of the particulate GC has been identified, GC-A, GC-B and GC-C, bearing receptor domains for ANP, CNP (C-type natriuretic peptide) and the heat stable toxin of E.coli (ST_A), respectively. ANP is principally synthetized in the heart, but distributed to many target tissues, whereas CNP is restricted to the brain and nervous tissue. This limited localization of CNP is in contrast with the more ubiquitous distributed GC-B receptor, suggesting that CNP might not be the (sole) agonist for GC-B. Thusfar, however, no other ligands for the GC-B receptor have been found.

The identity of GC-C as the receptor for ST_A in intestinal epithelium has been recently demonstrated by molecular cloning [Schultz et al., 1990]. No evidence for ST_A-stimulated GC activity in other tissue except opossum kidney [Garbers, 1991] has been reported sofar. ST_A is a low molecular weight (18 amino acids), heat stable peptide secreted by pathogenic strains of Escherichia coli upon colonization of the intestinal tract. Together with the heat labile, cholera toxin-like enterotoxin it constitutes a major cause of diarrhoeal disease among children in developing countries [Guerrant, 1985]. Very recently an endogenous peptide, designated guanylin, mimicking the action of ST_A on GC has been discovered [Currie et al., 1992]. Interestingly, the ANP-sensitive form of GC, GC-A, is expressed mainly in subepithelial cell types in the intestine, but not or to a very low extent in the Cl^- secreting epithelial cells, implying that ANP-provoked chloride secretion results from the release of an endogenous secretagogue/neurotransmitter in the submucosa rather than from a direct effect of ANP on mucosal cGMP levels [Vaandrager et al., 1992].

5.2 Protein kinase G

Protein kinase G (PKG) is found in a soluble form (type I) and a membrane associated form (type II). The soluble form is a dimer of two identical subunits, of which two subspecies exist: Iα and Iß, probably formed by alternative splicing of a single gene. The distribution of these two isoforms differ: Iα is chiefly

found in the lung, Iß in smooth muscle and cerebellum. The α and ß forms also differ in affinities for several cGMP analogues. Binding of cGMP to PKG causes a conformational change, activating the enzyme by exposing the catalytic domains, rather than by dissociation of the subunits as found for PKA. Each monomer contains two cGMP binding sites (four per holoenzyme). The membrane bound form of PKG (type II) is monomeric and seems to be unique to the intestine. The amount of PKG-II decreases from the villus tip to the crypt. Autophosphorylation of PKG-I causes a 10-fold decrease in the Ka for cAMP. Thus, in vivo autophosphorylation might allow PKG-I to be activated by cAMP as well as by cGMP. The role of autophosphorylation in PKG-II function has not been established unequivocally. However, measurements of PKA versus PKG-II activation in rat intestinal brush border membranes following *in situ* exposure to either CT or ST_A virtually rule out the possibility that ST_A/cGMP is acting through PKA or that CT/cAMP is acting through PKG-II [Van Dommelen and De Jonge, 1986].

Naturally occurring inhibitors of PKG have not been identified. However, H7 and H8 are known inhibitors of both PKA and PKG (H8 being more specific for PKG). Intracellular effects of cGMP can be mimicked by using cGMP analogues, such as 8-Br-cGMP, without the need for receptor activation.

6.0 Role of PKA, PKG, PKC and calcium in intestinal chloride secretion.

A physiological role of PKA in Cl^- secretion was demonstrated by the finding that transfection of T84 cells with a mutant form of the regulatory subunit of PKA resulted in an inability to open Cl^- channels, despite an increase in cAMP levels [Rogers et al., 1990].

The intestine is the sole tissue in which cGMP-mediated Cl^- secretion occurs, presumably due to the unique expression of a different type of PKG (PKG-II) in this organ. Two lines of evidence have suggested a role for PKG-induced phosphorylation in Cl^- secretion. First, Ussing chamber measurements of intestinal stripped mucosa obtained from cystic fibrosis patients have demonstrated a defect in cGMP-induced chloride secretion, distal to PKG activation [De Jonge et al., 1987]. Secondly, an independent role for PKG was strongly suggested by the finding that ST_A- and 8-Br-cGMP-, but not cAMP- or Ca^{2+}-provoked Cl^- secretion could be inhibited by relatively low concentrations of the PK inhibitors H8 and staurosporin [De Jonge, unpublished results]. Nevertheless, PKG has been demonstrated to be a more potent inhibitor of sodium absorption (villus function) than a stimulator of chloride secretion (crypt function) in agreement with the

decreasing concentration gradient of PKG from villus to crypt.

The physiological role of PKC in intestinal ion transport has been deduced mainly from studies using phorbol esters. In rabbit and chicken ileum and in rat distal colon, the stimulatory effects of phorbol esters were found to be mediated by release of prostaglandins from sub-epithelial cells with subsequent cAMP elevation [Rao and De Jonge, 1990]. However, by using epithelial cell lines, PKC was also found to have a direct effect on Cl^- secretion: PKC stimulated and inhibited Cl^- secretion in the human colon carcinoma cell line HT29 and in cultures of tracheal epithelial cells [Chapter 5; Li et al., 1989]. Furthermore, a large prostaglandin-independent component of phorbol ester-provoked Cl^- secretion has been shown to exist in human ileal mucosa [De Jonge, manuscript in preparation]. The target protein for inhibition of Cl^- secretion is not necessarily the Cl^- channel itself, but can also be one or more of the other transport systems needed to induce Cl^- secretion. Indeed, PKC was found to inhibit $NaKCl_2$ cotransport in HT29 human colonic cells [Franklin et al., 1989].

An increase in calcium by Ca^{2+}-ionophore has been shown to stimulate Cl^- secretion both in intact epithelium and in epithelial cell lines. In two human colon carcinoma cell lines, T84 and HT29cl.19A, the Ca^{2+}-activated Cl^- conductance differed from that stimulated by cAMP in both anion selectivity and current-voltage relation [Cliff and Frizzell, 1990; Vaandrager et al., 1991]. In the HT29cl.19A cells, the distribution of the cAMP- and Ca^{2+}-activated Cl^- channels also differed: Ca^{2+} stimulated Cl^- efflux from both the apical and the basolateral membrane, whereas cAMP only stimulated apical Cl^- efflux. This difference in polarization might be the basis for the lack of Ca^{2+}-stimulated net Cl^- secretion in this particular cell line [Vaandrager et al., 1991; Chapter 5]. As calcium is a known activator of a variety of enzymes, e.g. calcium/calmodulin dependent protein kinases (CaCaMK) and phospholipases, the effects of Ca^{2+} could be either direct or mediated through activation of these Ca^{2+}-activated enzymes. In the T84 cells, Ca^{2+}-stimulated Cl^- secretion was mediated by CaCaMK-II [Worrell and Frizzell, 1991]. In this cell line, however, the role of CaCaMK-II-stimulated Cl^- channels in allowing net Cl^- secretion has been seriously questioned [Anderson and Welsh, 1991]. In human small intestine, circumstantial evidence exists for the coupling of Ca^{2+}, through CaCaMK-I, to a different type of Cl^- channel, i.e. CFTR [De Jonge, unpublished results]. Since CaCaMK-I is expressed in intestine, but not in most other epithelia [Rao and De Jonge, 1990], this might explain why Ca^{2+}-mediated Cl^- secretion is defective in CF ileum but not in trachea or sweat gland [Quinton, 1990].

7.0 Candidate target proteins for the induction of chloride secretion.

Although abundant evidence has indicated a role for PKA, PKG, calcium and PKC in the modulation of ion transport, the protein kinase substrates involved in ion transport regulation are not established yet. The targets of the protein kinases could be the ion channel or transporter itself, or regulatory proteins of these transport proteins. As cystic fibrosis is associated with a defect in epithelial Cl^- transport, the mutated gene product responsible for this defect could be the target for PKA, PKG and PKC, and in the intestine also for CaCaMK. The identification of this gene on chromosome 7 two years ago [Rommens et al., 1989; Riordan et al., 1989; Kerem et al., 1989] provided the tools to test this possibility. The gene product is called cystic fibrosis transmembrane conductance regulator or CFTR. Mutations in this gene were associated with cystic fibrosis. CFTR expression in the intestine decreases from crypt to villus, consistent with CFTR being responsible for Cl^- secretion in intestinal crypts [Trezise and Buchwald, 1991].

7.1 CFTR

Amino acid seqence analysis suggested that CFTR consists of two membrane spanning domains, each composed of six transmembrane regions; a R domain, which contains many potential consensus phosphorylation sites; and two nucleotide-binding folds or NBF's. Expression of CFTR induces cAMP-activated Cl^- channels in CF epithelial cells [Rich et al., 1990; Drumm et al., 1990] and in a variety of cell types normally lacking such channels [Anderson et al., 1991b; Kartner et al., 1991; Berger et al., 1991], suggesting that CFTR encodes a cAMP-activated Cl^- channel. However, these studies do not exclude the possibility that CFTR encodes a regulator of this channel, requiring that these cells have endogenous cryptic Cl^- channels stimulated by cAMP only in the presence of CFTR. The first option, CFTR encodes a Cl^- channel, has gained support by the observation that mutations in the proposed membrane spanning domains altered the anion selectivity of this CFTR expression-induced Cl^- channel [Anderson et al., 1991a]. Very recently, conclusive evidence for CFTR being a Cl^- channel was reached by the succesfull reconstitution of purified recombinant CFTR protein into proteoliposomes and the appearance of a cAMP/PKA regulated Cl^- channel upon fusion with planar lipid bilayers [Bear et al., 1992].

The presence of multiple consensus sequences for PKA, PKC and PKG [Kemp and Pearson, 1990] on the R domain of CFTR suggests that regulation of Cl^- secretion may occur by phosphorylation of CFTR. PKA-induced

phosphorylation of CFTR has indeed been found to regulate this Cl^- channel. Addition of the catalytic subunit of PKA to the cytosolic surface of excised patches activated the CFTR Cl^- channel [Berger et al., 1991; Tabcharani et al., 1991]. Addition of PKC also activated the CFTR channel, but this effect was small compared to the effect of cAMP. Interestingly, PKC potentiated the effect of cAMP (rate and magnitude) [Tabcharani et al., 1991]. Seven serines in the R domain were substrates for PKA *in vitro* and phosphorylation on four of these residues also occurred *in vivo* after an increase in cAMP levels [Cheng et al., 1991]. Replacement of these four serines (but not three or less) by alanine prevented cAMP dependent activation of the CFTR channel [Cheng et al., 1991]. A CFTR mutant lacking most part of the R domain had lost its regulation by PKA and generated Cl^- channels that were constitutively active even without an increase in cAMP [Rich et al., 1991]. These studies indicate an important role for the R domain in regulation of the CFTR channel. Nevertheless, most mutations in CFTR leading to cystic fibrosis are not in this R domain, but are in the nucleotide binding fold (NBF), particularly NBF1 [Kerem et al., 1989]. Recent studies in our laboratory have shown that purified PKG-II, but not PKG-I, is also able to activate the CFTR channel in excised patches of CFTR transfected epithelial cells [Bijman and De Jonge, in preparation]. Phosphorylation studies are in progress to learn whether CFTR itself or a regulatory protein in the same membrane is the target for PKG-II-catalyzed phosphorylation.

The most common mutation in the CF gene (75%) is a deletion of phenylalanine at position 508 in the NBF1 (ΔF508). This and other mutations in the NBF's prevent glycoprotein maturation, which potentially leads to defective membrane delivery of CFTR [Cheng et al., 1990; Gregory et al., 1990]. Those NBF mutants that were processed normally still failed to generate cAMP activated Cl^- channels [Gregory et al., 1990]. Despite the maturation defect found in ΔF508-CFTR mutants, some of these mutated molecules, although far less efficient than wild-type CFTR, still reached the plasma membrane [Dalemans et al., 1991]. Analysis of these mutants in the plasma membrane revealed a reduced activity of the cAMP-dependent Cl^- channel, due to an increase in closing time [Dalemans et al., 1991; Drumm et al., 1991]. The result of the ΔF508 mutation thus has two major consequences: 1) it prevents the maturation and normal subcellular localization of the protein in the plasma membrane and 2) those mutants that do reach the plasma membrane generate Cl^- channels with a reduced activity.

CFTR shows structural similarity with several members of the "ATP binding cassette" (ABC) superfamily of proteins, including the P-glycoprotein associated

with multidrug resistance. Several members of this family utilize the energy released during ATP hydrolysis to actively transport its substrate across the cell membrane. In analogy with these proteins, CFTR could also work as an energy driven pump for yet unidentified substrates. Alternatively, the NBF's could serve as regulators of CFTR function, rather than drive a pump. Recently, a regulatory role for ATP in CFTR function has been reported [Anderson et al. 1991]. Phosphorylation of CFTR by PKA was needed, but not sufficient to open the Cl^- channel. The phosphorylated CFTR additionally required ATP or its hydrolyzable analogues. However, the EC_{50} for ATP-induced Cl^- channel activity (± 300 μM) was much higher than that for most other nucleotide-binding proteins, such as PKA (3 μM), G-proteins (10 pM-100 μM) and Na^+/K^+-ATPases (0.2-2 μM), raising the possibility that CFTR might function as an energy sensor. By responding to cellular ATP levels, the Cl^- secretion rate is adjusted to the metabolic energy needed to perform transepithelial Cl^- transport. Nevertheless, these studies do not exclude the possibility that CFTR functions both as a Cl^- channel and as a pump for yet unidentified substrates. This possibility has gained interest by the recent finding that one of the members of the ABC family, MDR1, can also function as a volume-regulated Cl^- channel [Valverde et al., 1992]. The drug pump function and the Cl^- channel activity of MDR1 differed in that the drug pumping required hydrolyzable ATP in contrast to activation of the Cl^- channel. For CFTR such a difference is not likely, as ATP hydrolysis is apparantly needed for Cl^- channel opening.

Scope of this thesis

In this thesis the following aspects of ion transport regulation and transmembrane signalling in intestinal epithelial cells were studied:

1) the polarized distribution of G-protein subunits among the basolateral and the apical membrane, with emphasis on the possible occurrence of novel, epithelium-specific G-protein subspecies in the apical membrane that could play a role in the activation of the apical PI-cycle and/or apical Cl^- channels. The results of this study are described in Chapter 2.

2) the possible identification of apical Cl^- channels that are directly activated by G-proteins, in analogy to G-protein regulated Na^+ and Cl^- channels in the kidney [Light et al., 1989; Schwiebert et al., 1990] and cardiac Ca^{2+} and K^+ channels [Yatani et al., 1987; Yatani et al, 1988]. The results of this study are described in Chapter 3.

3) the possible existence of cross-talk between cyclic nucleotide-mediated

pathways and the PI-cycle. The apical PI-cycle could function as an amplification mechanism for neurohormonal signals generated at the basolateral membrane (e.g. cAMP, PKC, Ca^{2+}), or as a transmembrane signalling mechanism triggered by apical enterotoxin receptors (e.g. heat-stable E.coli toxin). The results of this study are summarized in Chapter 4.

4) the identification of protein kinase C isozymes in intestinal epithelial cells and the analysis of their possible role in ion transport regulation. The results of this study can be found in Chapter 5.

5) the identification of the targets for PKC-mediated effects on ion transport. These results are documented in Chapter 6.

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CHAPTER 2

ASYMMETRICAL DISTRIBUTION OF G-PROTEINS AMONG THE APICAL AND THE BASOLATERAL MEMBRANE OF RAT ENTEROCYTES.

The distribution of the α and ß subunits of guanosine nucleotide binding proteins (G-proteins) among the apical (BBM) and basolateral membrane (BLM) of polarized rat enterocytes was investigated by *in vitro* ADP-ribosylation assays and immunoblotting with G-protein subunit specific antisera. The enterocytes were found to express αi2, αi3, αs and ß-subunits, whereas αi1 and αo subunits could not be detected. The αi2 and the αi3 subunits were located predominantly in the basolateral membrane, in contrast to the αs and the ß subunits, which were distributed uniformly among both membranes. Furthermore, a 39 kDa and a 78 kDa protein, recognized by αi1,2 in contrast to αi1 and αi3 specific antisera but resistant to ADP-ribosylation by pertussis toxin, were localized exclusively at the apical border. These Gi-related proteins might represent novel members of the G-protein family. Activation of apical G-proteins by GTP or its analogs failed to release the αs, αi and ß subunits or the 39 kDa and 78 kDa αi-like proteins from the membrane, suggesting a functional role for these proteins in the apical membrane itself. Our recent finding of a GTP[S]-sensitive Cl^- conductance in the apical membrane of rat enterocytes suggests that one or more of these G-proteins may act as local regulators of specific apical transport functions.

INTRODUCTION

Guanine nucleotide binding regulatory proteins (G-proteins) are a family of receptor-coupled signal transducing proteins that regulate a variety of second messenger systems and ion channels. G-proteins are composed of three subunits designated α, ß and γ, with Mr of approximately 39-52 kDa, 35-36 kDa and 8-11 kDa, respectively. The α subunit of each G protein is unique, the ß and γ subunits

appear to be shared. The α subunit contains the functional domains for guanine nucleotide binding, GTPase activity, ADP-ribosylation by cholera and/or pertussis toxin, and interaction with specific receptor and effector proteins [1]. For some G-proteins multiple isoforms have been described, caused by alternative splicing of the same gene (e.g. Gs, 4 isotypes [2]) or by the existence of multiple genes, encoding for similar G-proteins, which form a subfamily (e.g. Gi1, Gi2 and Gi3) [3]. The biological importance of this diversity is as yet not fully understood, since all splice variants of Gs can both activate adenylate cyclase and activate Ca^{2+}-channels [2], and all Gi species can activate the G-protein gated atrial K^+-channel [4]. However, the inhibition of adenylate cyclase in NG108-15 cells was specifically transduced by Gi2 [5].

It has been generally accepted that G-proteins function mainly at cell surface membranes where they can associate with various transmembrane receptors and effector proteins. Therefore it is very likely that they are distributed similarly to the receptor/effector systems to which they are specifically coupled. In enterocytes, similar to other epithelial cell types, the plasma membrane is composed of at least two structurally and functionally distinct regions: the apical or brush border membrane and the basolateral membrane. Most hormone receptors and adenylate cyclase are distributed asymmetrically and are found to be localized predominantly at the basolateral membrane [6,7]. In contrast, the apical membrane of rat enterocytes was found to be enriched in components of the cGMP-signalling system [8,9], a G-protein-activated phosphoinositide cycle [7] and GTP[S]-sensitive Cl^--channels [10,11]. These findings prompted us to explore the possibility that at least some G-proteins might have an apical localization and might regulate brush border specific functions.

Our results indicate that some G-protein subunits (αs, ß) are distributed equally among the apical and the basolateral membrane, whereas others are segregated predominantly to the basolateral membrane (αi2, αi3) or are localized exclusively in the apical membrane (a 39 kDa and a 78 kDa αi-like protein). The potential biological implications of these findings are discussed.

MATERIALS AND METHODS

materials: Antiserum D43 (kindly provided by L. van der Voorn, Netherlands Cancer Institute, Amsterdam, The Netherlands) was raised in rabbits against a mixture of holomeric G-proteins purified from human brain and contained antibodies specifically recognizing the ß-subunits and the α-subunit of Go on western blots. The following antisera were kindly donated by Dr. G. Milligan, Glasgow [specificities and sensitivities are summarized in ref. 12 and 35]: antiserum IM1 was raised

in rabbits against the peptide NLKEDGISAAKDVK, corresponding to amino acids 22-35 of the α subunit of Go and specifically recognizes αo; antiserum SG1, produced in rabbits against a decapeptide (KENLKDCGLF) corresponding to the C-terminal amino-acid sequence of the α-subunit of transducin, specifically recognizes the αi1 and the αi2 subunits, but not the αi3 subunit; antiserum 13B was produced in rabbits against a peptide KNNLKECGLY corresponding to the C-terminal decapeptide of the α-subunit of the inhibitory G-protein Gi3 and specifically recognizes αi3, but not αi1 and αi2 subunits; antiserum I1C, specific for the αi1 subunit and not recognizing the αi2 and the αi3 subunits, was raised against the synthetic peptide LDRIAQPNYI, corresponding to amino acids 159-168 of the α subunit of the inhibitory G-protein, Gi1; antiserum CS1, produced in rabbits against a decapeptide (RMHLRQYELL) corresponding to the C-terminal amino-acid sequence of the α-subunit of the stimulatory G-protein and specifically recognizes the αs subunits;
Antiserum AS/7 (obtained from Du Pont), similar to antiserum SG1, was raised in rabbits against the decapeptide KENLKDCGLF and specifically recognizes the α subunit of transducin, Gi1 and Gi2.
^{32}P-NAD (specific activity 30 Ci/mmol) was obtained from New England Nuclear, Frankfurt, Germany; pertussis toxin was from List Biological Laboratories, Campbell, CA, USA; cholera toxin was obtained from Sigma; ^{14}C-labeled rainbow ^{TM} protein molecular weight markers were from Amersham, UK.

Preparation of basolateral membranes: Under light diethyl ether anaesthesia the small intestine of a rat was removed and rinsed twice with 20 ml of icecold saline (0,9 % NaCl). All further steps were performed at 0-4 °C. Inside out segments of rat jejunum were mounted on metal rods attached to a vibration apparatus (Vibro-Mixer E1 from Chemap A.G. Hännedorf ZN, Switzerland). Epithelial cells were released by mechanical vibration (50 Hz, amplitude 1.5 mm) for 30 minutes in 0.13 M NaCl, 5 mM EDTA, 10 mM Tris, pH 7.4 and isolated by low speed centrifugation at 345 g. The cells were washed twice in 20 ml 300 mM mannitol, 12 mM Tris-HCl, pH 7.4. Basolateral membranes were prepared essentially as described by Murer et al. [6]. Briefly, cells were homogenized in a Virtis blender and nuclei, cell debris and mitochondria were removed by differential centrifugation. The remaining membranes were pelleted and basolateral membranes were separated from apical membranes by sucrose gradient centrifugation. The basolateral membranes were recovered between 30-40% sucrose, pelleted and finally resuspended in 100 mM mannitol; 10 mM Hepes-Tris, pH 7.4. The membranes were freshly used or were frozen in liquid nitrogen and stored at -80°C.

Preparation of apical membranes: brush border caps and brush border membrane vesicles: Intact brush border (BB) caps from small intestinal epithelial cells were isolated by vibration of inside out gut segments in hypotonic EDTA (2.5 mM) as described by De Jonge [13] and finally resuspended in 20 mM Tris-HCl, pH 7.3. Brush border membrane vesicles (BBMV), hereafter referred to as KSCN-BBMV were prepared by exposing the brush border caps to a chaotropic agent (0.52 M KSCN) as described in detail by Hopfer et al. [14]. Vesicles prepared by this method are virtually depleted of cytoskeletal proteins and calmodulin [14,15]. Brush border membrane vesicles hereafter referred to as Mg-BBMV were prepared from isolated enterocytes by mechanical homogenization and differential Mg^{2+} precipitation as described [16]. The brush border membranes do not aggregate in the presence of divalent cations [17] in contrast to all other subcellular membranes.

ADP-ribosylation of membranes: Cholera toxin and pertussis toxin were activated by incubation

membrane fractions. As judged by the marker enzyme distribution, the basolateral membranes were relatively devoid of brush border membrane contamination and vice versa.

Identification of G_α-subunits by pertussis and cholera toxin catalyzed ADP-ribosylation. G-proteins can be distinguished by the ability to serve as a substrate for pertussis toxin (PT) catalyzed ADP-ribosylation (Gi, Go), cholera toxin (CT) catalyzed ADP-ribosylation (Gs), PT and CT catalyzed ADP-ribosylation (transducin) or by a lack of recognition sites for any of the toxins. These properties were exploited to identify G-protein species in the enterocyte. Incubating BB caps, KSCN-BBMV, Mg-BBMV and BLM with ^{32}P-NAD and PT revealed the presence of a 41 kDa protein in all membrane fractions (Fig.1). This 41 kDa protein was tentatively identified as the α subunit of Gi, since the other PT substrate transducin is uniquely present in rods and cones and αo specific antiserum failed to detect αo subunits in these membrane fractions (Fig.3D). CT catalyzed ADP-ribosylation enabled the detection of two other proteins with Mr of 46 and 42 kDa, tentatively identified as splice variants of the α subunits of Gs (Fig.2) [3], which were likewise present in all membrane fractions. These subunits sometimes appeared as doublets, possibly as a result of covalent modifications.

Identification of G-protein subunits by specific antisera. A variety of α and ß subunit specific antisera were used to further identify the different G-proteins. Antiserum 13B, specific for the αi3 subunit, identified a protein with Mr of 41 kDa predominantly in the basolateral membrane, although a faint staining could be detected in the apical membrane fractions (Fig.3A). Antiserum SG1, which specifically recognizes both αi1 and αi2 subunits, also showed a substantial enrichment of a 41 kDa protein in the basolateral membrane compared to the apical membrane (Fig.3B). Since antiserum I1C, specific for the αi1 subunit, did not react with a 41 kDa protein in any of the membrane fractions (results not shown), the protein recognized by antiserum SG1 is tentatively identified as the αi2 subunit. As indicated by combined ^{32}P-NAD labeling and immunoblotting, the PT sensitive 41 kDa protein shown in Fig.1 comigrated with the proteins identified by antisera SG1 (Fig 4A,B) and 13B (not shown) and with purified G-proteins from human and bovine brain (Fig.5,6), further confirming their identity as αi subunits. Antiserum SG1 additionally recognized a 39 kDa protein, which was detected only in the BB caps and occasionally in KSCN-BBMV, but not in the Mg-BBMV or the BLM (Fig.3B) and was clearly distinguishable from the 41 kDa band (Fig.4B,5). In

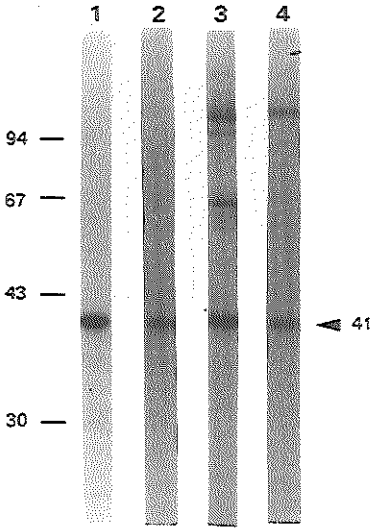

FIGURE 1. Pertussis toxin dependent ADP-ribosylation of intestinal epithelial membrane proteins. Autoradiographic profiles of ^{32}P-labeled proteins (10 μg/lane) after incubation of BLM (lane 1), Mg-BBMV (lane 2), BB caps (lane 3) and KSCN-BBMV (lane 4) with ^{32}P-NAD, preactivated pertussis toxin and 0.1 % saponin. Experimental details of the ADP-ribosylation assay are as in Methods. Migration of molecular standards expressed in kDa is as indicated. The ADP-ribosylation of the 41 kDa protein was observed only in the presence of PT (not shown). The results are representative for at least three other experiments.

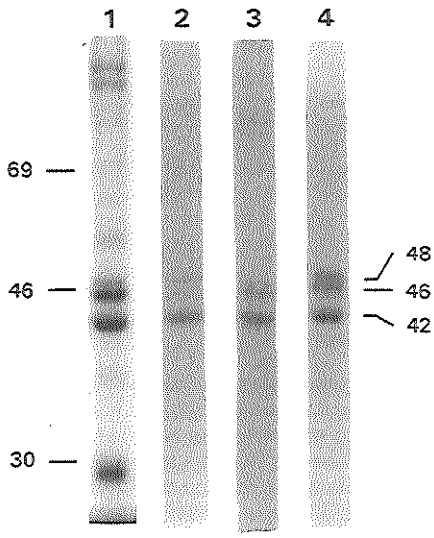

FIGURE 2. Cholera toxin dependent ADP-ribosylation of intestinal epithelial membrane proteins. Autoradiographic profiles of ^{32}P-labeled proteins (10 μg/lane) after incubation with ^{32}P-NAD, preactivated cholera toxin and 0.1 % saponin of BLM (lane 1) and Mg-BBMV (lane 2), after incubation with ^{32}P-NAD and preactivated cholera toxin of BB caps (lane 3) and after incubation of ^{32}P-NAD, preactivated cholera toxin and 25 μg alamethicin of KSCN-BBMV (lane 4). Experimental details of the ADP-ribosylation assay are as in Methods. Migration of molecular mass standards expressed in kDa is as indicated. The ADP-ribosylation of the 46 and 42 kDa proteins was only observed in the presence of CT (not shown). The results are representative of three or more experiments.

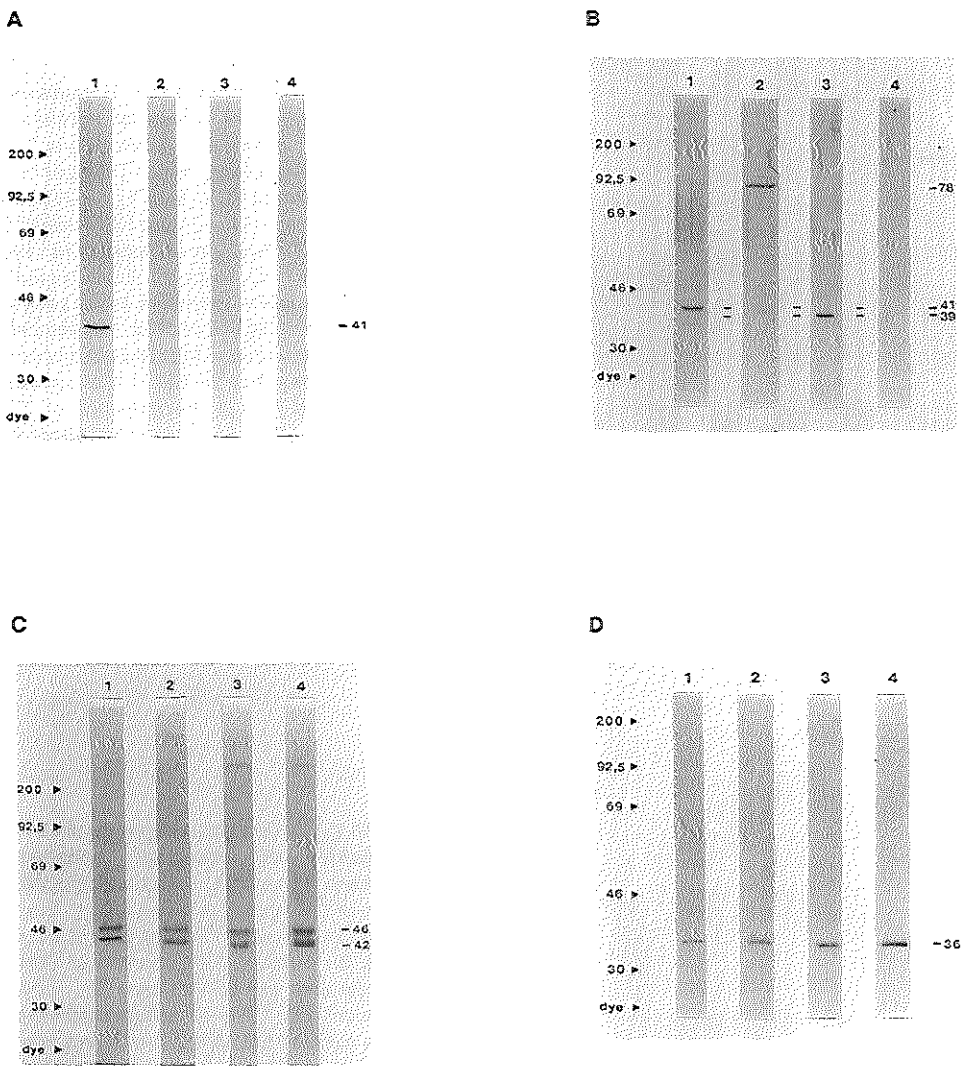

FIGURE 3. Detection of G-protein subunits with subunit-specific antisera in intestinal epithelial membrane fractions. 5 μg of BLM (lane 1), Mg-BBMV (lane 2), BB caps (lane 3) and KSCN-BBMV (lane 4) proteins were separated by SDS-PAGE. Immunoblotting was performed as described in the Methods. A) Antiserum 13B, αi3 specific, dilution 1:200. B) Antiserum SG1, αi1,2 specific, dilution 1:200. C) Antiserum CS1, αs specific, dilution 1:200. D) Antiserum D43, αo and β specific, dilution 1:1000. Migration of the molecular mass standards expressed in kDa is as indicated. The results shown are representative for at least three other experiments.

contrast to the 41 kDa αi subunit, this 39 kDa protein was not a substrate for pertussis toxin catalyzed ADP-ribosylation (Fig.1 and 4). This antiserum also detected a 78 kDa protein predominantly present in Mg-BBMV, although additionally found in BB-caps and BLM, but not in KSCN-BBMV (Fig.3B). The staining of the 41, 39 and 78 kDa proteins was blocked by the peptide to which the antiserum was raised, arguing against non-specific binding (Fig.6). Similar results were obtained with a different antiserum (AS/7) raised against the same conjugate (not shown).

Antiserum CS1, specific for the αs subunits, identified two proteins with Mr of 46 and 42 kDa (which sometimes appeared as doublets) in both the basolateral and the apical membrane fractions (Fig.3C). The 46 kDa and 42 kDa proteins comigrated with the ADP-ribosylated cholera toxin substrates shown in Fig.2 and are likely to represent two species of αs formed by alternative splicing [2]. The membranal rather than cytoskeletal origin of αs was indicated by its relative abundance in cytoskeleton-free KSCN-BBMV in comparison with Mg-BBMV and BB caps (Fig.3C). The distribution of the αs subunit among the apical and the basolateral membrane was virtually symmetrically (Fig.3C, lane 1 and 4).

Antiserum D43, specific for the αo and the ß subunits, identified only a 36 kDa band, which comigrated with the ß-subunit, in all membrane fractions (Fig.3D), albeit slightly enriched in the apical membrane. This shows that the ß-subunit is symmetrically distributed in enterocytes. The αo subunit could not be detected in any of these membrane fractions, neither by antiserum D43 (Fig.3D), nor by a second antiserum, IM1 (not shown). In contrast, both antisera were found capable of detecting αo subunits in crude membrane fractions of brain (D43, results not shown) or of NG108-15 cells (IM1, [35]).

G-protein subunit release experiments. To examine whether apical G-proteins function at the apical membrane or are released and translocated to other subcellular regions, *in vitro* release studies were performed using the non-vesiculated BB caps as a G-protein source. First we investigated if PT and CT catalyzed ADP-ribosylation could trigger release of αi and αs, respectively, from the open BB caps. As shown in Fig.4A, the ADP-ribosylated αi subunit remained confined to the 100.000 g particulate fraction and did not translocate to the supernatant. This was confirmed by immunoblotting the ^{32}P-labeled BB caps with αi1,2 (Fig.4B) and αi3 specific antisera (not shown). Also incubation of the BB caps with GTP[S] or p[NH]ppG uptill 60 min. at 30°C did not trigger αi release, either alone or in combination with ADP-ribosylation (results not shown). The ADP-

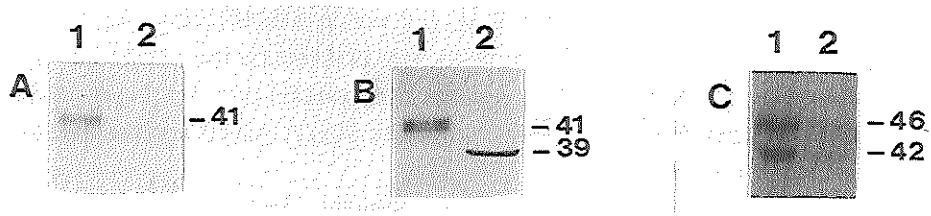

FIGURE 4. Distribution of ADP-ribosylated substrates in particulate and soluble fractions of brush border caps. 150 ug brush border caps were ADP-ribosylated by pertussis toxin or cholera toxin as described in the methods without addition of detergent and the soluble and particulate fractions were prepared. Both fractions were solubilized in 70 μl SDS sample buffer and analyzed on SDS-PAGE. Following electrophoresis, the ADP-ribosylated proteins were transferred to nitrocellulose paper and identified by autoradiography. A) Particulate (lane 1) and soluble (lane 2) fraction of pertussis toxin catalyzed ADP-ribosylated BB caps. B) Treatment of the same blot with antiserum SG1 (αi1,2 specific; dilution 1:200) following decay of ^{32}P radioactivity. Detection was done with ^{125}I-Protein A and autoradiography. C) Particulate (lane 1) and soluble (lane 2) fraction of cholera toxin catalyzed ADP-ribosylated BB caps. The results shown are representative for three experiments.

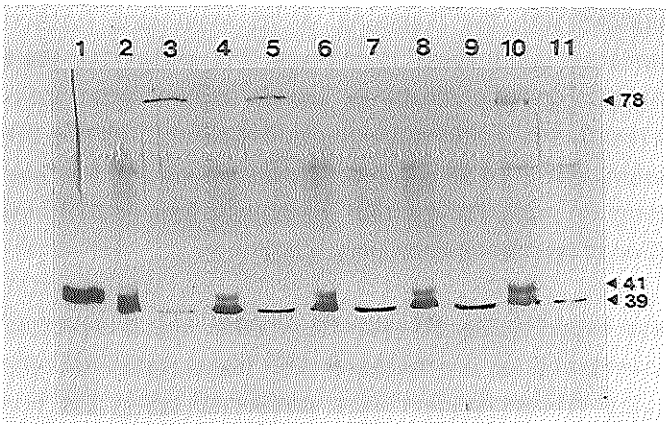

FIGURE 5. Release of the 39 kDa αi-like protein from brush border caps. Suspensions of 300 μg brush border caps were incubated for 0, 5, 15 and 30 minutes at 30°C and then separated into particulate (lanes 2,4,6,8,10) and soluble (lanes 3,5,7,9,11) fractions. In lanes 10 and 11, the incubation was carried out for 30 minutes at 30°C in the presence of protease inhibitors (see METHODS). Particulate and soluble fractions were solubilized in 100 μl SDS sample buffer and analyzed on SDS-gels. Proteins were detected with antiserum SG1 (αi1,2 specific, dilution 1:200), followed by alkaline phosphatase-conjugated second antibody. As a marker, a purified G-protein mixture from bovine brain was loaded (lane 1; 0.2 μg). The results are representative for at least three other experiments.

ribosylated αs subunits were predominantly recovered in the particulate fraction, although some αs subunits were detected in the soluble fraction (Fig.4C). Immunoblotting with αs specific antiserum confirmed these data and further showed that this release occurred also in the absence of CT and was therefore not triggered by the ADP-ribosylation assay (not shown). Incubation of the BB caps with GTP[S] or p[NH]ppG uptill 60 minutes at 30°C did not enhance its release (alone or in combination with ADP-ribosylation) nor did GDP[S] inhibit its basal release (not shown), suggesting that the small basal release was aspecific and not induced by activation of Gs.

In contrast to the αi and αs subunits, the 39 kDa αi-like protein recognized by antiserum SG1 was readily released after incubation of the membranes at 30°C. This release was independent of guanine nucleotides and could not be inhibited by GDP[S]. Its solubilization was already detectable after 5 min. of incubation and increased in time (Fig.5, lanes 2-9). The addition of protease inhibitors prevented the solubilization (Fig.5, lanes 10 and 11). This suggests that the release of the 39 kDa protein is triggered by endogenous proteases that become activated/solubilized during incubation of the BB caps at higher temperature. As its solubilization is not accompanied by a measurable shift

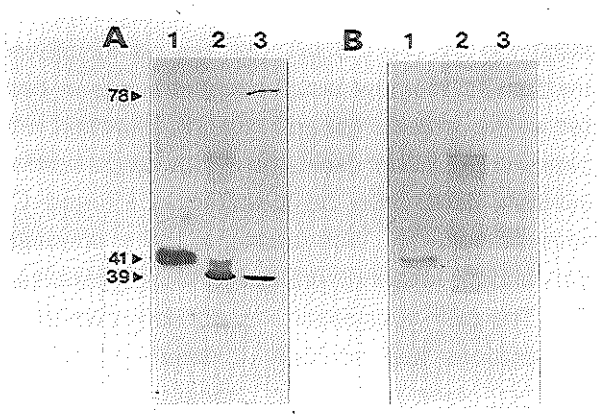

FIGURE 6. Peptide displacement of immunoreactive bands in brush border caps recognized by αi1,2 specific antiserum. 300 μg brush border caps were incubated for 5 minutes at 30°C and then separated in a particulate and a soluble fraction (lanes 2 and 3, resp.). Both fractions were solubilized in 100 μl SDS sample buffer and analyzed on gel. Lane 1 contains 0.2 μg of a purified G-protein mixture from bovine brain. Proteins were detected with antiserum SG1 (αi1,2 specific; dilution 1:200), followed by alkaline phosphatase conjugated second antibody. A) SG1. B) SG1, presaturated with the peptide to which it was raised (0.5 mg/ml, 5 min. incubated at 20°C) and subsequently diluted 1:200).

in molecular size of the 39 kDa protein (Fig.5), the release mechanism is likely to involve the proteolysis of another protein to which the 39 kDa protein is coupled. The fraction of 39 kDa protein releasable in 15-30 minutes varied per experiment and is most likely dependent on the protease content of the isolated membranes, which can vary per BB batch and may include both intrinsic proteases and contaminating pancreatic enzymes. In some experiments a complete release was found in 15-30 minutes (Fig.4B). Immunoblotting did not reveal solubilization of the ß-subunit (results not shown), confirming the hydrophobic nature of this protein. Addition of 0.1 % BSA as a carrier protein before precipitation with trichloroacetic acid did not influence the results (not shown).

DISCUSSION

Membrane associated G-proteins generally act as signal transducers, coupling receptors for hormones, neurotransmitters or light to effectors, such as adenylate cyclase, phospholipases, ion channels and phosphodiesterases [1]. Considering the asymmetrical distribution of most, if not all, G-protein coupled hormone receptors at the basolateral pole of the enterocyte [7], the G-proteins are likewise expected to reside preferentially in this subcellular region. In our immunochemical experiments such a distribution pattern could be substantiated for some of the G-proteins, notably the αi2 and αi3 subunits, but not for others: the αs and the ß subunits were present in about equal concentrations in both membranes. Two newly detected αi-like proteins (39 and 78 kDa), identified with antiserum raised against αi1,2, were found almost exclusively in the apical border.

The αi and the αs subunits could also be detected in PT and CT catalyzed ADP-ribosylation assays, respectively. With respect to the αi subunits, however, the ADP-ribosylation data and the immunoblotting data were qualitatively but not quantitatively comparable. The ADP-ribosylation reaction is known to be critically dependent on assay conditions and addition of detergent. To our experience, the labeling of each membrane preparation changed dramatically when different detergents were used and their order of potency varied per membrane species. To ensure optimal assay conditions, different detergents were therefore selected for a comparison between the various membrane preparations examined (Fig.2). As the ADP-ribosylation under our assay conditions is presumably incomplete and variable between different preparations, these data are unsuitable for quantitative analysis.

Besides the classical G proteins, Gi2, Gi3 and Gs, two possibly novel Gi-

like species were detected immunochemically in the intestinal membranes. The 39 kDa protein, detected by αi1/αi2, but not by αi1 and αi3 specific antisera, differed from the 41 kDa αi subunits in that: 1) its molecular mass was slightly lower; 2) it did not serve as a substrate for PT catalyzed ADP-ribosylation; 3) it was found only in BB caps and (at a low level) in KSCN-BBMV, but not in BLM; 4) it was readily released after incubation of BB caps at 30°C. As the N-terminus of several α subunits is known to be important for β/γ interaction and membrane attachment [36], one could argue that this 39 kDa protein originates from αi2 through limited proteolysis of a N-terminal segment. Since the N-terminus of αi appears also essential for recognition of the α subunit by pertussis toxin [36], its removal could in principle account for the lack of PT catalyzed ADP ribosylation of the 39 kDa protein shown in Figs.1 and 4. Such a hypothesis would imply that (1) proteolysis is limited and restricted to a small N-terminal fragment, because the 39 kDa protein is still recognized by antiserum SG1 (raised against the C-terminal decapeptide); (2) the C-terminus of αi (essential for recognition of the α subunit by pertussis toxin [26]) must be altered, causing the lack of pertussis-toxin-catalysed ADP-ribosylation of the 39 kDa protein shown in Figs.1 and 4, however without disturbing antiserum recognition; (3) the protease responsible for this cleavage is located exclusively at the apical border; no evidence for a 41 to 39 kDa protein conversion was found in purified BLM; (4) proteolysis must have occurred *in vivo* or during preparation of the BBM; in contrast, several other peripherous membrane proteins known to be sensitive to proteolysis (e.g. cGMP dependent protein kinase; villin) do not undergo proteolytic modification during isolation of brush border membrane caps [37]. However, if the 39 kDa protein arises by proteolysis of the 41 kDa αi2 subunit, it remains unclear why prolonged incubation of the brush borders, leading to a time dependent increase in solubilization of the 39 kDa band, does not significantly diminish the intensity of the 41 kDa band, in clear contrast to the immunoreactive 78 kDa band (Fig.5). Alternatively, the 39 kDa protein may originate at least in part from proteolysis of the 78 kDa protein, which in some (Fig.5), although not all experiments decreased when release of the 39 kDa protein increased. Although its size suggests that it might be a dimer of the αi2 subunit or of the αi-like 39 kDa protein, no dissociation into 39-41 kDa subunits was observed following N-ethylmaleimide treatment or boiling in the presence of 2-mercapto-ethanol. Similarly to the 39 kDa protein, the 78 kDa protein is readily released from the membrane and its release could be prevented by protease inhibitors (Fig.5). Its distribution is different from both the αi2 and the αi-like 39 kDa protein in that it is highly enriched in the Mg-

BBMV (Fig.3B). A similar high molecular weight protein was also detected by the same antiserum in rat liver membranes [32]. The rat liver 78-90 kDa protein was likewise enriched in fractions that contained little or no αi subunits [32]. The function of this protein is not yet known, but its molecular weight argues against a role as a classical heterotrimeric G-protein.

Assuming that the 39 kDa protein is a novel member of the αi family, its ability to be readily released from the BB by proteolysis makes it difficult to draw conclusions about its intracellular localization. It remains possible that this protein is more uniformly distributed among basolateral and apical membranes in the intact cell, but is lost during the preparation of the basolateral membranes and the Mg-BBMV, but not of the BB caps. This possibility is rather remote since Mg-BBMV's, in contrast to open BB caps, are formed immediately after shearing the cells and are resealed tightly in a right side out orientation preventing the release of intramicrovillar proteins [16]. A more likely possibility is that this protein is associated with the terminal web structure which is recovered only in BB caps, but not in Mg-BBMV or cytoskeleton depleted KSCN-BBMV. Such a localization might have important implications for its physiological function. It could be involved in endo- or exocytotic pathways or in the regulation of the organization of the cytoskeleton as described for G-proteins in neutrophils [29]. Assuming however that the 39 kDa protein is a proteolytic fragment of the 78 kDa protein, the unequal distribution of these proteins among Mg-BBMV (enriched in 78 kDa protein) and BB caps (enriched in 39 kDa protein) could reflect the protection of intravesicular proteins in Mg-BBMV to degradation by extravesicular proteases, preventing a possible conversion of the 78 kDa into the 39 kDa protein [cf. refs. 16 and 37]. Clearly, further studies are needed to purify and characterize both the 78 kDa and the 39 kDa αi-like proteins in order to identify their possible relationship and their physiological significance.

The targets for the apical G-proteins do not need to be in the apical membrane itself. It has already been described that G-protein α subunits can be released from the membrane upon activation by GTP, GTP analogs or ß-adrenergic receptor activation [25,26,27]. This is of particular interest in understanding the mechanism of action of cholera toxin. Enterocytes are the target for this toxin *in vivo*, causing secretory diarrhoea. It has always been intriguiing how cholera toxin, entering the enterocyte from the luminal site, is able to activate adenylate cyclase which is located at the basolateral membrane. Dominguez et al. [28], who were the first to demonstrate apical localization of αs subunits in rabbit enterocytes by the use of non-immunological techniques, have

suggested that cholera toxin activates αs in the apical membrane, which then translocates and couples to adenylate cyclase in the basolateral membrane. However, in this *in vitro* study we could not detect release of αs, αi or ß subunits from the apical membrane, even after prolonged exposure to GTP or its analogs or following cholera and pertussis toxin catalyzed ADP-ribosylation. Under similar conditions a slow and partial release of αs subunits has been described in rat glioma membranes [26]. The reason for this tissue difference is unclear. Although the absence of G-protein subunit translocation needs to be confirmed for intact epithelium, our data support the concept that the apical G-proteins fulfill a functional role in the same membrane to which they are targetted. In that regard, at least part of the cellular content of Gi and Gs is expected to be localized in the basolateral membrane considering their known interaction with adenylate cyclase in this tissue. Our study shows that this is indeed true for rat enterocytes.

Sofar no physiological activators or inhibitors of apical G-proteins in epithelial cells have been defined. Possible candidates include unidentified receptor proteins in the luminal membrane, second messengers activated by hormone receptors in the basolateral membrane or, in analogy with Go in the growth cone [30], a GAP-43 like protein, which can intracellularly activate G-proteins, without the necessity of a G-protein coupled receptor. A polarized distribution of G-protein subunits was also found in the rat renal cell-line LLC-PK1 by Ercolani [31]. In the renal epithelial cells, in contrast to the enterocyte, αi3 was localized exclusively in the apical membrane where it is possibly involved in the activation of amiloride sensitive Na^+-channels [33] and apical Cl^--channels [34]. A similar role for G-proteins in the apical membrane of intestinal epithelial cells has been suggested by our recent identification of a novel type of Cl^--channel in this membrane, that was activated by GTP and GTP analogs and inhibited by GDP[S] in the absence of cytoplasmic messengers [10,11]. The presence of a G-protein activated PI cycle in the apical membrane [7] also suggests a role for apically localized G-proteins. The αs subunits and the 39 kDa/78 kDa αi-like proteins are possible candidates for both Cl^--channel and phospholipase C activation in contrast to the αi subunits which were localized almost exclusively in the basolateral membrane.

We thank Dr. G. Milligan for generously supplying the specific antisera to αi1, αi1,2, αi3, αo and αs. We are also grateful to L. van der Voorn for her gift of the specific antiserum to αo/ß and the G-protein mixture purified from human and bovine brains. This study was supported by the Netherlands Organization for the Advancement of Scientific Research (NWO).

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CHAPTER 3

G-PROTEINS MEDIATE INTESTINAL CHLORIDE CHANNEL ACTIVATION

The localisation of several GTP-binding regulatory proteins in the apical membrane of intestinal epithelial cells has prompted us to investigate a possible role for G-proteins as modulators of apical Cl^--channels. In membrane vesicles isolated from rat small intestine or human HT29cl.19A colon carcinoma cells, the entrapment of GTP[S] led to a large increase in Cl^- conductance, as evidenced by an increased $^{125}I^-$-uptake and faster SPQ-quenching. The enhancement was observed in the presence, but not in the absence of the K^+-ionophore valinomycin, indicating that the increased Cl^- permeability is not secondary to the opening of K^+-channels. The effect of GTP[S] was counteracted by GDP[S] and appeared to be independent of cytosolic messengers, including ATP, cAMP and Ca^{2+}, suggesting that protein phosphorylation and/or phospholipase C activation is not involved. Patch clamp analysis of apical membrane patches of HT29cl.19A colonocytes revealed a GTP[S] activated, inwardly rectifying anion selective channel with a unitary conductance of 20 ± 4 pS. No spontaneous channel openings were observed in the absence of GTP[S], while the open-time probability (P_o) increases dramatically to 0.81 ± 0.09 upon addition with GTP[S]. Since the electrophysiological characteristics and regulatory properties of this channel are markedly different from those of the more widely studied cAMP/A-kinase operated channel, we propose the existence of a separate Cl^- selective ion channel in the apical border of intestinal epithelial cells. Our results suggest an alternative regulatory pathway in transepithelial salt transport and a possible site for anomalous channel regulation as observed in cystic fibrosis patients.

INTRODUCTION

Chloride fluxes across biological membranes are involved in a number of important physiological processes, including osmo-regulation, salt absorption and

secretion, neurotransmission and intracellular pH regulation. Among these, transepithelial salt secretion is of a particular interest, since anomalous regulation of the Cl^--channel involved leads to severe pathological conditions like secretory diarrhoea (cholera) and cystic fibrosis [1-3]. Although little is known of the physical structure of epithelial Cl^--channels [4], their mode of activation has been analyzed in more detail. On the basis of electrophysiological techniques (e.g. patch clamp, planar lipid bilayers, Ussing chamber) several intracellular regulatory mechanisms are emerging. These include: 1) channel activation through cAMP- and, in intestine, cGMP-dependent protein phosphorylation [1,2]; 2) activation through Ca^{2+}-mobilizing hormones such as acetylcholine, bradykinin or histamine [1,2] and 3) modulation of channel activity by protein kinase C [5,6].

According to the classical concept, regulation is initiated by hormone-receptor activation of GTP-binding regulatory proteins (Gs, Gp) in the basolateral membrane, activation of adenylate cyclase or phospholipase C and subsequent generation of cytosolic messengers (cAMP, Ca^{2+}) capable of communicating with the apical membrane [1,2]. However, recent studies have demonstrated that G-proteins are also capable of modulating ion channel activities through a direct or lipid metabolite-derived mechanism, independent of cytosolic messengers. This has been demonstrated definitely for cardiac Ca^{2+}-, neuronal K^+-and epithelial Na^+- and Cl^--channels [7-11], but G-protein involvement in intestinal Cl^--channel regulation has not been reported previously. We have recently found that a number of GTP-binding proteins, including Gs, Gp and p21 *ras*, are abundant in apical membranes of intestinal epithelial cells, despite the absence of any known G-protein-coupled hormone receptors ([12] and footnote 1). Since intestinal Cl^--channels are localized preferentially in the same membranes [1,2], a possible role for apical G-proteins as modulators of intestinal Cl^- secretion was studied. In this report, we present data illustrating that activation of G-proteins with GTP or GTP[S][2] enhances the Cl^- conductance of vesiculated intestinal membranes and unmasks the existence of a novel type of Cl^--channels in apical membrane patches from human colonocytes.

MATERIALS AND METHODS

Materials: Human HT29cl.19A colonocytes were generously donated by Dr. Laboisse, Unite 239 INSERM, Paris, France. All nucleotides were purchased from Boehringer Mannheim (FRG). [^{125}I]-iodine was obtained from Amersham Ltd. and SPQ from Calbiochem.

Cell culture: HT29cl.19A colonocytes were routinely grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Gibco). Cells were harvested 3-5 days after reaching confluency.

Vesicle preparation: Right-side out apical membrane vesicles from human HT29cl.19A cells were prepared from cell homogenates by differential Mg^{2+}-precipitation in the presence of trypsin-inhibitor (50 μg/ml), PMSF and 2 mM EGTA as described [1]. Thereafter, the vesicles were resuspended in a buffer containing 20 mM Tris/HEPES (pH = 7.0), 300 mM mannitol, 150 mM KCl, 6 mM $MgSO_4$, 3 mM Ca/EGTA (pCa = 6) and 1 mM $NaVO_3$, supplemented with either 150 mM KCl (iodine uptake assays) or 20 mM SPQ (Cl^--influx assays). Various nucleotides were trapped into the vesicles through a single cycle of freeze-thawing in liquid nitrogen. Rat brushborder membrane vesicles ('Mg/EGTA-BBMV') were prepared as previously described [13] and guanine nucleotides were trapped as described above.

Iodine uptake assay: Chloride efflux was quantitated using a ^{125}I-uptake assay analogous to the $^{23}Na^+$-assay developed by Garty et al. [14]. In short, extra-vesicular Cl^- was removed by eluting the vesicles through a DOWEX 1X8 column (gluconate form, 700 μl bedvolume) with 150 mM potassium gluconate. Assays were started by mixing the vesicles suspension with tracer amounts of radioactive iodine (3 μCi/mg of protein) in the absence or presence of the K^+-ionophore valinomycin (10 μM). Uptake was terminated by rapid elution of the vesicle suspension through a second DOWEX 1X8 column (formate form, 400 μl bedvolume), to remove external $^{125}I^-$, followed by γ-scintillation counting of the eluate. Background radioactivity (usually less than 1000 c.p.m), as measured by the amount of $^{125}I^-$ in the eluate in the absence of membrane vesicles, was subtracted.

SPQ-quenching: Extravesicular fluorochrome was removed prior to the experiments, by washing three times with excess (> 10 volumes) buffer. Vesicles were added to the cuvette at a concentration of approx. 100 μg protein/ml and continously stirred. Fluorescence was monitored at 350 nm (excitation) and 450 nm (emission) as described [15], using a Perkin Elmer LS-3B fluorescence spectrometer. Chloride uptake was started by adding KCl (final concentration: 25 mM) to the cuvette. After 3 - 5 minutes, trapped SPQ was released by addition of 10 μl 10% (v:v) Triton X-100, in order to achieve maximal quenching. The rate of salt-induced SPQ quenching was determined by fitting the change of fluorescence to $F(t) = A_0 + A_1 / [1 + KC_{Cl}(1 - e^{-k1t})] + k_0 t$ [16]. In this equation, $F(t)$ represents fluorescence at time t, A_0 and A_1 the residual fluorescence and the amplitude of quenching, respectively. k_1 represents the rate constant and the term $k_0 t$ was used to correct for linear, Cl^--channel independent quenching [16]. KC_{Cl} is the product of the Stern-Volmer constant (K; 92 M^{-1}) and the final [Cl^-] (25 mM).

Patch clamp analysis: Single channel recordings from apically localized channels were obtained from confluent monolayers of HT-29cl.19A cells using a List EPC7 amplifier. Signals were filtered at 3 kHz, digitized and stored on video tape prior to data analysis. Pipette solution contains 140 mM NaCl, 5 mM KCl, 5 mM HEPES (pH = 7.4), 1.2 mM $MgCl_2$ and 1 mM Ca/EGTA (pCa = 8), whereas bathing solution was identical except that 10 μM GTP[S] was added. GTP[S]-activated Cl^--channels were identified by the expected shift in voltage-current relationship observed after increasing the NaCl concentration in the bath to 420 mM. The channel incidence was approx. 25% (n = 98).

RESULTS

In an initial series of experiments, Cl^--channel activation was studied in a suspension of KCl-loaded plasma membrane vesicles obtained from human HT29cl.19A cells, a Cl^--secreting subclone isolated from the original HT29 colon carcinoma cell line [17]. Conductive chloride efflux was measured by quantitating Cl^--diffusion potential-driven uptake of tracer amounts of radio-labelled iodine. We found this method superior to $^{36}Cl^-$-influx assays because: 1) epithelial anion channels prefer I^- over Cl^- ($P_I/P_{Cl} > 1$) [17], 2) $^{125}I^-$ is available at higher specific radioactivity, and 3) iodine is accumulated exclusively into intact vesicles containing functional Cl^--channels (c.f. ref. [14]), resulting in diminished background labelling and increased sensitivity. Addition of trace amounts of radio-labelled iodine to KCl^--loaded vesicles results in a rapid increase in intravesicular $^{125}I^-$ (Fig.1). As compared to control preparations, $^{125}I^-$ accumulation is much larger (approx. 2-3 times control values) in GTP[S] containing vesicles, suggesting that

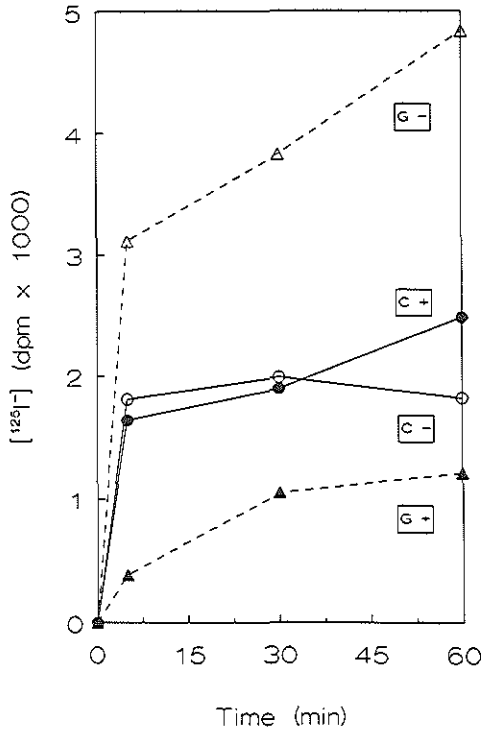

FIGURE 1. [^{125}I]uptake in apical membrane vesicles from HT-29cl.19A colonocytes. Time-course of radiolabelled iodine uptake in control (O) and 100 μM GTP[S]-containing (Δ) vesicles in the absence (open symbols) and presence (closed symbols) of valinomycin (10 μM). Data points are all derived from one representative experiment (n = 3).

GTP[S] induces a sustained increase in transmembrane chloride permeability. Disturbing the Cl^--diffusion gradient, by adding the K^+-ionophore valinomycin (10 μM), eliminates the GTP[S]-stimulation of $^{125}I^-$ uptake (Fig.1.), confirming its identification as conductive Cl^--efflux. In contrast, $^{125}I^-$-uptake in control preparations was not affected. In the presence of valinomycin, $^{125}I^-$ accumulation is somewhat lower in GTP[S]-containing relative to control vesicles (Fig.1). This phenomenon is probably an artifact of the experimental procedure used, since increased anion permeability, as occurs in GTP[S] containing vesicles, may facilitate exchange of iodine from the vesicle to the Dowex column during elution. Although considerable variation in basal $^{125}I^-$ uptake between the various vesicle preparations was noted (approx. 2000 - 7000 c.p.m.), the GTP[S]-induced increase in $^{125}I^-$ accumulation appeared highly reproducable (1.51 ± 0.12 times basal uptake at t = 30; mean ± S.E.M. for n = 3).

The accumulation of $^{125}I^-$ in GTP[S]-containing vesicles persists for at least 30-60 minutes. At least two phenomena may underly this sustained enhancement of intravesicular $^{125}I^-$ uptake. First, the electrogenic efflux of Cl^- generates a sustained inside-positive diffusion potential, which acts as a driving-force for prolonged $^{125}I^-$ accumulation (c.f. see: ref.[14]). Secondly, the contribution of an additional subpopulation of vesicles which accumulate $^{125}I^-$ solely in the presence of guanine nucleotides may give rise to a further increase in the equilibrium concentration of intravesicular $^{125}I^-$. As expected for right-side out vesicles, no effects of GTP[S] were observed when added after vesicle resealing (not shown), demonstrating that GTP[S] acts on the cytoplasmic side of the membrane.

TABLE 1. Effects of GDP[S] on GTP[S]-induced transmembrane Cl^- fluxes.

	k_1 of SPQ quenching (% of control)
Control	100
GDP[S]	50 ± 11*
GTP[S]	316 ± 15*
GDP[S] + GTP[S]	209 ± 8*

SPQ-quenching experiments were performed as described in 'Materials and Methods'. Rate constants (k_1) were calculated and expressed as percentage relative to control preparations (mean ± SEM for 3 independent experiments). k_1 in control preparations was 0.6 ± 0.1 min^{-1}. Concentrations used: GDP[S], 5 mM and GTP[S], 100 μM. Asterisk indicates $P < 0.05$ relative to the control.

To further substantiate our finding that GTP[S] increases Cl^--conductance, we measured Cl^--uptake using the chloride sensitive fluorescent probe SPQ. Recent studies have revealed that this water-soluble dye, whose fluorescence is quenched in the presence of Cl^-, is a suitable and sensitive probe for chloride transport assays in biological systems [15]. As shown for rat small intestinal brushborder membrane vesicles (Fig.2), addition of KCl (25 mM final concentration) to SPQ-loaded vesicles rapidly quenches fluorescence, indicative of an increasing intravesicular Cl^--concentration. In the presence of valinomycin, i.e. when generation of a counteracting membrane potential is inhibited, SPQ quenching is much faster in GTP[S]-loaded relative to control vesicles (Fig.2 and Table 1). Apparently, the GTP[S]-sensitive Cl^- conductance is not confined to human colon carcinoma cells, but occurs also in the apical membranes of non-neoplastic enterocytes. Qualitatively similar results were obtained with vesicle preparations from cultured HT29cl.19A cells (not shown). Importantly, no difference between control and GTP[S]-containing vesicles was observed in the absence of valinomycin (Fig.2), indicating that increased Cl^--conductance is not secondary to GTP-induced opening of K^+-channels. The notion that GTP-binding proteins may be involved in stimulating Cl^--conductance is further supported by our observation that GTP-induced acceleration of transmembrane Cl^--influxes is diminished in the presence of trapped GDP[S], a well known inhibitor of GTP-binding proteins (Table 1).

TABLE 2. Effects of guanine nucleotides on SPQ quenching.

	k_1 of SPQ quenching (min^{-1})		
Control	0.64 ± 0.08 (10)	GTP	1.08 ± 0.18 (3)*
ATP (1 mM)	0.70 ± 0.09 (5)	GTP - Vanadate	0.69 ± 0.17 (3)
ATP[S]	0.69 ± 0.17 (3)	GTP[S]	1.52 ± 0.20 (10)*
cAMP	1.63 ± 0.10 (3)*	GTP[S] + cAMP	2.17 ± 0.12 (2)*
GDP	0.65 ± 0.02 (3)	GMP-PNP	2.03 (1)
GDP[S]	0.90 ± 0.05 (3)*		

Various guaninine nucleotides (100 μM), ATP[S] (200 μM) and cAMP (1 mM) were trapped into rat brushborder membrane vesicles as described in Figure 2. Effects of cAMP ± GTP[S] were tested in the presence of 1 mM ATP. Rate of SPQ quenching was determined as described under 'Materials and Methods' and expressed as mean ± SEM. Number of independent experiments is given between parentheses. Asterisk indicates $P < 0.05$ relative to the control.

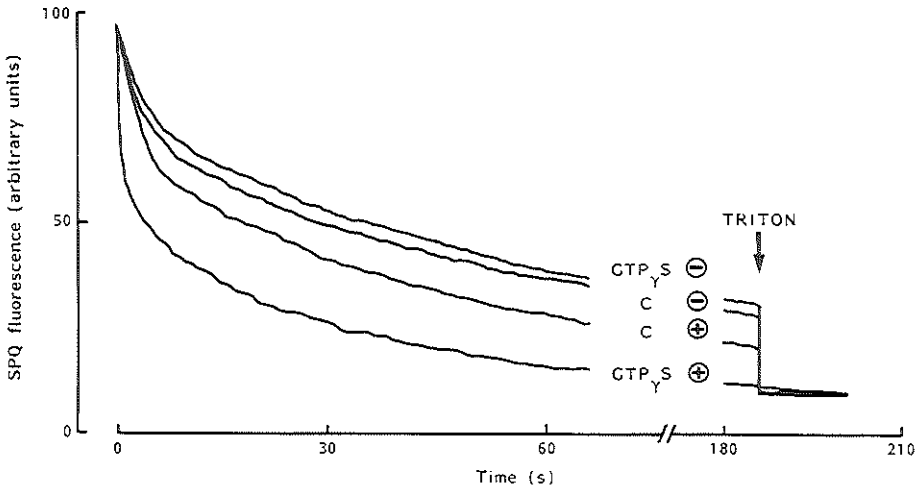

FIGURE 2. Rate of SPQ quenching. Cl^--influx in control (C) and GTP[S]-containing rat brushborder membrane vesicles was determined in the absence (-) or presence (+) of valinomycin (10 μM) using the Cl^--sensitive probe SPQ.

We have studied the effects of various nucleotides by trapping equimolar (100 μM) concentrations into rat intestinal brushborder membranes vesicles. As clearly shown in Table 2, non-hydrolysable GTP-analogues such as GTP[S] and GMP-PNP are potent activators of conductive Cl^--influx. GTP activates chloride transport also, but only when GTPase inhibitor vanadate [19] is present (Table 2). Other guanine nucleotides, including GMP and GDP, were always ineffective. A small increase in SPQ-quenching, however, was observed in GDP[S]-containing vesicles (Table 2), suggesting the possible existence of a channel inhibitory G-protein in brushborder membranes. ATP (1 mM) or ATP[S] (200 μM) alone did not induce Cl^--influx (Table 2), nor did it potentiate the response to GTP[S] (notshown). This suggests that protein phosphorylation and/or ATP-binding is not essential for a GTP-mediated increase in Cl^- conductance. In the presence of 1 mM ATP, cAMP was found to stimulate transmembrane ion fluxes additional to the response to GTP[S] (Table 2), most likely through protein kinase A mediated protein phosphorylation [1].

Chloride selective anion channels have been previously studied in detail in various human tissues, including nasal polyps [20], tracheal cells [21, 22], sweat gland duct cells [3] and renal epithelium [11], as well as in a number of tumour cell lines such as T84 [23] and HT29 intestinal cells [24]. We have studied

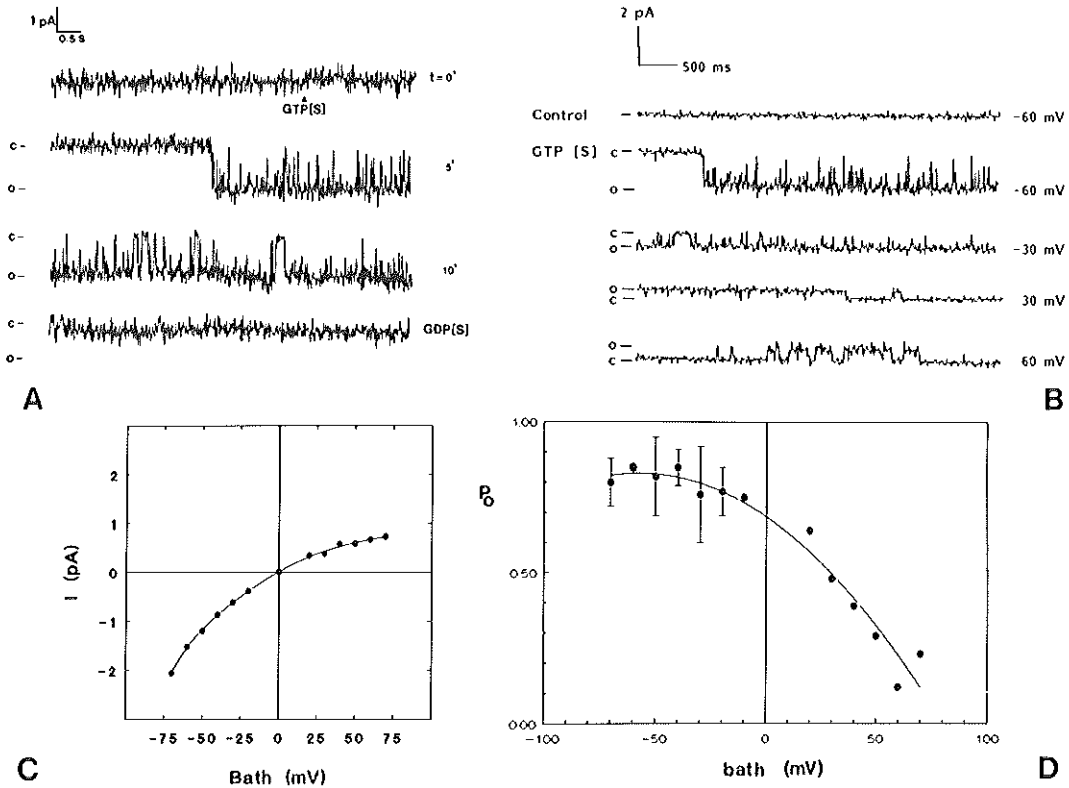

FIGURE 3. Patch clamp analysis of GTP[S]-activated Cl^--channels. A) Activation of Cl^--channels by GTP[S] (10 μM) in apical membrane patches obtained from HT-29cl.19A cells. Closed (c) and open (o) states of the channel are indicated. GDP[S] represents a recording of a GTP[S] activated channel 2 minutes after addition of 2 mM GDP[S]. **B)** Patch clamp recordings of a GTP[S]-stimulated Cl^--channel at various holding potentials. **C)** Voltage/current relationship of GTP[S] activated Cl^--channels. **D)** Open state probability of GTP[S] activated Cl^--channels. Data are expressed either as mean ± S.D. for 4 determinations or as the mean of a duplicate observation.

the biophysical characteristics of GTP[S]-activated Cl^--channels in HT29cl.19A colonocytes using patch clamp analysis, enabling a comparison with the well documented cAMP-activated Cl^--channel.

Upon exposure of inside-out apical membrane patches to 10 μM GTP[S], highly frequent channel openings were observed within 2 - 20 minutes (Fig.3A). The channels were anion-selective ($P_{Cl}/P_{Na} > 8$), as determined by changing the ionic composition of bathing solutions, and showed the permeability sequence $NO_3^- > Br^- \simeq I^- > Cl^- > F^-$. Since addition of GDP[S] (2 mM, Fig. 3A) blocks the channel rapidly, we suggest that they are identical to those involved in the GTP[S]-induced increase in transmembrane Cl^--flux (c.f. Fig.1 and 2). GTP-activated Cl^--channels display an average single channel conductance of 20 ± 4 pS and exert a prominent, inward rectification (Fig.3B,C). At physiological membrane potential, the open-state probability (P_o) is 0.81 ± 0.09 (n = 10), which tends to decrease by depolarization (Fig.3D). Interestingly, this particular channel was only found after stimulation with GTP[S] and, in contrast to the cAMP/protein kinase A activated channel, never occurred spontaneously or following membrane depolarization.

Electrical properties of the GTP[S]-activated channels differ markedly from the cAMP/protein kinase A activated Cl^--channels, which have a somewhat larger unitary conductance and, most importantly, were found to be outward rectifying [21-24]. This suggests that, in addition to cAMP-activated channels, a different class of Cl^--selective channels exists, which are coupled to G-proteins. This notion is supported by our observation that, in vesicle preparations, co-stimulation with GTP[S] (100 μM) and cAMP (1 mM), in the presence of ATP, results in an additional rather than synergistic, increase in the rate of SPQ quenching (Table 2).

DISCUSSION

The co-existence of an activating G-protein and ion channel in the apical membrane of a polarized epithelium is not unprecedented: the activity of amiloride-sensitive Na^+-channels and of Cl^--channels in renal apical membranes has likewise shown to be regulated by GTP and GTP[S], presumably acting through αi3 [9-11]. Although the renal Cl^--channel has characteristics very different from the GTP[S]-activated channel in intestinal epithelia (e.g. single channel conductance of 305 pS, activation by protein kinase C and Ca^{2+}, frequent spontaneous openings [11]), these findings suggest a more wide spread role for

G-protein operated anion channels in transepithelial ion fluxes. Presently, little is known about the identity of the channel-associated G-protein or about its cellular activators. According to the classical concept of cholera toxin-provoked intestinal salt and water secretion, the A1 subunit of the toxin is released at the apical membrane and, upon translocation to the basolateral membrane, activates adenylate cyclase through ADP-ribosylation of a basolateral pool of the stimulatory G-protein Gs [25]. Subsequently, cAMP triggers the opening of apical Cl^--channels through protein kinase A-mediated phosphorylation [1]. The recent demonstration of a distinct pool of Gs (Gs^{ap}) in the apical membranes [26] has led to the speculation that αs^{ap}, rather than A1, translocates to the basolateral membrane following its ADP-ribosylation at the apical border [25, 26]. Alternatively, it remains possible that the inward-rectifyer functions as a stress- or swelling-activated Cl^--channel recently identified in epithelial tissues, including intestine [27, 28].

Coupling of the G-protein to the Cl^--channel may either be direct, as shown previously for the action of several hormones and neurotransmitters on cation channels [7-11], or indirect, possibly through locally produced autacoids, as demonstrated earlier for cardiac muscarinic K^+-channels [29, 30]. Our experimental conditions however, exclude the possibility that GTP[S] acts indirectly through the generation of cytosolic messengers such as cAMP and Ca^{2+}. Furthermore, the co-localization of the GTP-receptor and the Cl^--channel in the apical membrane patches and vesicles virtually rules out a channel recruitment model in which the G-protein, analogous to the G_E postulated for exocytosis [31], would trigger the fusion of channel-containing vesicles with the apical membrane (c.f. ref. [32]). As yet, no information is available about the molecular structure of GTP-activated Cl^--channels. A putative structure of the cAMP/protein kinase A-activated Cl^--channel has recently been proposed from the nucleotide sequence of CFTR, the cystic fibrosis transmembrane conductance regulator [33]. Because CFTR contains two nucleotide binding folds [33], capable of binding both ATP and GTP [34], we cannot exclude that GTP[S] increases Cl^--conductance through CFTR. However, in view of the prominent differences between cAMP- and GTP[S]-sensitive Cl^--channels (I/V characteristic, voltage activation; c.f. ref [1]), the existence of a different class of anion-selective channels seems more likely. Interestingly, an apically localized, inwardly rectifying Cl^--channel has recently been reported for gastric oxyntic cells in amphibians [35]. Although this particular channel has electrical characteristics very similar to the GTP[S]-activated channels, they are certainly not identical, since marked differences exist in sensitivity for cAMP-dependent protein phosphorylation and membrane

depolarization. Therefore, it is important to establish whether or not G-protein coupled Cl^--channels are functional in cystic fibrosis patients, because the presence of functional GTP-activated channels may imply a possible route for bypassing the defect in cAMP-, cGMP and Ca^{2+} activation of Cl^- secretion [2, 36-38], most plausibly the primary lesion in this disease.

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FOOTNOTES

1. N.v.d.Berghe et al., in preparation.

2. Abbreviations used: ATP[S], adenosine 5'-O-(3-thiophosphate); CFTR, Cystic Fibrosis transmembrane conductance regulator; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; GDP[S], guanosine 5'-O-(2-thiophosphate); GMP-PNP, guanylyl-imidodiphosphate; GTP[S], guanosine5'-O-(3-thiophosphate);HEPES,4-(2-hydroxyethyl)-1-piperazineethanesulfonicacid;PMSF, phenyl-methyl-sulfonyl-fluoride; P_o, open time probability; P_{Cl}, channel permeability to Cl^--ions; P_I, channel permeability to I^--ions; P_{Na}, channel permeability to Na^+-ions; SPQ, 6-methoxy-N-(3-sulfopropyl)-quinolinium.

CHAPTER 4

PHORBOL ESTER AND cAMP INHIBIT THE CARBACHOL-INDUCED CALCIUM RESPONSE AT DIFFERENT TARGETS IN HT29cl.19A CELLS.

Our recent identification of a GTP-activated phospholipase C in the apical membrane of rat enterocytes, despite the lack of Ca^{2+}-linked hormone receptors in this subcellular region [Vaandrager et al. Am.J.Physiol. 259: G410-G419, 1990] and the apparent role of Ca^{2+} as a third messenger in cyclic nucleotide-induced inhibition of Na^+ absorption [Semrad et al., J.Clin.Invest. 86: 585-591, 1990], prompted us to examine possible cross-talk between second messengers generated at the basolateral membrane (cAMP, Ca^{2+}, protein kinase C) and the PI-cycle in HT29cl.19A and T84 colonocytes. In HT29cl.19A cells, Ca^{2+}-ionophore caused a slight stimulation of InsPn production. However, HPLC analysis revealed a rise in InsP1, rather than Ins(1,4,5)P3, arguing against a role for Ca^{2+} as a trigger of apical or basolateral PIP2 hydrolysis. Short-term treatment with phorbol ester (PMA) did not affect basal InsPn levels but inhibited the carbachol-induced rise in InsPn and calcium, indicating negative feedback by PKC. Downregulation of PKC α resulted in recovery of both responses. In contrast, cAMP had no effect on the production of InsPn, either alone or in the presence of carbachol. However, cAMP strongly inhibited the carbachol-induced calcium response, without affecting basal calcium levels. Furthermore, heat-stable Escherichia coli toxin (ST_A), a cGMP-linked agonist, was likewise unable to affect InsPn levels in T84 colonocytes. In conclusion, we found no evidence for a role of calcium as third messenger in the action of cyclic nucleotides in the colonic cell lines. In addition, no evidence was obtained for a physiological role of cAMP, cGMP, protein kinase C or calcium as triggers of (apical) PI-derived signals. Instead, PMA and cAMP were found to inhibit the carbachol-activated calcium response, by acting on different targets: PMA at a step upstream, cAMP downstream of InsPn formation.

INTRODUCTION

In the intestine, at least three signal transduction pathways are involved in the regulation of transepithelial salt and water transport (10). These pathways include protein phosphorylation through cAMP- and cGMP-dependent kinases and activation of phospholipase C (9,10). Since intestinal epithelial cells are exposed to multiple stimuli *in vivo* (4), several different pathways may become activated simultaneously. Cross-talk between second messenger-generating pathways has been described in several other tissues and appears to affect multiple cellular functions (see below).

The mechanism of cross-talk between cyclic nucleotides and the PI-cycle varies between the cell types studied. Inhibition of the PI-cycle by cyclic nucleotides has been described to occur upstream of phospholipase C (PLC), through phosphorylation of a 24 kDa G-protein (23) or the 50 kDa VASP protein (32,36) as well as downstream of PIP2 hydrolysis through phosphorylation of a brain InsP3 receptor (31) or inhibition of calcium entry and/or activation of the Ca^{2+}-ATPase (11,13). In addition, stimulation of the PI-cycle by cyclic nucleotides has also been demonstrated both upstream (15) and downstream (5,7,22,24,27) of PLC activation.

Apart from this modulatory role of cyclic nucleotides, the PI-cycle is also known to be under inhibitory control of protein kinase C (PKC). The targets for PKC-induced inhibition can be the enzyme itself (PLC ß, 28) or the coupling between the G-protein and PLC (18). Furthermore, PKC was found to inhibit Ins(1,4,5)P3-induced calcium mobilization by enhancing the metabolism of Ins(1,4,5)P3 (16) and by inhibiting the Ins(1,4,5)P3 operated calcium channel (37).

In intestinal epithelial cells, a potential interaction between cyclic nucleotide-mediated pathways and the PI-cycle is of a particular interest. First, in chicken enterocytes, cAMP- and cGMP-induced sodium absorption was mediated through a rise in cytosolic calcium (6,29,30). In the human colon carcinoma cell line HT29cl.19A, however, cAMP could, but a rise in calcium by itself could not, provoke chloride secretion (35), indicating that cAMP can not solely act through calcium. However, a role for calcium as a permissive factor in cAMP-induced chloride secretion can not be excluded. Whether or not cAMP can mobilize calcium in the colonic cell lines is therefore important to examine.

In addition, while all known Ca^{2+}-mobilizing hormone receptors are localized basolaterally, enterocytes contain a GTP-activated form of PLC in both the basolateral and the apical membrane (34). Cross-talk between second

messengers elicited at the basolateral (cAMP, PKC, Ca^{2+}) or the apical (cGMP) membrane and the apical PI-pool may constitute a hypothetical mechanism for regulation of InsPn release and Ca^{2+}-signalling at the apical membrane.

In the present study, the effects of hormone- or enterotoxin-generated second messengers on PLC activation were studied by measuring the formation of inositol phosphates and calcium mobilization in the absence and presence of carbachol. This report shows that: i) cAMP does not stimulate InsPn production nor the mobilization of calcium in HT29cl.19A cells, suggesting that cAMP-induced calcium mobilization in the intestine is a unique feature of the absorptive villous cells; ii) PMA and cAMP inhibited the carbachol-activated calcium response, by acting on different targets, upstream and downstream of InsPn formation, respectively.

MATERIAL AND METHODS

Materials: Cell culture media and fetal calf serum were from Gibco. Forskolin, carbachol and PMA were from Sigma and A23187 and 8-Br-cAMP from Boehringer, Germany. Myo-[2-^{3}H]Inositol was obtained from NEN-Dupont, Germany. Indo-1 acetoxymethylester was from Molecular Probes. AG1 X8 Dowex 100-200 mesh was obtained from Bio-Rad, Eugene, OR.

Cell culture: HT29cl.19A cells (kindly donated by Dr. C.L. Laboisse, INSERM, Paris, France) and T84 cells were routinely grown in cell culture flasks (Falcon) under a humidified 95% air, 5% CO_2 atmosphere at 37°C. HT29cl.19A medium contained: Dulbecco's modified Eagles medium (DMEM), 10% fetal calf serum (FCS), 40 mg/l penicillin and 90 mg/l streptomycin. T84 medium contained: DMEM/Ham's (1/1), 5% FCS, 5% HEPES and penicillin and streptomycin. Cells were subcultured at a surface ratio of 1:7 (HT29cl.19A) or 1:5 (T84) after trypsinization every 7 days, when they had reached 70-80 % confluence. The passage number of the cells used in this study varied between 11 and 30 (HT29cl.19A) and between 20 and 40 (T84).

Determination of total inositol phosphates. Cells grown to 70-80% confluency on 6 well cell culture plates were labeled for 48 hours with [^{3}H]Inositol (2μCi/ml) in inositol free medium, supplemented with 5% fetal calf serum under an atmosphere of 95% air, 5% CO_2 at 37°C. At 1 h before stimulation, the cells were shifted to serum-free DMEM containing 10 mM HEPES (pH 7.4). The cells were stimulated with carbachol for various periods in the presence of 10 mM LiCl and incubations were terminated by replacing the medium by 1 ml of 10% ice-cold trichloroacetic acid. After 10 minutes, extracts were collected and trichloroacetic acid was removed by washing with diethylether. The samples were neutralized with 1 M Tris, pH 8.5 and processed for analysis of the total [^{3}H]inositol phosphate containing fraction (InsPn) by anion exchange chromotography on AG1 X8 Dowex columns (formate form) as previously described (33). In some experiments, long-term pretreatment with PMA (27 h) decreased the total water-soluble [^{3}H]inositol uptake (as determined by radioactivity present before separation of the inositol phosphates). This has also been described for vascular smooth muscle cells (26). The reason for this PMA related decrease in inositol uptake is unclear. In

our calculations corrections were applied for these differences in total amount of radioactivity per sample.

Separation of inositol phosphates by HPLC. HT29cl.19A cells were labeled with 20 μCi [^{3}H]inositol/ml for 48 h. At 1 h before stimulation, the cells were shifted to serum free DMEM containing 10 mM HEPES and then stimulated with carbachol for 10 sec. The incubation was stopped by adding trichloroacetic acid. After diethylether extraction, the samples were neutralized with Tris-base and inositol phosphates were separated on a Partisil Sax column (250.4.6 mm; Whatman) at a flow rate of 1.25 ml/min. The gradient used was as previously described (33). Nucleotide standards (2.5 mM AMP, ADP and ATP) were added. For quantification of the various inositol phosphates, 0.5 min (625 μl) fractions were collected and ^{3}H radioactivity was determined.

Calcium measurements. HT29cl19A cells were grown to 70-80% confluency on cover slips (9.21 mm). The cover slips were loaded with Indo 1-AM (5 μM, 45 min.) in culture medium at 37°C, 5% CO_2. Extracellular Indo 1-AM was removed by three washes with calcium buffer (120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 20 mM HEPES, 10 mM glucose, pH 7.4 adjusted with Tris) and incubations were carried out at 37°C in the same buffer. Calibration of the responses was performed by adding 10 mM $CaCl_2$ and 0.1 mg/ml digitonin (Fmax), followed by addition of 2.5 mM $MnCl_2$ (Fmin). The intracellular calcium concentration was calculated using the following equation: $[Ca^{2+}]_i = K_d$ (F-Fmin/Fmax-F). The K_d value used was 250 nM (14).

RESULTS

Carbachol-induced production of inositol phosphates. Carbachol induced a dose-dependent rise in inositol phosphate (InsPn) formation in both the HT29cl.19A cells (Fig.1) and the T84 cells (not shown). Half-maximal release occurred at 10 μM, whereas maximal release was found at 100 μM carbachol. The formation of InsPn was linear for up to 30 minutes in both cell lines (Fig.2, HT29cl.19A cells). A HPLC profile of the carbachol-induced release of InsPn in HT29cl.19A cells showed a rise of Ins(1,4,5)P3 and of its breakdown products InsP1, InsP2, Ins(1,3,4)P3 and Ins(1,3,4,5)P4 (Fig.3).

Effects of cAMP, cGMP and calcium on the formation of inositol phosphates. A possible modulation of InsPn production by cAMP or calcium was tested in HT29cl.19A colonocytes. Since the intestinal isoforms of guanylate cyclase and cGMP dependent protein kinase are no longer expressed in this subclone (De Jonge, unpublished observations), effects of cGMP were studied in the T84 cells. As indicated in Table 1, the cell-permeable analogue of cAMP, 8-Br-cAMP and the specific activator of intestinal guanylate cyclase, heat stable E.coli enterotoxin (ST_A), added alone or in combination with carbachol had no effect on the

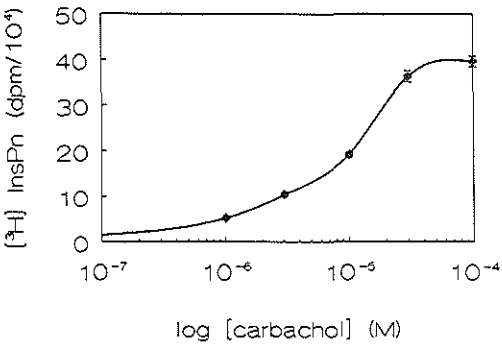

FIGURE 1. Dose dependency of carbachol-induced inositol phosphate accumulation. [^{3}H]Inositol labeled HT29cl.19A cells were incubated with the indicated concentrations of carbachol for 30 min in the presence of 10 mM LiCl. The incubations were terminated with trichloroacetic acid and inositol phosphates were analyzed on Dowex columns as described in Material and Methods. Data are expressed as mean ± S.D. for three determinations.

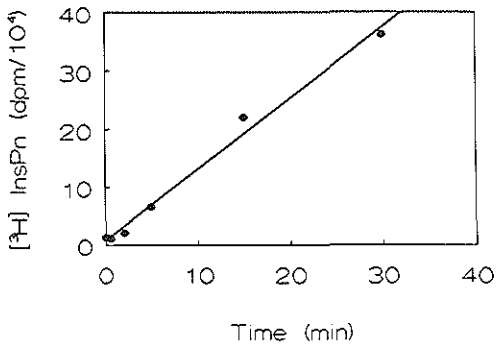

FIGURE 2. Time-course of the carbachol-induced inositol phosphate formation. [^{3}H]Inositol labeled HT29cl.19A cells were incubated with 100 μM carbachol for the indicated time periods. All incubations were performed in the presence of 10 mM LiCl during 40 min. After termination with trichloroacetic acid, inositol phosphates were analyzed on Dowex columns as described in Material and Methods. Data are representative for three determinations.

production of InsPn. Calcium ionophore A23187 alone slightly activated the release of InsPn, but did not significantly affect the carbachol-induced release of InsPn (Table 1). Since HPLC analysis revealed an increase in InsP1 (1.6 ± 0.2; n=3) but not in Ins(1,4,5)P3 (0.9 ± 0.1; n=3), the effect of Ca^{2+}-ionophore is most likely the result of calcium activated hydrolysis of PI, rather than activation of PIP2 breakdown by phospholipase C (20).

Inhibition of carbachol-induced InsPn formation by protein kinase C. Effects of protein kinase C were studied by using the tumor promotor phorbol 12-myristate 13-acetate (PMA). Pretreatment of HT29cl.19A cells for 10 min. with 5.10^{-7} M PMA resulted in a strong inhibition of the carbachol induced release of InsPn (Fig.4). Pretreatment of the cells for 27 h with 5.10^{-7} M PMA, which is known to result in a complete downregulation of PKC α, but not of the other PKC isozymes (β1, γ, η and ζ) expressed in this cell type (35) was associated with a full recovery of the carbachol-induced PIP2 hydrolysis (Fig.4). PMA alone did not affect basal InsPn levels (Table 1).

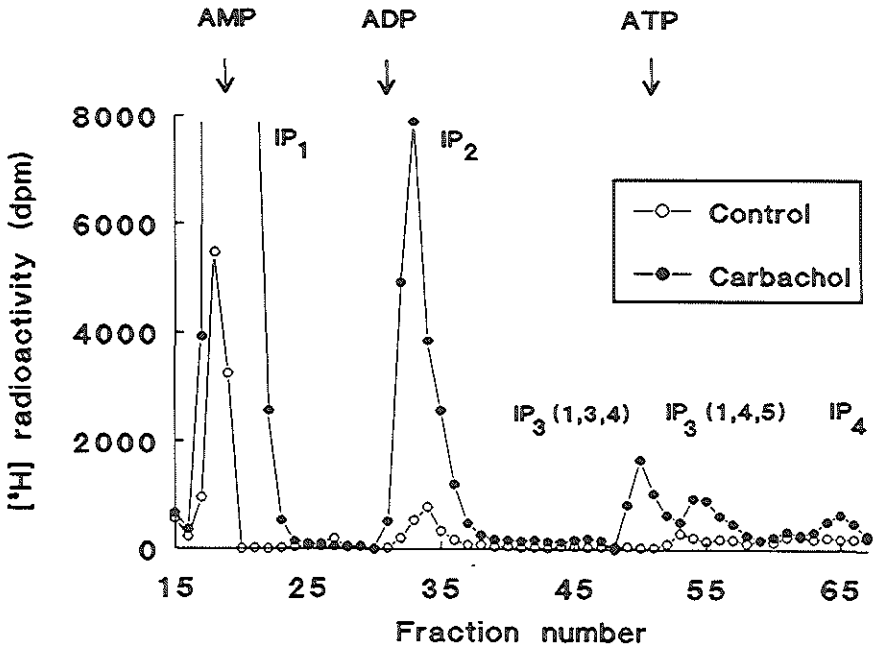

FIGURE 3. HPLC profile of [^{3}H]Inositol phosphates obtained from control and carbachol-stimulated HT29cl.19A cells. Carbachol was added (100 μM, 10 sec) to [^{3}H]Inositol labeled cells and after termination of the incubation by trichloroacetic acid, inositol phosphates were separated on a Partisil Sax column as described in Material and Methods. Closed symbols, control; open symbols, carbachol stimulated cells.

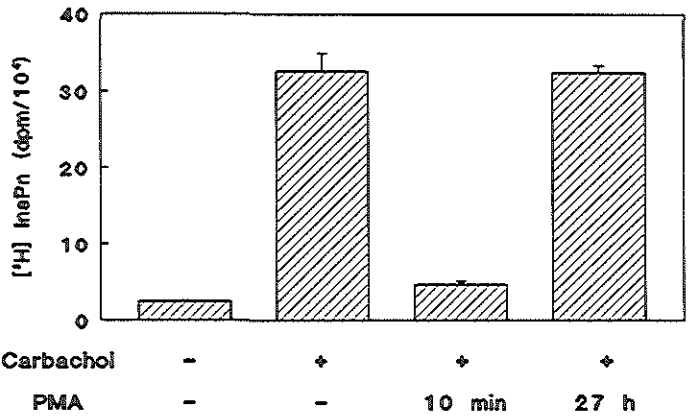

FIGURE 4. Effects of PMA pretreatment on the carbachol-induced inositol phosphate accumulation. [^{3}H]Inositol labeled HT29cl.19A cells were pretreated for 10 min or 27 h with 5.10^{-7} M PMA, before addition of 250 μM carbachol. After 30 min incubation in the presence of 10 mM LiCl, reaction was stopped by adding trichloroacetic acid. Inositol phosphates were analyzed on Dowex columns as described in Material and Methods. Data are expressed as mean ± S.D. for three experiments in triplo.

TABLE 1. Effects of carbachol, cAMP, ST_A (cGMP) and Ca^{2+} on [^{3}H]InsPn formation in HT29cl.19A and T84 cells.

	HT29cl.19A	T84
a) Control	1.0	1.0
b) Carbachol	4.7 ± 0.9 (10)	6.3 ± 1.1 (3)
c) 8-Br-cAMP	1.1 ± 0.1 (4)	ND
d) A23187	1.5 ± 0.3 (5)	ND
e) ST_A	ND	0.9 ± 0.1 (3)
f) Carbachol + 8-Br-cAMP	4.6 ± 1.0 (3)	ND
g) Carbachol + A23187	4.2 ± 1.1 (3)	ND
h) Carbachol + ST_A	ND	6.0 ± 0.9 (3)
i) PMA	1.1 ± 0.1 (5)	ND

Cells were preincubated for 10 minutes with 10 mM LiCl (a,b,c,d,e,i) or LiCl with 8-Br-cAMP (f) or A23187 (g) or ST_A (h) and carbachol (b,f,g,h), 8-Br-cAMP (c) or A23187 (d) were added for another 10 min. The incubations were terminated with trichloroacetic acid and [^{3}H]inositol phosphates were analyzed on Dowex columns as described in Materials and Methods. Data are expressed as factor stimulation of inositol phosphates production compared to control cells (mean ± SD). Number of experiments are indicated between parentheses. The concentrations used are: carbachol 100 μM, 8-Br-cAMP 1 mM, A23187 10 μM, ST_A 300 Units/ml, PMA 5.10^{-7} M. ND, not determined.

Measurements of $[Ca^{2+}]_i$. Cross-talk between the PI-cycle and cyclic nucleotides has been described to occur not only at the level of phospholipase C activation, but also at the level of calcium release. Therefore, we subsequently examined the effects of PMA and cAMP on intracellular calcium levels, by using indo 1-AM as a fluorescent calcium probe. The adenylate cyclase activator forskolin was used as a cAMP raising agent, since 8-Br-cAMP was autofluorescent at the wavelengths used for Indo 1-AM calcium measurements. Basal calcium levels were 159 ± 24 nM (mean ± SD, n=23). Carbachol induced a transient rise in calcium with a peak value of 832 ± 343 nM (n=8; Fig.5A). Both PMA and forskolin failed to induce calcium release (Fig.5B,C). However, 10 minutes pretreatment of the cells with PMA or forskolin resulted in a strong inhibition of the carbachol-induced calcium response down to a level of 275 ± 42 nM (n=6) and 302 ± 82 nM (n=6), respectively (Fig. 5B,C). In PKC α depleted cells, the carbachol-triggered calcium release showed a partial recovery (546 ± 155 nM; n=5; Fig.5D). In two experiments, a similar decrease in calcium mobilization was found when cells were pretreated with 8-Br-cAMP (the data were corrected for autofluorescence caused by 8-Br-cAMP addition), indicating that the inhibition is most likely mediated by cAMP and not due to non-specific effects of forskolin.

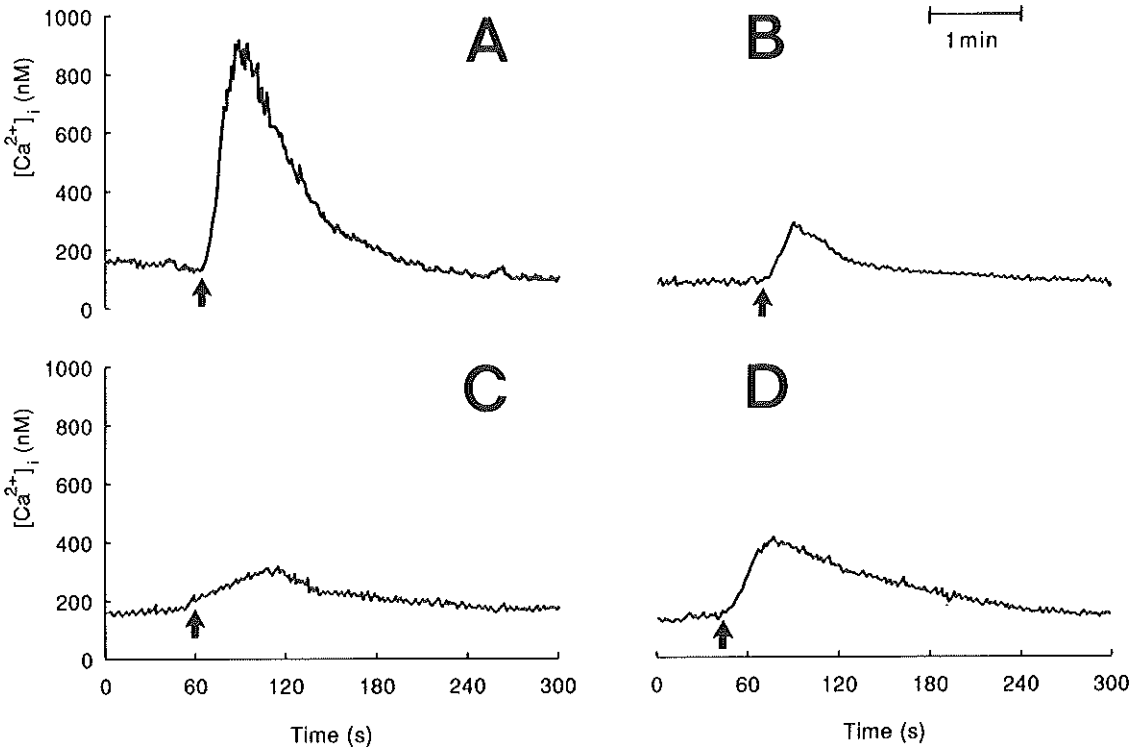

FIGURE 5. Effects of PMA pretreatment and cAMP on the carbachol-induced Ca^{2+}-rise. HT29cl.19A cells were loaded with indo 1-AM and changes in intracellular calcium levels were monitored in a fluorimeter as described in Material and Methods. (A) response to carbachol (250 μM). B) response to carbachol after 5 min pretreatment of the cells with 5 μM forskolin C) response to carbachol after 10 min pretreatment with 5.10^{-7} M PMA. D) carbachol response in cells pretreated for 27h with 5.10^{-7} M PMA. The tracings shown are representative for 5-8 experiments. Addition of carbachol is indicated with an arrow.

DISCUSSION

In this study cultures of intact colonic epithelial cells were exploited to investigate the possibility of cross-talk between cyclic nucleotides or other second messengers and the phosphoinositide (PI)-cycle. As enterocytes contain a PI-cycle at both the basolateral and the apical membrane, despite the lack of G-protein-coupled hormone receptors at the luminal membrane, cross-talk could be a physiologically important mechanism in the activation of the apical PI-pool. Hypothetically, PI-derived messengers at the apical membrane may serve as local regulators of cellular functions, e.g. Cl^- transport across apical Cl^- channels. However, our measurements of inositol phosphate (InsPn) accumulation and calcium signalling in human colonocytes did not provide evidence in favour of this model. In contrast, our data revealed the existence of a negative feedback mechanism by protein kinase C (PKC) on the carbachol-stimulated InsPn production and calcium release in HT29cl.19A cells. Cyclic AMP also partially inhibited the carbachol-induced calcium response, however without inhibition of the InsPn formation, indicating cross-talk between PI- and cAMP-mediated pathways. In chicken enterocytes, cAMP and calcium routes did also interact, but in an opposite manner: cAMP alone stimulated the mobilization of calcium (29). These differences between chicken enterocytes and human colonocytes might be species-specific. More plausibly, however, they may reflect regional differences in regulatory mechanisms along the lenght of the intestine: calcium was found to serve as an independent activator of Cl^- secretion in human ileum, but not in human and rabbit colon (2,8,12). A similar difference between chicken enterocytes and human colonocytes was found with respect to cGMP effects on Ca^{2+} mobilization: whereas the cGMP-linked agonists atrial natriuretic factor (ANF) and heat stable E.coli toxin (ST_A), have been shown to exploit Ca^{2+} as a third messenger in chicken and rat enterocytes, respectively (17,30), no evidence was found for a role of InsPn (present study) or Ca^{2+} (19) in the action of ST_A in T84 colonocytes.

The mechanism underlying PMA-inhibition of the carbachol-induced InsPn production is unknown, but inhibition of PLC activity or the coupling of this enzyme to a G-protein, as has been described to occur in other cells (18,28), is most likely. Surprisingly, a similar decrease in InsPn formation could not be demonstrated following activation of PKC by carbachol itself, since (i) the production of InsPn by carbachol was linear for up to 30 minutes (Fig.2), and (ii) downregulation of PKC did not result in potentiation of the InsPn release by

carbachol (Fig.4). The different efficacy of PMA- and carbachol-activated PKC is unlikely to result from the involvement of different PKC isozymes: carbachol-induced chloride secretion and PMA-induced inhibition of carbachol-activated InsPn release are both mediated by PKC α (35). As PMA is an irreversible activator of PKC, whereas carbachol transiently activates PKC (measured by PKC translocation, Ref.35), this might explain the observed differences. Such a discrepancy between PMA and endogenously activated PKC was not found universally in all cell types: both carbachol and PMA could inhibit Ins(1,4,5)P3-induced calcium release in pancreatic acinar cells (37).

Possible mechanisms for the cAMP-induced inhibition of the calcium mobilization may include: (i) a decrease in the activity of Ins(1,4,5)P3 to release calcium; (ii) inhibition of cellular calcium entry mechanisms; or (iii) activation of the Ca^{2+}ATPase. Examples of each of these mechanisms can be found in the literature (11,13,31). Which one of these molecular targets is predominant in the HT29cl.19A cells needs further study.

In renal epithelial cells, cGMP-provoked inhibition of the Na^+-channel is suggested to be mediated through protein kinase C (25). A similar intermediary role of PKC in the mechanism of action of cGMP-linked secretagogues (e.g. ST_A) could be postulated in intestinal epithelial cells. However, since ST_A was apparently unable to trigger InsPn release, either in intact colonocytes (this study) or in isolated apical membranes (34), the possibility that PKC is activated by diacylglycerol derived from PIP2 breakdown can be ruled out. Nevertheless, it remains possible that ST_A is capable of activating PKC independently of InsPn and calcium release, e.g. by activating a phosphatidylcholine specific phospholipase C (3) or by releasing diacylglycerol from yet unknown sources as has been reported for tracheal epithelial cells in response to ß-agonists (1).

In conclusion, we found no evidence for our hypothesis, that the intestinal apical PI-pool may serve as an amplification step in the regulation of chloride secretion by cAMP, cGMP, calcium or PKC. Although a predominant contribution of the basolateral PI-pool to InsPn generation and calcium mobilization may have masked minor effects of second messengers on the apical PI-pool, this possibility seems rather remote: at least in mature enterocytes, the capacity of the apical membrane to release InsPn exceeded the capacity of the basolateral membrane (34). The mode of regulation of the apical PI-cycle therefore still remains to be elucidated. The outcome of this study warrants a further search for agonists and receptors in the apical membrane, capable of activating apical G-proteins and phospholipase C and modulating apical transport systems through the local

generation of PKC activating and calcium mobilizing second messengers. In tracheal epithelial cells, calcium-linked P2-purinergic receptors have been identified in both the apical and the basolateral membrane (21). However, the relative contribution of the apical versus the basolateral membrane receptors to InsPn formation and Ca^{2+}-signaling was not assessed separately in this study.

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CHAPTER 5

DUAL ROLE FOR PROTEIN KINASE C α AS A REGULATOR OF ION SECRETION IN THE HT29cl.19A HUMAN COLONIC CELL LINE.

The involvement of protein kinase C (PKC) in the regulation of intestinal ion secretion was studied in polarized monolayers of the HT29cl.19A human colon carcinoma cell line. Carbachol, phorbol ester (PMA/PDB) and 8-Br-cAMP induced chloride secretion as measured by a rise in the short circuit current (I_{sc}). The electrical response to carbachol coincided with a transient translocation of PKC α from the soluble to the particulate fraction. The carbachol-, PDB- and 8-Br-cAMP-induced I_{sc} responses were inhibited by pretreatment of the cells with PMA (5.10^{-7} M) for 2h, a time period in which PKC α, ß1 and γ levels were not changed. As shown by $^{86}Rb^+$ and $^{125}I^-$ efflux studies, the main targets for this inhibition were basolateral K^+ transporters rather than apical Cl^--channels. Prolonged exposure to PMA (24 h) led to a 60% recovery of the 8-Br-cAMP response, but not of the carbachol- or PDB-provoked secretion. As shown by immunoblotting with PKC isozyme specific antisera, the recovery of the 8-Br-cAMP response coincided with the downregulation of PKC α, whereas the levels of PKC ß1 and γ were unmodified. These results suggest that PKC α, but not PKC ß1 or γ, is involved in both acute stimulation and chronic inhibition of ion secretion in the HT29cl.19A colonic cell line.

INTRODUCTION

The protein kinase C (PKC) family is known to consist of multiple isozymes encoded by a family of genes (Nishizuka, 1989; Rao and De Jonge, 1990). PKC α, ß1, ß2 and γ are calcium and phospholipid dependent kinases, with only minor differences in their dependence on diacylglycerol and their substrate specificity (Huang et al., 1988; Marais and Parker, 1989). PKC ε is a calcium independent, but phospholipid dependent kinase with a different substrate

specificity (Schaap and Parker, 1990). The more recently cloned PKC δ and ζ are not fully characterized yet (Ono et al., 1988). The physiological role of PKC isozymes in general and in epithelial ion transport in particular is still unknown. Since PKC α, ß and γ are described to be differentially downregulated by phorbol ester, this phenomenon could possibly be used as a tool to study isozyme specific effects (Isakov et al., 1990).

Sofar studies to determine the role of PKC in epithelial ion transport have been carried out with purified PKC, consisting of a mixture of isotypes (Hwang, 1989; Li et al., 1989), or with PKC inhibitors (staurosporine, H7, peptide pseudosubstrates) and PKC activators (phorbol esters, diacylglycerol analogues), which are unable to discriminate between distinct PKC isozymes (Rao and De Jonge, 1990). In membrane patches of tracheal cells, PKC provoked chloride channel activation at low calcium levels and chloride channel inactivation at high calcium levels, suggesting a model in which ion secretion in epithelial cells is controlled in an opposite direction by calcium dependent and calcium independent PKC isoforms (Li et al., 1989).

Current information on the role of PKC in intestinal ion secretion is based mainly on studies in rabbit and chicken ileum and in rat distal colon, in which PMA-induced Cl^- secretion is mediated predominantly by prostaglandin synthesis in sub-epithelial cell types (Smith et al., 1982; Chang et al., 1985; Donowitz et al., 1986; Musch et al., 1990). Recent studies in a Cl^- secreting subclone of the human colonic cell line, HT29cl.19A, however show that phorbol esters and cAMP trigger ion secretion also in the presence of prostaglandin inhibitors and suggest that both secretory pathways share the same transport systems required to allow net chloride secretion, i.e. apical chloride channels and basolateral localized K^+-channels, $NaKCl_2$-cotransporters and Na^+/K^+-ATPase (Vaandrager et al., 1992; Bajnath et al., 1991). Hence, the major difference between the stimulus-secretion pathways activated by phorbolesters and by cAMP appears to reside in the exploitation of different protein kinases, rather than different transport systems.

In the present study PKC isozyme specific antisera were used to examine the effects of phorbol ester treatment on the levels and possible translocation of each isozyme in HT29cl.19A cells. The effects of PKC activation and downregulation on intestinal ion transport pathways were monitored in parallel by a combination of short circuit current (I_{sc}) and isotope efflux measurements ($^{125}I^-$ and $^{86}Rb^+$). The results suggest a key role for PKC α, but not PKC ß1 or γ, in both acute stimulation and chronic inhibition of ion secretion in this cell line.

MATERIALS AND METHODS

Materials: Cell culture media and fetal calf serum were from Gibco. Forskolin, carbachol, PMA and PDB were from Sigma (St Louis, U.S.A.) and A23187 and 8-Br-cAMP from Boehringer, Mannheim, Germany. $Na^{125}I$ (14 Ci/mg), $^{86}RbCl$ (5 mCi/mg) and ^{14}C-labeled Rainbow ^{TM} protein molecular weight markers were from Amersham (England). Myo-[2-^{3}H]Inositol was obtained from NEN-Dupont, Germany. The rabbit polyclonal anti-peptide antibodies used in this study were raised and characterized earlier in the laboratory of Dr.P.J.Parker (Marais and Parker, 1989). These antibodies were found capable of specifically recognizing PKC isozymes and did not show cross-reactivity on immunoblots with other isoforms of PKC (see Marais and Parker, 1989, Figure 5).

Cell culture: HT29cl.19A cells (kindly donated by Dr. C.L. Laboisse, INSERM, Paris, France) were grown in cell culture flasks (Falcon) under a humidified 95% air, 5% CO_2 atmosphere at 37 °C in Dulbecco's modified Eagles medium, 10 % fetal calf serum, 40 mg/l penicillin and 90 mg/l streptomycin. Cells were subcultured at a surface ratio of 1 : 5 after trypsinization every 5 days, when they had reached 70-80 % confluence. The passage number of the cells used in this study varied between 11 and 30.

Measurements of short circuit current and transepithelial resistance: HT29cl.19A cells were grown to confluency (12-14 days after plating at a 1 : 2.5 surface ratio) on tissue culture treated polycarbonate filters firmly attached to a polystyrene ring (6.4 mm diameter, Transwell, Costar). The filter was placed in a tightly fitting insert separating the serosal and mucosal compartment of an Ussing chamber. The bathing solution was a modified Meyler buffer (108 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, 20 mM $NaHCO_3$, 0.8 mM Na_2HPO_4, 0.4 mM NaH_2PO_4, 20 mM Na-HEPES, 10 mM glucose, pH 7.4), which was aerated by a gas lift system using 95% O_2 and 5% CO_2. Short circuit current (I_{SC}) was measured with two platinum current electrodes in direct contact with the bath, and two calomel electrodes connected to the bath by salt bridges. The transepithelial electrical resistance of the filter-grown HT29cl.19A monolayers, as calculated from voltage deflections induced by positive and negative current pulses with a duration of 1 sec., was 108 ± 27 $\Omega.cm^2$ (mean ± S.D.; n=24) in the absence of secretagogues.

SDS-PAGE and immunoblotting: SDS-polyacrylamide gel electrophoresis was performed according to Laemmli, 1970. Samples were dissolved in SDS sample buffer (0.1 M Tris, 1.7 % SDS, 6.7 % glycerol, 0.7 M ß-mercapto-ethanol, 0.1 % bromophenol blue, pH 7), boiled for three minutes and separated on 7.5 % polyacrylamide slab gels. HT29cl.19A cells used for both the Ussing chamber measurements and immunoblotting were solubilized in boiling SDS sample buffer to prevent proteolytic degradation of PKC. After SDS-PAGE, proteins were electroblotted onto nitrocellulose paper (0.1 μM, Schleicher and Schuell) in 25 mM Tris, 192 mM glycine and 20% methanol. The blots were then incubated with 3% gelatin in Tris buffered saline (TBS: 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl) to block non-specific binding. After 2 washes with TTBS (0.05% Tween in TBS; 5 min./wash), the blots were incubated with antisera (1:500 in TTBS) for 60 minutes at room temperature. The blots were washed again in TTBS (3 times, 10 min./wash) and incubated with goat-anti-rabbit antiserum conjugated to alkaline phosphatase for 60 minutes at room temperature (1:1000 in TTBS). After five washes with TTBS and two with TBS (10 minutes/wash), 0.8 mg 5-Bromo-4-Chloro-3-

Indolylphosphate and 1.65 mg Nitro Blue Tetrazolium in 5 ml 0.2 M Tris, 10 mM $MgCl_2$, pH 9.1 was added for colour development. The reaction was stopped in H_2O.

PKC translocation assay: HT29cl.19A cells were grown to 90-100 % confluency in 6 well cell culture plates. One hour before the experiment started, the medium was replaced by a modified Meyler buffer. Incubation of the cells was done at room temperature for the indicated time period in fresh Meyler. The reaction was stopped in ice and the cells were immediately washed twice with icecold buffer A (25 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 2,5 mM Mg-acetate, 2 mM DTT), followed by a wash with lysis buffer at 4°C (buffer A + 2.5 mM EGTA). The cells were then scraped in lysis buffer containing protease inhibitors (leupeptin 10 μg/ml, PMSF 100 μM), sonicated (3 bursts of 5 sec.) and the particulate and the soluble fractions were separated in an airfuge (3 min., 100.000 g, 4°C). The particulate fraction was resuspended in lysis buffer containing protease inhibitors and sonicated (3 burst of 5 sec.). After protein determination, the fractions were solubilized in SDS sample buffer to a final concentration of 1 mg/ml or if necessary, concentrated by TCA precipitation (10% TCA, 10 min. on ice) and then solubilized in SDS-sample buffer. The pH of the samples was adjusted with 1 M Tris, pH 8.5. The recovery of the proteins was 80-100 %. The samples were separated by SDS-PAGE and, after transfer to nitrocellulose paper, incubated with PKC α, β1 and γ specific antisera.

Isotope efflux experiments: HT29cl.19A cells were grown to 70-80 % confluence in 6 well cell culture plates. Cells were loaded for 1.5 hour under a humidified 95% air, 5% CO_2 atmosphere at 37 °C in 0.5 ml of a modified Meyler solution containing the isotopes (3 μCi/ml $^{125}I^-$ or 1 μCi/ml $^{86}Rb^+$). Extracellular isotope was removed within 1 minute by washing three times with 3 ml modified Meyler solution at room temperature. The cells retained 0.31±0.4 % of the added $^{125}I^-$ and 23±4 % of the added $^{86}Rb^+$ (n=20). The isotope efflux was determined at 37 °C by addition and consecutive replacements of 1 ml of a modified Meyler solution at 1 to 2 minute intervals. The cells were kept under a 95% O_2 and 5% CO_2 atmosphere, except during the replacements. The residual isotope at the end of the experiment was determined by addition of 1 ml 1 M NaOH. Due to its relatively high uptake, $^{86}Rb^+$ could be determined together with $^{125}I^-$ in a gamma counter. To compare the isotope efflux at the various time points we expressed the data as the fractional efflux per minute i.e. the amount of released isotope in an interval as percentage of the amount of cell-associated isotope present at the beginning of that interval and divided by the length of that interval (c.f. Clancy et al., 1990).

Determination of total inositol phosphates: Cells grown to 70-80% confluency on 6 well cell culture plates were labeled for 48 hours with [^{3}H]Inositol (2μCi/ml) in inositol free medium, supplemented with 5% fetal calf serum under an atmosphere of 95% air, 5% CO_2 at 37°C. At 1 h before stimulation, the cells were shifted to serum-free DMEM containing 10 mM HEPES (pH 7.4). Control and PMA (27h, 5.10^{-7} M) pretreated cells were stimulated with 250 μM carbachol for 30 minutes in the presence of 10 mM LiCl. Incubations were terminated by replacing the medium by 1 ml of 10% ice-cold trichloroacetic acid. After 10 minutes, extracts were collected and trichloroacetic acid was removed by washing with diethylether. The samples were neutralized with 1 M Tris, pH 8.5 and processed for analysis of the total [^{3}H]inositol phosphate containing fraction (InsPn) by anion exchange chromotography on AG1 X8 Dowex columns (formate form) as previously described (Tilly et al., 1990). In some experiments, long-term pretreatment with PMA (27 h) decreased the total

water-soluble [^{3}H]Inositol uptake (as determined by radioactivity present before separation of the inositol phosphates). This has also been described for vascular smooth muscle cells (Pfeilschifter et al., 1989). The reason for this PMA related decrease in inositol uptake is unclear. In our calculations corrections were applied for these differences in total amount of radioactivity per sample.

Protein determination: Protein was determined by the protein assay kit from Pierce, Rockford Illinois.

RESULTS

Ion transport measurements. The effects of carbachol, Ca^{2+}-ionophore, phorbol esters and 8-Br-cAMP on the short circuit current (I_{SC}) in filter grown monolayers of HT29cl.19A colonocytes were measured in a modified Ussing chamber. In the HT29cl.19A subclone the I_{SC} mainly reflects electrogenic Cl^- secretion to the mucosal side, as demonstrated earlier by transepithelial and intracellular potential measurements (Bajnath et al., 1991) and isotope flux studies (Augeron et al., 1986). Muscarinic stimulation by carbachol induced a transient rise in I_{SC}, followed by a sustained second phase (Fig.1A; Table 1). The phorbol esters PDB (Fig.1B; Table I) and PMA (data not shown) induced a sustained response (EC_{50}: 5.10^{-8} M), either in the presence or absence of Ca^{2+}-ionophore (Fig. 1B, Table 1). Ca^{2+}-ionophore A23187 did not induce an electrical response itself (Fig.1B,C; Table 1), nor did it enhance the PDB-induced secretion (Table 1). The most potent secretagogue, 8-Br-cAMP, considered as a non-PKC linked agonist (Rao and De Jonge, 1990), induced a large and sustained rise in the I_{SC} that was considerably enhanced in the presence of Ca^{2+}-ionophore (Fig.1C; Table 1).

Addition of PDB to 8-Br-cAMP pretreated colonocytes had an additive but transient effect on the I_{SC}, followed by an inhibition down to basal levels within a time frame of 2 hours (Table 1; IC_{50}: 4.10^{-8} M). In the presence of Ca^{2+}-ionophore, the PDB inhibition of cAMP-provoked secretion was potentiated and basal I_{SC} values were reached as early as 60-80 minutes (Fig.1C). Prolonged exposure of the colonocytes to PMA (2-5h) resulted also in a strong inhibition of the response to carbachol and PDB (Fig.1A,B; Table 1). Chronic treatment of the cells with PMA (24 h) resulted in a 60% recovery of the 8-Br-cAMP response, whereas the carbachol response and the responses to PDB remained inhibited (Fig.1A,B,C; Table 1).

Both the stimulatory and inhibitory effects of PMA on the I_{SC} were not seen in response to the inactive phorbol ester analogue 4α-phorbol 12,13

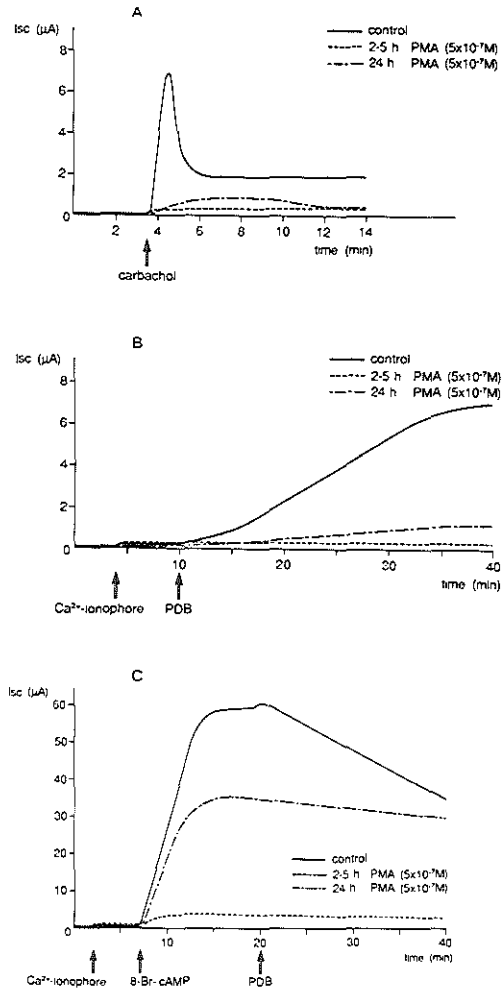

FIGURE 1. Stimulatory and inhibitory effects of phorbolesters on net ion secretion in HT29cl.19A colonocytes. Cells were grown as monolayers on filters and short circuit measurements were performed in a modified Ussing chamber. Panel A: Changes in the short circuit current (I_{SC}) provoked by carbachol (250 μM, ———) and its inhibition after 2-5 h (– – – –) and 24 h (·—·—) pretreatment with 5.10^{-7} M PMA. Panel B: Changes in I_{SC} provoked by addition of calcium ionophore (10 μM) and PDB (5.10^{-7} M; ———) and its inhibition after 2-5 h (– – – –) and 24 h PMA (·—·—) pretreatment of the cells with 5.10^{-7} M PMA. Panel C: Changes in I_{SC} provoked by sequential addition of calcium ionophore (10 μM), 8-Br-cAMP (0.7 mM) and PDB (5.10^{-7} M) in control (———) and PMA pretreated (– – –,·—·—) HT29cl.19A monolayers. The tracings shown are representative for at least three experiments (see table 1) and are expressed as I_{SC} per filter (0.33 cm^2). 2-5h PMA: full inhibition was already manifest at 2h PMA and persisted for the following 3h.

TABLE 1. Short circuit current measurements.

	I_{SC} (μA / cm^2)		
	Control	2-5 h PMA *	24 h PMA
Basal	0.2 ± 0.2 (10)	0.3 ± 0.3 (4)	0.3 ± 0.3 (4)
Carbachol	21.0 ± 8.4 (9)	0.6 ± 0.3 (3)	2.4 ± 1.8 (4)
Ca^{2+}-ionophore	0.3 ± 0.3 (7)	0.6 ± 0.6 (3)	3.6 ± 0.6 (3)
PDB	21.3 ± 4.2 (6)	ND	ND
Ca^{2+}-ionophore + PDB	21.6 ± 4.5 (6)	0.6 ± 0.6 (3)	0.6 ± 0.6 (3)
8-Br-cAMP	94.2 ± 31.8 (5)	6.6 ± 4.2 (3)	50.0 ± 12.3 (3)
8-Br-cAMP + Ca^{2+}-ionophore	178.2 ± 42.9 (3)	8.4 ± 5.3 (3)	104.7 ± 29.9 (6)
8-Br-cAMP + PDB	115.2 ± 35.3 (3)	6.6 ± 4.2 (3)	50.0 ± 12.3 (3)
8-Br-cAMP + Ca^{2+}-ionophore + PDB	181.8 ± 43.2 (5)	8.4 ± 5.3 (3)	104.7 ± 29.9 (6)

HT29cl.19A cells were grown to confluency on filters and the short circuit current (I_{SC}) was measured in a modified Ussing chamber with and without PMA pretreatment for 2-5 h and 24 h. The data are expressed in $\mu A/cm^2$ (mean ± SD) and represent maximal values. The concentrations used are: carbachol, 100 μM, Ca^{2+}-ionophore, 10 μM, PDB/PMA 5.10^{-7} M and 8-Br-cAMP, 0.7 mM. The number of experiments are indicated between parentheses. Values of transepithelial electrical resistance (mean ± SD; Ωcm^2) in control, 2-5 h PMA pretreated and 24 h PMA pretreated monolayers were 108 ± 27 (n=24), 119.5 ± 26.6 (n=8) and 142.1 ± 24.7 (n=9), respectively. ND, not determined. * Full inhibition was already manifest at 2h and persisted for the following 3h.

didecanoate (5.10^{-7} M). The transport inhibition was not accompanied by significant changes in electrical resistance (Table 1, legend), arguing against a possible impairment of cell viability or monolayer architecture. These data suggest a dual role for protein kinase C (PKC) in acute stimulation (Fig.1A,B; Table 1) and chronic inhibition (2-5h PMA; Fig.1A,B,C; Table 1) of ion secretion in HT29cl.19A cells.

Iodide and rubidium efflux measurements. Earlier studies have shown that the transport system responsible for PMA-induced activation of the I_{SC} is a chloride channel in the apical membrane of the HT29cl.19A colonocytes (Vaandrager et al., 1992). In the present study, the transport systems serving as possible targets for PMA-induced inhibition were studied in more detail by measuring the effects of PMA on iodide and rubidium efflux rates, mainly reflecting the activity of apical chloride channels and basolateral potassium transporting systems, respectively (Clancy et al., 1990; Venglarik et al., 1990; Vaandrager et al., 1991). Forskolin, a specific activator of adenylate cyclase, was used instead of 8-Br-cAMP as a cAMP

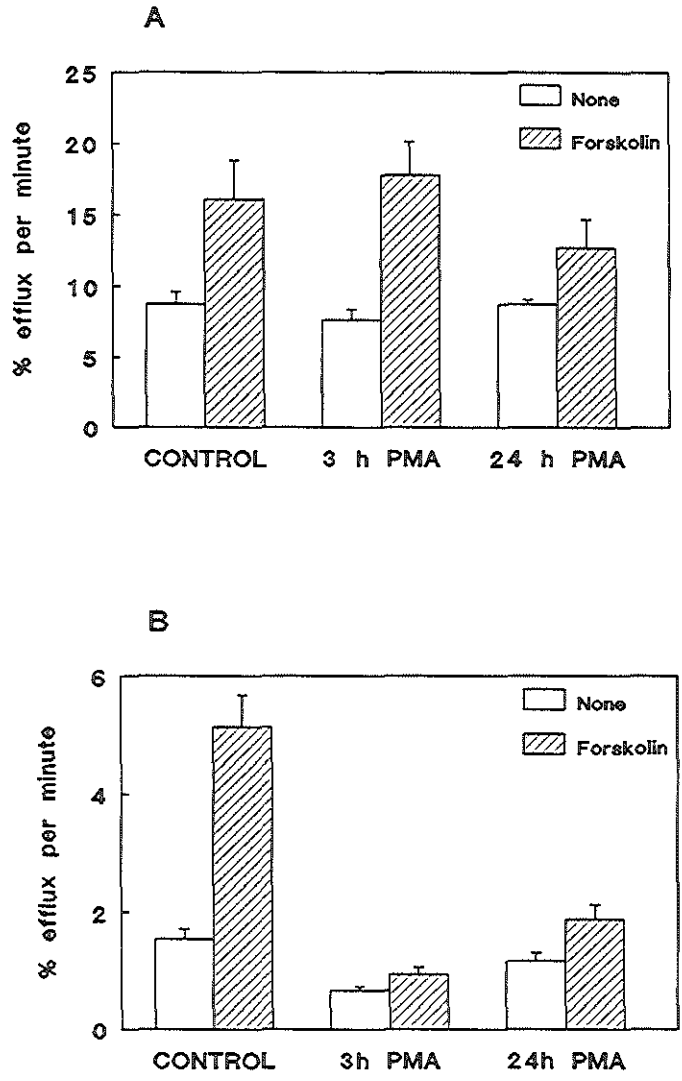

FIGURE 2. Effects of PMA on forskolin induced ^{86}Rb and ^{125}I efflux. The iodide (panel A) and rubidium (panel B) efflux in response to forskolin (5 μM) was studied in HT29cl.19A cells with and without PMA pretreatment for 3 and 24 hours. Data are expressed as maximal values (mean ± SD) and were obtained after 5 minutes forskolin stimulation. The data are representative for at least two other experiments. The rubidium efflux following 24 h PMA pretreatment was significantly different from the values obtained after 3 h PMA exposure in both control ($p < 0.05$, Student's t-test) and forskolin treated cells ($p < 0.05$, Student's t-test).

raising agent, since the response to 8-Br-AMP was relatively slow and therefore difficult to measure accurately in the efflux assay.

As shown in Fig.2A, PMA treatment of the cells for three hours did not inhibit the forskolin-induced iodide efflux, whereas only a slight inhibition was found after 24 hours of PMA exposure. In contrast, the forskolin-induced rubidium efflux was strongly inhibited after 3 hours of PMA exposure and showed a partial recovery (30%) after 24 hours of PMA pretreatment (Fig.2B). Since the potassium transport systems seem to be shared by the different secretion pathways, we conclude that the inhibition of the 8-Br-cAMP-, carbachol- and PDB-induced I_{sc} responses after 2-5 hours PMA pretreatment apparently results from the inhibition of a potassium transport mechanism rather than a chloride channel.

Effects of PMA on PKC isozyme levels. In order to identify the type of isozyme(s) of PKC involved, possible effects of PMA pretreatment on the cellular content of PKC isozymes were tested by immunoblotting with PKC isozyme specific antisera. Filter grown monolayers of HT29cl.19A cells were pretreated for various time periods with PMA, monitored in the Ussing chamber and immediately boiled in SDS-sample buffer to prevent proteolytic degradation of PKC. Immunoblotting with PKC isozyme specific antisera showed a complete downregulation of PKC α following 24 hours exposure to 5.10^{-7} M PMA (Fig.3A). No downregulation was detectable at early time points up to 5 hours (Fig.3A). In contrast, the levels of PKC ß1 and PKC γ remained unaffected following PMA treatment for up to 24 hours (Fig.3B,C). No significant levels of PKC ß2 or PKC ϵ could be detected by the immunoblotting technique (results not shown). Reactivity with PKC α, ß1 and γ (both bands) could be blocked by presaturating the antisera with the antigenic peptide (results not shown). As in leukemic T cells (Isakov et al., 1990), PKC γ was manifest as a doublet in both HT29cl.19A homogenates and in two batches of freshly purified PKC mix (Fig.3). However, a single band is found in most other studies (see Marais and Parker, 1989). Whether the doublet reflects a covalent modification or different subtypes of PKC γ is presently unknown. The selective downregulation of PKC α after 24h PMA pretreatment coincided with the partial (60%) recovery of the 8-Br-cAMP response in the Ussing chamber, suggesting a role for PKC α in the inhibition of the I_{sc} responses following 2h PMA pretreatment.

Carbachol-induced PKC α translocation. The sustained loss of PDB- and carbachol-induced ion secretion in PKC α depleted cells suggest that PKC α

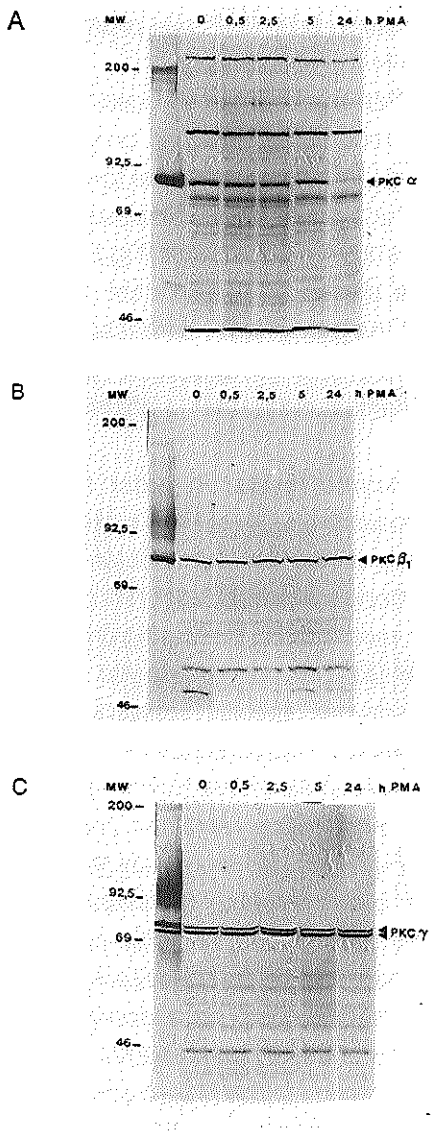

FIGURE 3. Effects of PMA pretreatment on levels of PKC α, β1 and γ. HT29cl.19A cells were pretreated for several hours with 5.10^{-7} M PMA. Samples of cell homogenates (15 μg protein) were separated by SDS-PAGE, transferred to nitrocellulose paper and incubated with PKC isozyme specific antisera. A) Antiserum specific for PKC α (1:500). B) Antiserum specific for PKC β_1 (1:500). C) Antiserum specific for PKC γ (1:500). The first lane contains a purified PKC mix (0.5 μg) from human brain together with rainbow marker proteins. Similar results were obtained in two other experiments.

might act as a mediator of carbachol- and PDB-induced ion secretion. To test this possibility, PKC translocation assays were performed using PKC isozyme specific antisera. Plastic-grown monolayers of HT29cl.19A colonocytes were exposed for 0-10 min. to carbachol or 10 min. to PMA or Ca^{2+}-ionophore and the particulate and the soluble fractions were separated. Immunoblotting with PKC isozyme specific antisera identified a transient PKC α redistribution in response to carbachol: PKC α appeared in the particulate fraction at 2-5 minutes following carbachol addition and disappeared again after 10 minutes of carbachol exposure (Fig.4). The fraction of PKC α translocated in response to carbachol was apparently too small to reveal a significant fall of PKC α levels in the soluble fraction. Despite the presence of PKC α in the particulate fraction at 5 min., a fall in the carbachol-dependent I_{sc} was manifest. This observation suggests that 1) incomplete translocation of PKC is apparantly sufficient to reach the I_{sc} peak value and 2) a separate negative feedback signal (e.g. protein dephosphorylation) is generated which is clearly different from reversed translocation of PKC α from the particulate to the soluble fraction. PMA induced a similar but persistent translocation of PKC α, whereas Ca^{2+}-ionophore had no effect on PKC α

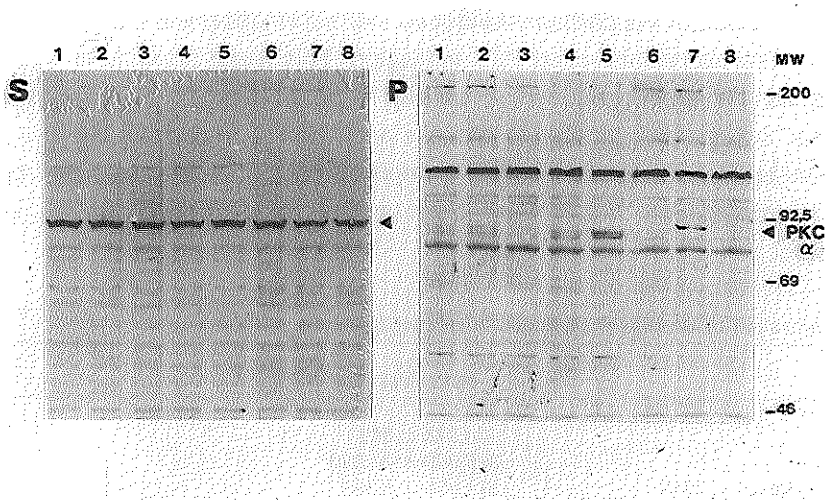

FIGURE 4. Carbachol induced PKC α translocation. HT29cl.19A cells were exposed for several time periods to carbachol (100 μM), Ca^{2+}-ionophore (10 μM) or PMA (5.10^{-7} M). Soluble (S) and particulate fractions (P) were separated by centrifugation and samples of each fraction (20 μg protein) were subjected to SDS-PAGE, transferred to nitrocellulose and incubated with PKC α specific antiserum (1:500). Lane 1: control, lane 2-6: carbachol 1, 1.5, 2, 5 and 10 minutes, resp., lane 7: PMA 10 minutes, lane 8: Ca^{2+}-ionophore 10 minutes. Similar results were obtained in two other experiments.

redistribution (Fig.4), supporting the concept that PKC α is a mediator of the PMA- and carbachol-induced ion secretion.

In most experiments, the PMA- and carbachol-induced PKC redistribution differed in that carbachol caused the appearance of a doublet of PKC α in the particulate fraction, which migrated faster than the single band of PKC α observed in PMA treated cells (Fig.4). However, in some experiments (2 out of 6) a doublet was also found in response to PMA, whereas occasionally (1 out of 3 experiments) a single band was detected in response to carbachol (results not shown). The doublet presumably reflects phospho- and dephosphoforms of the enzyme (Parker et al., 1987).

Since PKC β1 and PKC γ were located predominantly in the particulate fraction, translocation of these isozymes could not be studied (results not shown).

Carbachol-induced inositol phosphate formation. The loss of carbachol-induced ion secretion in PKC α depleted cells could in principle also result from the inhibition of phospholipase C (PLC) by a downregulation resistant form of PKC. To test this possibility, we measured the formation of inositol phosphates (InsPn) in the absence and presence of carbachol in control and PMA pretreated cells (27h). As shown in Table 2, chronic PMA pretreatment did not lead to a significant reduction of the carbachol-induced InsPn formation and thus does not interfere with carbachol-provoked PLC activation. This result strongly suggests that (i) the diacylglycerol produced, in spite of its possible action as activator of PKC β1 or γ, is apparantly unable to provoke active ion secretion; (ii) the loss of carbachol-induced ion secretion in PKC α depleted cells most likely results from PKC α downregulation rather than from a lack of activating signal, i.e. diacylglycerol.

TABLE 2. Formation of inositol phosphates in HT29cl.19A cells.

Addition	InsPn (dpm)
a) None	24,872 ± 923
b) Carbachol	325,989 ± 23,090
c) Carbachol after 27 h PMA	324,955 ± 8,928

[^{3}H]Inositol labeled HT29cl.19A cells were incubated for 30 min in the presence of 10 mM LiCl alone (a), LiCl and 250 μM carbachol (b) or were pretreated for 27 h with 5.10^{-7} M PMA, before addition of carbachol and LiCl (c). The reaction was stopped by adding trichloroacetic acid. Inositol phosphates (InsPn) were analyzed on Dowex columns as described in Material and Methods. Data are expressed as mean ± SD and are representative for three experiments done in triplicate.

DISCUSSION

Expression pattern of PKC isozymes in HT29cl.19A colonocytes. In the present work, using PKC isozyme specific antisera, the HT29cl.19A cells were shown to express three PKC isozymes, α, ß1 and γ. Two other PKC isotypes, ß2 and ϵ could not be detected immunologically. To our knowledge, the expression of PKC γ in non-neuronal tissue has not been described before. However, since intestinal and neuronal tissues are embryologically related, this could account for the unexpected detection of PKC γ in HT29cl.19A colonocytes. This study presents evidence for a complex role of PKC α, but not PKC ß1 or γ, as a regulator of intestinal ion transport. Although PKC ß1 and γ are also expressed in significant quantities, no clue was provided concerning their function.

Role of PKC α as an ion secretion inhibitor. The possible target(s) for PMA induced inhibition of the I_{SC} responses was investigated by a separate assessment of Cl^- and K^+ channel activity, based on measurement of iodide and rubidium efflux rates, respectively. Surprisingly, no evidence was found for an inhibitory action of PKC activators on forskolin-(i.e. cAMP-) provoked Cl^--channel activation under conditions in which the cAMP-provoked I_{SC} was completely abolished (Fig. 2A). In this respect the HT29cl.19A colonocytes clearly differ from tracheal epithelium, in which PMA was shown to inhibit cAMP-provoked iodide efflux in intact cells and to block protein kinase A-activated Cl^--channels in excised membrane patches in the presence of purified PKC (Li et al., 1989). The molecular basis for this tissue difference in Cl^--channel regulation is presently unknown, but may reside in possible differences in PKC isozyme pattern/localization, in Cl^--channel subtypes or in other regulatory factors.

Instead, 3h exposure of the HT29cl.19A colonocytes to PMA led to an almost complete inhibition of the forskolin-induced rubidium efflux, indicating the inhibition of a Rb/K transport system by PKC. In the parental HT29 cell line, PMA has been reported to inhibit the bumetanide sensitive $NaKCl_2$-cotransport activity with a similar time course (Franklin et al., 1989). Since the PMA-induced inhibition is relatively slow, the mechanism most likely involves a reduction in the number of K^+ transporters, e.g. by endocytosis or proteolytic breakdown, as has been described for the $NaKCl_2$-cotransporter (Franklin et al., 1989). This process may be triggered by PKC-catalyzed phosphorylation of the K^+ transporters theirselves or may possibly involve different (regulatory) phosphoproteins.

Our conclusion that PKC α rather than any of the other isozymes

expressed in this cell line (i.e. PKC ß1 and γ) is responsible for transport inhibition is based on our finding that part of the cAMP-induced I_{sc} (60%, Fig.1C) and ^{86}Rb efflux rate (30%, Fig.2B) is restored following 24 hours PMA treatment. At this time point, the cellular level of PKC α was completely downregulated, whereas PKC ß1 and γ remained resistant to downregulation (Fig.3). The finding that the recovery of the I_{sc} response is less than 100% can be explained in several ways. For example, the cAMP-induced ^{86}Rb efflux apparently recovers very slowly and might remain rate limiting for net ion secretion. Alternatively, a fraction of the 8-Br-cAMP induced ion secretion may be mediated via PKC α. This possibility has gained interest in view of the recent finding that forskolin is able to elevate diacylglycerol levels in tracheal cells (Anderson and Welsh, 1990). It is also possible that expression of the cAMP-activated apical Cl^--channel is impaired following chronic PMA treatment. The recent findings that (i) the cystic fibrosis gene encoded protein, CFTR, is most likely identical to the cAMP-activated Cl^--channel itself (Anderson et al., 1991; Kartner et al., 1991) and (ii) expression of the CF gene is indeed strongly inhibited by phorbol ester treatment (Trapnell et al., 1990) lends some support to the latter explanation.

Role of PKC α as a mediator of ion secretion. The carbachol- and PDB-provoked rise in short circuit current (I_{sc}) shown in Fig.1 and the rapid increase in fractional efflux of ^{36}Cl and ^{125}I occurring across the apical membrane as observed in a previous study (Vaandrager et al., 1992) suggest that their common mediator, PKC, exerts an acute stimulatory effect on apical Cl^- channels in the HT29cl.19A colonocytes. The PKC α subtype is likely to play a crucial role in the activation process for the following reasons. First, both carbachol and PMA were found to induce a rapid translocation of a small fraction of the cellular PKC α pool from the cytosolic to the particulate fraction. The carbachol-provoked translocation is apparently triggered by diacylglycerol formation rather than calcium signalling: Ca^{2+}-ionophore alone does not provoke net ion secretion (Fig.1) and does not translocate PKC α (Fig.4), although in other cell types Ca^{2+}-ionophore induced PKC translocation has been described (Ho et al., 1988). Secondly, the initial phase of the carbachol induced I_{sc} response (Fig.1) and the PKC α translocation (Fig.4) were both transient (lasting 2-5 minutes), whereas phorbol ester-provoked changes in I_{sc} (Fig.1) and PKC α translocation (Fig.4) were sustained for at least 10 minutes. Thirdly, our finding of a selective recovery of cAMP-, but not of carbachol/PDB-provoked ion secretion in PKC α downregulated cells, under conditions in which all other PKC isozymes were normally expressed, also

strongly supports an obligatory role for PKC α in carbachol- and PDB-triggered ion secretion. Other possible explanations for a selective inhibition of carbachol/PDB-induced secretion, i.e. PMA-induced uncoupling of the muscarinic receptor from phospholipase C or a complete PMA-induced downregulation of any of the participating transport systems itself, are rather remote, considering our observations that: (i) the production of inositol phosphates by carbachol is not inhibited after 27h PMA treatment (Table 2), and (ii) the same transporters which fail to respond to carbachol/PMA-induced signals are still able to respond to cAMP-induced signals.

In summary, this study presents evidence for a complex role of PKC α as a regulator of intestinal ion transport. Among the various PKC isozymes detected immunologically in the HT29cl.19A colonocytes (α, β1, γ) only the α form showed rapid translocation in response to PKC activators (PMA, carbachol) and became downregulated by chronic exposure to PMA (24h; 5.10^{-7} M) [1]. Measurements of ion transport activities carried out in parallel suggest a dual role for PKC α as a stimulator and inhibitor of intestinal ion secretion. $^{86}Rb^+$ and $^{125}I^-$ efflux studies showed that inhibition was relatively slow and targetted on basolateral potassium transport systems, while the stimulation was acute and exerted at the level of an apical chloride channel. Extrapolation of these results to the intestinal epithelium *in vivo* would imply that the action of intestinal secretagogues capable of provoking prolonged PKC activation (similar to PMA) would be self-limiting (i.e. lasting < 5h) and would bring about a state of resistance to other secretagogues, including microbial toxins (cholera toxin, heat-stable E.coli toxin) acting through different intracellular signals (cAMP, cGMP). Attempts to substantiate this prediction in an animal model are in progress in our laboratory.

FOOTNOTE

[1] Preliminary results, using PKC δ, ζ and η specific antisera, indicate that PKC ζ and η (but not PKC δ) are also expressed in the HT29cl.19A cells, but do not downregulate in response to PMA.

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CHAPTER 6

PHORBOL ESTERS STIMULATE AND INHIBIT Cl^- SECRETION BY DIFFERENT MECHANISMS IN A COLONIC CELL LINE.

Phorbol 12-myristate 13-acetate (PMA) was found to increase both the short-circuit current (I_{sc}) and the efflux of $^{125}I^-$ or $^{36}Cl^-$ in the colonic epithelial cell line HT29cl.19A. Neither the PMA-provoked rise in I_{sc} nor the stimulation of $^{125}I^-$ efflux was affected by the cyclooxygenase inhibitor indomethacin. The PMA-induced increase in Cl^- efflux was not accompanied by a rise in cAMP levels. A prolonged incubation with PMA (3 h), however, inhibited the PMA- and the cAMP-stimulated I_{sc} by > 90%, whereas the cAMP-provoked $^{125}I^-$ and $^{36}Cl^-$ efflux was not inhibited. The long-term PMA treatment was found to inhibit the basal and cAMP-provoked $^{86}Rb^+$ efflux by 65 ± 9% and 86 ± 7% respectively. A 3 h incubation with PMA also strongly inhibited the Ca^{2+} ionophore A23187-induced increase in $^{86}Rb^+$ efflux, whereas the A23187-stimulated $^{125}I^-$ efflux was only marginally inhibited. These data suggest that phorbol esters, presumably by activation of protein kinase C, can provoke Cl^- secretion in HT29cl.19A colonocytes independently of a prostaglandin- or cAMP-mediated pathway. Prolonged exposure to PMA, however, causes an inhibition of net electrogenic Cl^- secretion by downregulation of the activity of K^+ transporters.

INTRODUCTION

Activation of protein kinase C is thought to be a key step in the mechanism of action of various intestinal secretagogues (6,7,11-13,21). In excised patches of Cl^--secreting tracheal epithelial cells, protein kinase C and cAMP-dependent protein kinase were shown to directly activate apical Cl^- channels (15,17). From these studies it was suggested that protein kinase C stimulates Cl^- secretion independently of cAMP-dependent protein kinase by activating apical Cl^- channels.

The phorbol esters, phorbol 12-myristate 13-acetate (PMA) and phorbol 12,13-dibutyrate (PDB), are known to activate protein kinase C and to reproduce the effects of diacylglycerol (DAG), the main second messenger involved in the protein kinase C signal-transduction pathway (20,21). In rabbit and chicken ileum (7,19), and in rat distal colon (12) the phorbol ester-provoked electrogenic Cl^- secretion was found to be mediated largely by prostaglandins, known activators of adenylate cyclase. However, the presence of sub-epithelial cells in the intestinal preparations, which were found to be the main sources of the phorbol ester-generated prostaglandins (19,23) may mask direct effects of the activation of protein kinase C on epithelial cells.

The present study was aimed to investigate the effects of the phorbol esters on Cl^- secretion in a homogenous population of colonic epithelial cells. The subclone 19A of the human colon carcinoma cell line HT29 was selected as a model system because it responds to both cAMP-linked (e.g. forskolin) and Ca^{2+}-linked secretagogues (e.g. carbachol). It also forms tight monolayers when grown on filters, enabling electrical measurements in an Ussing chamber (2-5).

Both the acute and the long-term effects of PMA on Cl^- secretion were monitored, considering the opposite effects of both treatments reported for other epithelial cells. After a stimulatory phase, PMA was found to inhibit cAMP-activated Cl^- efflux in tracheal cells (17) and to diminish Na-K-Cl_2 cotransport activity in HT29 colonocytes (14).

We report here that phorbol esters stimulate Cl^- secretion in HT29cl.19A colonocytes, independently of prostaglandin synthesis and without a detectable increase in the cAMP levels. Prolonged incubation with PMA did not inhibit the cAMP- or Ca^{2+}-activated Cl^- conductance but suppressed net electrogenic Cl^- secretion by inhibiting K^+ transport pathways.

MATERIALS AND METHODS

Materials: HT29cl.19A cells were kindly donated by Dr. C.L. Laboisse (Institut National de la Sante et de la Recherche Mediacale, Paris). Cell culture media and fetal calf serum were derived from GIBCO. Forskolin, carbachol, PMA, PDB and 1-oleyl-2-acetylglycerol (OAG) were obtained from Sigma Chemical (St Louis, MO) and A23187 and 8-Br-cAMP from Boehringer (Mannheim, Germany). $H^{36}Cl$ (19 μCi/mg), $Na^{125}I$ (14 Ci/mg), and $^{86}RbCl$ (5 mCi/mg) were purchased from Amersham (UK).

Cell culture: HT29cl.19A cells were grown in cell culture flasks (Falcon) under a humidified 95% air-5% CO_2 atmosphere at 37°C in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum. The medium additionally contained 40 mg/l penicillin and 90 mg/l streptomycin. Cells

were subcultured at a surface ratio of 1 : 5 after trypsinization every 5 days, when they had reached 70-80 % confluence. The passage number of the cells used in this study varied between 11 and 30.

Measurements of short-circuit current: HT29cl.19A cells were grown to confluence (7-9 days after plating at a 1:2.5 surface ratio) on tissue culture-treated polycarbonate filters firmly attached to a polystyrene ring (6.4 mm diameter, Transwell, Costar). The ring containing the filter was placed in a tightly fitting insert separating the serosal and mucosal compartment of an Ussing chamber. The bathing solutions consisted of a modified Meyler buffer [(in mM) 108 NaCl, 4.7 KCl, 1.3 $CaCl_2$, 1.0 $MgCl_2$, 20 $NaHCO_3$, 0.8 Na_2HPO_4, 0.4 NaH_2PO_4, 20 Na-N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and 10 glucose, pH 7.4], which were perfused and aerated by a gas-lift system using 95% O_2-5% CO_2. The Cl^--free medium consisted of (in mM) 128 Na-gluconate, 4.7 K-gluconate, 1.3 Ca-cyclamate, 1.0 $MgSO_4$, 0.8 Na_2HPO_4, 0.4 NaH_2PO_4, 20 Na-HEPES and 10 glucose, pH 7.4, aerated with 100% O_2. I_{SC} was measured with two platinum current electrodes in direct contact with the bath, and two calomel electrodes connected to the bathing media by salt bridges. The electrical resistance of the filter-grown HT29cl.19A monolayers, as determined at the beginning of the I_{SC} measurements, was 115 ± 27 (SD) $\Omega.cm^2$ (n = 18).

Isotope efflux experiments: HT29cl.19A cells were either grown to 70-80% confluence (4-5 days after plating) in six-well cell culture plates (diam wells 34 mm; Nunc) or to full confluence on a filter (diam 24 mm, Transwell, Costar) similarly as described for the determination of I_{SC}. Cells were loaded for 1.5 h under a humidified 95% air-5% CO_2 atmosphere at 37°C in 0.5 ml (wells) or 2.5 ml (filters) of a modified Meyler solution containing the isotopes (5 μCi/ml $^{36}Cl^-$, 3 μCi/ml $^{125}I^-$ or 1 μCi/ml $^{86}Rb^+$). The extracellular isotope was removed within 1 min with three washes of 3 ml modified Meyler solution at room temperature. The cells in the wells were found to retain 0.31 ± 0.4% of the added $^{125}I^-$, 0.23 ± 0.04% of the added $^{36}Cl^-$ and 23 ± 4% of the added $^{86}Rb^+$ (n = 20). For the filter-grown cells these figures were 0.14 ± 0.02% for $^{125}I^-$, 0.09 ± 0.02% for $^{36}Cl^-$ and 8.9 ± 1.0% for $^{86}Rb^+$ (n = 12). The isotope efflux from the cells grown in wells was determined at 37 °C by addition and consecutive replacements of 1 ml of a modified Meyler solution at 1 to 2 min intervals. The cells were kept, except during the replacements, under a 95% O_2-5% CO_2 atmosphere. The residual isotope at the end of the experiment was determined by addition of 1 ml 1 M NaOH. The efflux into the upper compartment (mucosal side) of the cells grown on filters was determined in a similar way, whereas the efflux into the serosal bath was monitored simultaneously by transferring the filters at the same time points into consecutive wells containing 2 ml of modified Meyler. $^{125}I^-$ was measured simultaneously with $^{36}Cl^-$ by β-scintillation counting, whereas $^{86}Rb^+$, due to its relatively high uptake, could be determined together with $^{125}I^-$ in a gamma counter.

To render the isotope efflux at the various time points more comparable we expressed the data as the fractional efflux per min, i.e. the amount of isotope lost from the cells in the interval preceding a time point expressed as a percentage of the amount of isotope associated with the cell layer at the beginning of that interval and divided by the length of that interval (24).

cAMP determination: HT29cl.19A cells were cultured in six-well plates as described for the isotope efflux experiments. The cells were preincubated in 1 ml of a modified Meyler solution for 30 min at 37°C under a 95% O_2-5% CO_2 atmosphere, and consecutively incubated in a fresh Meyler solution (1 ml) for 10 min (control, PMA) or 3 min (forskolin). The incubation was terminated by removal of

the Meyler solution and addition of 0.25 ml of ice-cold 0.1 M HCl to the wells. The cAMP levels were determined with a commercial test kit from Amersham.

RESULTS

Short term effects of PMA on Cl^- secretion. In HT29cl.19A cells, the secretagogue-induced increase in transepithelial electric current was found to be caused predominantly by the opening of apical Cl^- conductances, and is thus indicative for electrogenic Cl^- secretion (3-5). After a lag phase of 5-10 min, PMA (0.5 μM) was found to provoke a clear increase in I_{sc} across a monolayer of filter-grown HT29cl.19A cells (Fig.1, Table 1). We also observed a slight increase in the transepithelial resistance after addition of PMA. The delay in the response might be caused by a slow penetration of PMA into the colonocytes, as the more permeant phorbol ester PDB (0.1 μM) induced a similar rise in I_{sc} without a significant lag time (results not shown). As shown in Table 1, OAG (0.1 mM), a membrane permeant analog of the physiological activator of protein kinase C DAG, was able to mimic the effect of PMA on I_{sc}. The PMA-induced rise in I_{sc} was not observed in Cl^--free medium and was inhibited by subsequent serosal addition of bumetanide (0.1 mM), an inhibitor of Na-K-Cl_2 cotransport, or barium (5 mM), an inhibitor of K^+ channels by 86 ± 5% and 92 ± 4% (n=3) respectively.

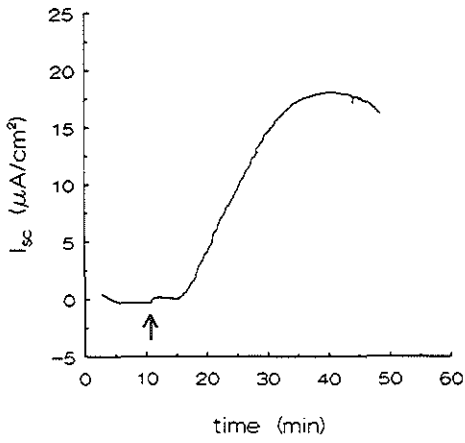

FIGURE 1. Phorbol 12-myristate 13-acetate (PMA) provoked rise in short-circuit current (I_{sc}) in HT29cl.19A cells. Confluent monolayers of HT29cl.19A cells grown on filters were placed in Ussing chamber. I_{sc} was monitored as described in MATERIALS AND METHODS. PMA (0.5 μM) was added to both mucosal and serosal bath at timepoint indicated with arrow. Shown is representative tracing from 6 experiments.

Because the activity of both cotransporters and basolateral K^+ channels is required during sustained Cl^- secretion (6), these data also indicate that the stimulation of I_{sc} by PMA represents an increase in electrogenic Cl^- secretion.

TABLE 1. Effects of PMA on I_{SC} in HT29.cl19A colonocytes.

Addition	I_{SC} max ($\mu A/cm^2$)	
	control	3 h PMA
Basal	0.3 ± 0.3*	
PMA (0.5 μM)	19.8 ± 8.7	1.5 ± 0.8
OAG (100 μM)	16.3 ± 4.9	ND
PMA (0.5 μM) + indomethacin (20 μM)	24.8 ± 7.2	ND
8-Br-cAMP (1mM)	94.0 ± 32	6.6 ± 3.2

Values are means ± SD; n=6 experiments. Confluent monolayers of HT29cl.19A cells grown on filters were placed in Ussing chamber. Short-circiut current (I_{SC}) was monitored as described in MATERIALS AND METHODS. Phorbol 12-myristate 13 acetate (PMA) and 1-oleyl-2-acetylglycerol (OAG) were added to both mucosal and serosal bath, wheras 8-bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP) was added to serosal bath only, because no further changes were detected after subsequent addition to the mucosal bath in initial experiments. Indomethacin (20 μM) was added to both bathing solutions 20 min before the addition of PMA. Prolonged PMA treatment was perfomed by exposing the filters to 0.5 μM PMA for 3 h in the cell culture medium in incubator and subsequent addition of PMA to bath solutions after filters were placed in Ussing chamber. Maximal value of I_{SC} was reached 25-30 min after addition of PMA and OAG or 10-15 min after addition of 8-Br-cAMP. ND, not determined. *Data from Ref 24.

To test whether the PMA-provoked rise in I_{SC} in HT29cl.19A cells was mediated by prostaglandins, we repeated these experiments in the presence of the cyclooxygenase inhibitor indomethacin. As shown in Table 1, the PMA-induced rise in I_{SC} appeared unaffected by indomethacin at a concentration up to 20 μM.

To ascertain that the PMA-provoked increase in I_{SC} resulted from the activation of a Cl^- conductance pathway, we measured the effect of PMA on the efflux of radiolabeled I^- and Cl^-. Efflux measurements of $^{125}I^-$ and $^{36}Cl^-$ have been advocated recently as a convenient method for studying Cl^- conductances in epithelial cells (8,24,26). PMA present in the efflux medium caused a small but significant increase in $^{125}I^-$ and $^{36}Cl^-$ efflux from HT29cl.19A cells grown on plastic (Fig.2). Because the unstimulated fractional efflux rate decreased with time, precise quantitation of the PMA effect was difficult (cf.Ref 24). The initial increase (determined at 3 min after the addition of PMA) in fractional $^{36}Cl^-$ efflux was found to be 2.2 ± 0.6-fold larger than the increase in fractional $^{125}I^-$ efflux. The observed $^{36}Cl^-$ to $^{125}I^-$ ratio of the PMA stimulated efflux resembled that of the cAMP-activated anion efflux (1.91 ± 0.45), but was dissimilar to the Ca^{2+} ionophore-induced efflux ratio (0.36 ± 0.14) determined under similar conditions (24). The difference in Cl^- to I^- efflux ratio between the cAMP- and Ca^{2+}-stimulated anion efflux suggests that these second messenger activate different types of Cl^-

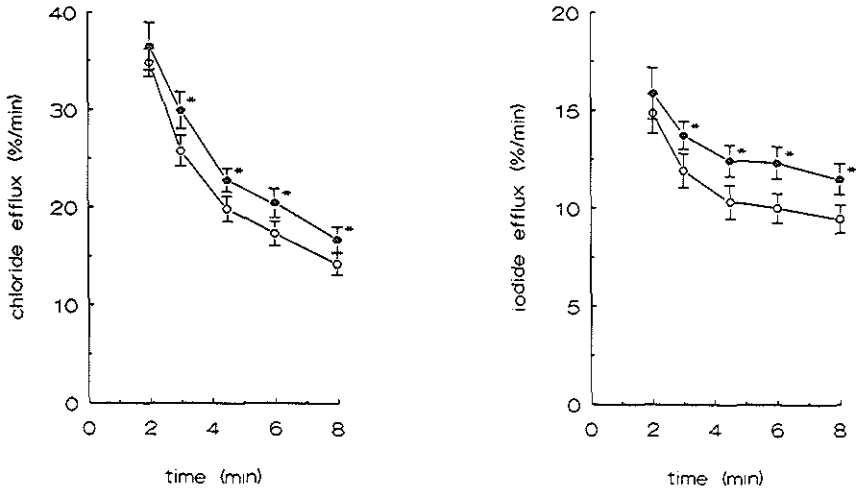

FIGURE 2. PMA stimulates $^{36}Cl^-$ and $^{125}I^-$ efflux in HT29cl.19A colonocytes. HT29cl.19A cells were grown in 6-well cell culture plates to 70-80% confluence. Cells were loaded with both $^{36}Cl^-$ and $^{125}I^-$ and fractional $^{36}Cl^-$ (left) and $^{125}I^-$ (right) efflux was determined simultaneously as described in MATERIALS AND METHODS. PMA (0.5 μM; ●) was included in efflux medium at t=0 and kept present throughout experiment. Control cells (○) received only vehicle [0.1% dimethyl sulfoxide (DMSO)]. Data are means ± SD (n=5 experiments). *Different from paired controls, P<0.01.

conductances in HT29cl.19A cells (cf.Ref.9). The PMA-induced rise in $^{125}I^-$ efflux from plastic-grown HT29cl.19A cells could be mimicked by PDB (0.5 μM), and was not inhibited by indomethacin (20 μM, 20 min preincubation; results not shown).

We also determined the effect of PMA on the $^{125}I^-$ efflux from filter-grown monolayers of HT29cl.19A cells. As shown in Fig.3, PMA merely increased the $^{125}I^-$ efflux across the apical border, but had no effect on basolateral anion efflux. The cAMP-linked secretagogue forskolin showed a similar although larger increase of $^{125}I^-$ efflux solely into the mucosal bath (Fig.3). In contrast, Ca^{2+} ionophore A23187 was found to provoke a rise in $^{125}I^-$ efflux across both the apical and the basolateral membrane (24). As shown in Fig.3, PMA, like forskolin, also caused a significant increase in $^{86}Rb^+$ efflux rate into the serosal bath. The increase in serosal K^+ efflux follows the increase in apical efflux of Cl^- and may assist in maintaining a sustained Cl^- secretion (cf.Ref.6). The stimulation of the $^{125}I^-$ and $^{86}Rb^+$ efflux by PMA seemed slightly faster than the stimulation of I_{sc} determined in the Ussing chamber. Presumably PMA action may have been more efficient in

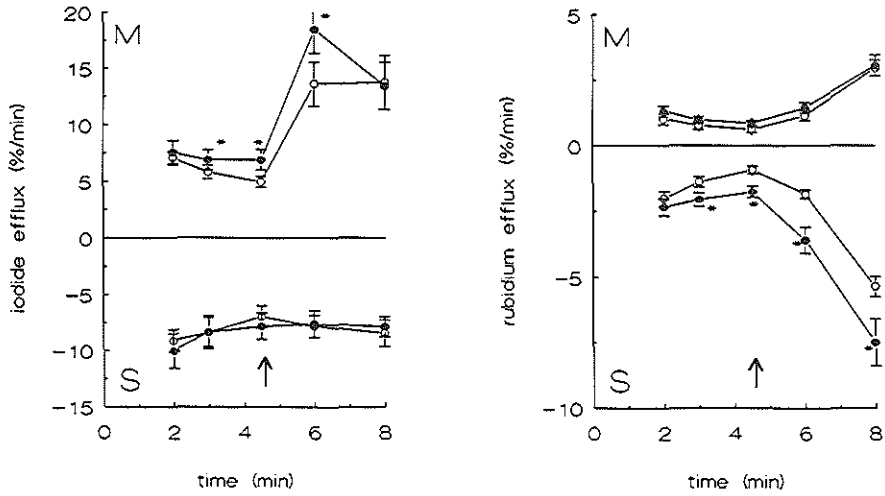

FIGURE 3. PMA stimulates apical $^{125}I^-$ and basolateral $^{86}Rb^+$ efflux from HT29cl.19A colonocytes. HT29cl.19A cells were grown on tissue culture-treated polycarbonate filters to full confluence. Cells were loaded with both $^{125}I^-$ and $^{86}Rb^+$. Fractional $^{125}I^-$ (left) and $^{86}Rb^+$ (right) efflux was determined simultaneously as described in MATERIALS AND METHODS. Forskolin (5 μM) was added to both PMA-treated and control cells 4.5 min after start of efflux experiment as indicated by arrow. PMA (0.5 μM; ●) was added at t=0 and kept present throughout experiment. Control cells (o) received only vehicle (0.1% DMSO). Sign of positive fractional efflux into serosal bath was arbitrarily reversed into negative sign, to discriminate serosal efflux graphically from mucosal efflux. M, mucosal side; S, serosal side. Data are means ± SD (n=3 experiments) *Different from paired controls, P<0.01.

the efflux experiments because in this assay cells were exposed to a premixed PMA solution, whereas PMA in the Ussing chamber was added as a bolus injection from a concentrated stock solution.

Because the PMA-induced anion efflux resembled the forskolin-stimulated efflux, a possible role of cAMP as a modulator of the PMA-induced Cl^- secretion was investigated further by direct measurements of cAMP levels. However, a 10 min exposure of plastic-grown HT29cl.19A colonocytes to PMA (0.5 μM), under similar condition as used for the $^{125}I^-$ efflux determinations, did not lead to a significant difference in cAMP levels as compared to control cells (stimulation factor 0.92 ± 0.12, n=4). For comparison, forskolin (5 μM, 3 min incubation) was found to raise the cAMP levels in the same series of experiments 5.9 ± 1.6 fold (n=4).

As shown in Fig.3, a short (4.5 min) preincubation with PMA had no significant effect on the forskolin induced increase in $^{125}I^-$ and $^{86}Rb^+$ efflux from filter-grown HT29cl.19A cells.

Long-term effects of PMA on Cl^- secretion. Prolonged incubation up to 3 hour in the presence of 0.5 μM PMA resulted in a return of the I_{sc} to a value which was only slightly above the value of unstimulated HT29cl.19A cells. Furthermore a 3 h preincubation with PMA caused a 93 ± 4% inhibition of the I_{sc} response to 8-Br-cAMP (Table 1). The electrical resistance of the PMA-treated monolayers (119 ± 25 Ω.cm^2; n=12) was not significantly different from control cells (115 ± 28 Ω.cm^2; n=18), excluding the possibility that PMA inhibited the I_{sc} by affecting the resistance of the monolayers. The PMA-provoked inhibition of the I_{sc} might result from inactivation of apical Cl^- channels, or from inhibition of transport pathways necessary for the maintenance of electrogenic Cl^- secretion, e.g. basolateral K^+ channels, or Na-K-Cl_2 cotransporters (6,18). To discriminate between these possibilities a more direct method of determining the activity of Cl^- or K^+ transporters was used, based on the efflux rates of radiolabeled ions (8,24,26).

The loading of $^{125}I^-$ in the long-term PMA-treated cells as determined after the washing procedure (0.42 ± 0.04% of the added isotope; n=5) was 30% higher than into untreated cells (see MATERIALS AND METHODS). This difference in loading is not expected to have large effects on the fractional anion efflux, as this

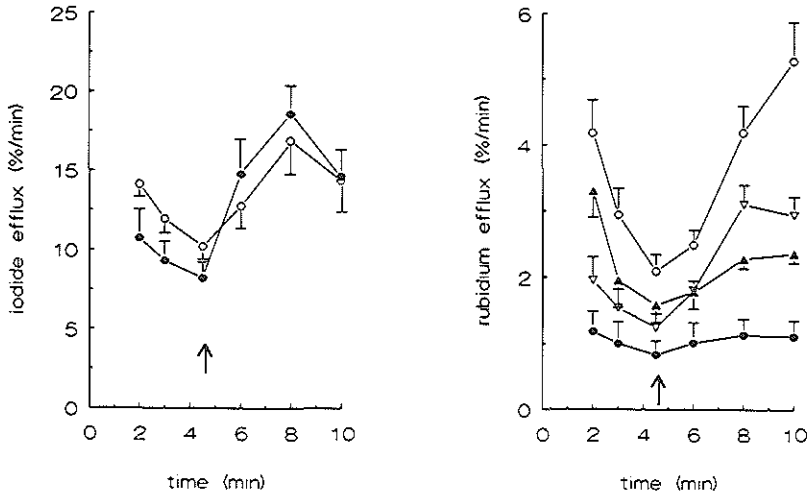

FIGURE 4. Prolonged PMA treatment inhibits basal and forskolin-stimulated $^{86}Rb^+$ efflux but not $^{125}I^-$ efflux; comparison with K^+ transport inhibitors. HT29cl.19A cells were grown in 6-well cell culture plates to 70-80% confluence. Cells were loaded with both $^{125}I^-$ and $^{86}Rb^+$, and fractional $^{125}I^-$ (left) and $^{86}Rb^+$ (right) efflux was determined simultaneously as described in MATERIALS AND METHODS. Forskolin (5 μM) was added to different incubations 4.5 min after start of efflux experiment as indicated by arrow. PMA (0.5 μM; o) was added to cells 3 h before start of efflux determination and was also included in efflux medium. Control cells (o) received only vehicle (0.1% DMSO). Barium (2 mM; Δ) or bumetanide (100 μM; ∇) was included in efflux medium at t=0 min. Data are means ± SD (n=3 experiments).

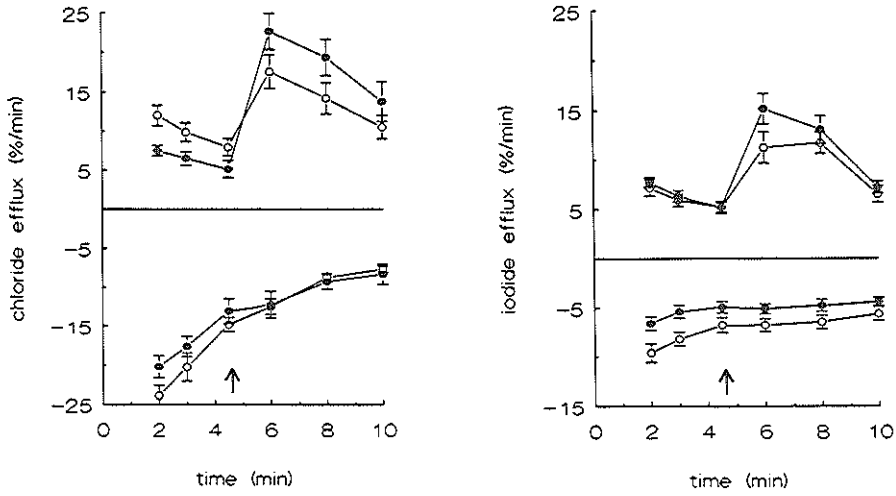

FIGURE 5. Prolonged PMA treatment does not inhibit forskolin-stimulated $^{36}Cl^-$ or $^{125}I^-$ efflux from HT29cl.19A colonocytes. HT29cl.19A cells were grown on tissue culture-treated polycarbonate filters to full confluence. Cells were loaded with both $^{36}Cl^-$ and $^{125}I^-$, and fractional $^{36}Cl^-$ (left) and $^{125}I^-$ (right) efflux was determined simultaneously as described in MATERIALS AND METHODS. Forskolin (5 μM) was added to both PMA-treated and control cells 4.5 min after start of efflux experiment as indicated by arrow. PMA (0.5 μM; o) was added to cells 3 h before start of efflux determination and was also included in efflux medium. Control cells (o) received only vehicle (0.1% DMSO). Sign of positive fractional efflux into serosal bath was arbitrarily reversed into negative sign, to discriminate serosal efflux graphically from mucosal efflux. Data are means ± SD (n=3 experiments).

value is corrected for the amount of radioactivity present in the cells. As shown in Fig.4, plastic-grown HT29cl.19A cells showed a 20-25% lower fractional efflux rate of $^{125}I^-$ after the 3 h incubation with PMA compared to control cells. In contrast, the forskolin-stimulated $^{125}I^-$ efflux showed a slight increase in response to the prolonged PMA treatment. A similar response of both basal and forskolin-stimulated $^{125}I^-$ and $^{36}Cl^-$ efflux to a prolonged PMA incubation was observed in filter-grown HT29cl.19A colonocytes (Fig.5). As also shown in Fig.5, PMA inhibited the basal efflux of radiolabeled anions into the serosal as well as the apical bath. The increase in $^{125}I^-$ efflux induced by 8-Br-cAMP (1 mM), which was 2-3 fold smaller than the increase in $^{125}I^-$ efflux after addition of forskolin, appeared likewise insensitive to a 3 h PMA preincubation (results not shown). These data indicate that the PMA-provoked inhibition of the cAMP-induced rise in I_{sc} is not caused by inhibition of the cAMP-activated apical Cl^- channel.

However, a 3 h exposure of plastic-grown HT29cl.19A cells to PMA was found to inhibit the basal $^{86}Rb^+$ efflux by 65 ± 9% (n=5) and the forskolin

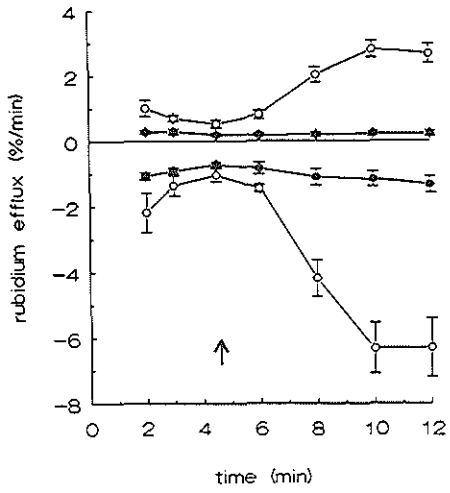

FIGURE 6. Prolonged PMA treatment inhibits $^{86}Rb^+$ efflux from HT29cl.19A colonocytes grown on permeable support. HT29cl.19A cells were grown on tissue culture-treated polycarbonate filters to full confluence. Cells were loaded with $^{86}Rb^+$. Fractional $^{86}Rb^+$ efflux was determined as described in MATERIALS AND METHODS. Forskolin (5 μM) was added to both PMA-treated and control cells 4.5 min after start of efflux experiment as indicated by arrow. PMA (0.5 μM; o) was added to cells 3 h before start of efflux experiment and kept present in efflux medium throughout experiment. Control cells (o) received only vehicle (0.1% DMSO). Sign of positive fractional efflux into serosal bath was arbitrarily reversed into negative sign, to discriminate serosal efflux graphically from mucosal efflux. Data are means ± SD (n=4 experiments).

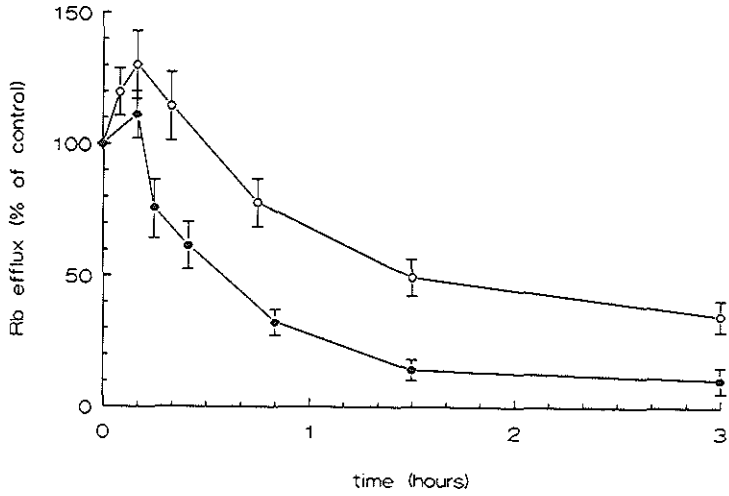

FIGURE 7. Time course of PMA inhibition of basal and forskolin-stimulated $^{86}Rb^+$ efflux. HT29cl.19A cells were grown in 6-well cell culture plates to 70-80% confluence. Fractional $^{86}Rb^+$ efflux was determined as described in MATERIALS AND METHODS. Forskolin (5 μM) was included in efflux medium 5 min after start of efflux experiment. PMA (0.5 μM) was added to cells at various time points before start of efflux determination and was also included in efflux medium. PMA effects on basal $^{86}Rb^+$ efflux (o) were determined by comparing fractional $^{86}Rb^+$ efflux of PMA-treated and control cells (incubated in presence of vehicle, 0.1% DMSO) at 5 min after start of efflux experiment. Effects on forskolin-stimulated efflux (o) were determined by comparing fractional $^{86}Rb^+$ efflux of PMA-treated and control cells at 5 min after addition of forskolin (when $^{86}Rb^+$ efflux was maximal) after subtraction of $^{86}Rb^+$ efflux in absence of forskolin. Data are means ± SD (n=3 experiments).

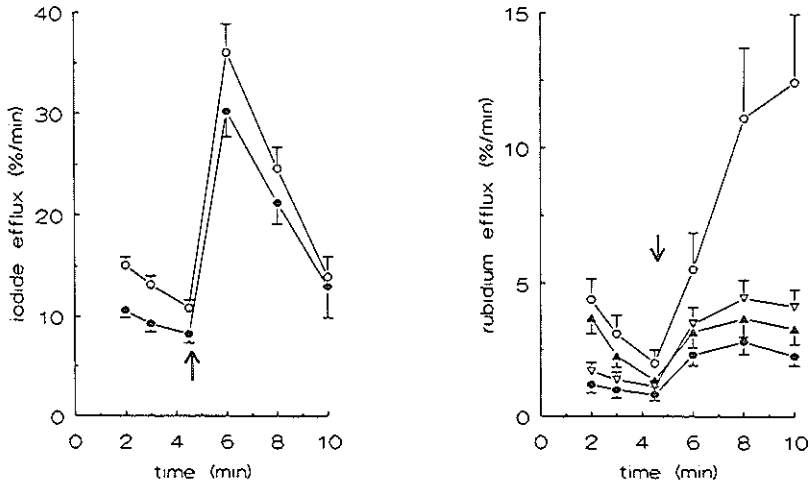

FIGURE 8. Prolonged PMA treatment inhibits Ca^{2+} ionophore-stimulated $^{86}Rb^+$ efflux but not $^{125}I^-$ efflux; comparison with K^+ transport inhibitors. HT29cl.19A cells were grown in 6-well cell culture plates to 70-80% confluence. Cells were loaded with both $^{125}I^-$ and $^{86}Rb^+$, and fractional $^{125}I^-$ (left) and $^{86}Rb^+$ (right) efflux was determined simultaneously as described in MATERIALS AND METHODS. Ca^{2+} ionophore A23187 (10 μM) was included in efflux medium 4.5 min after start of efflux experiment as indicated by arrow. PMA (0.5 μM; o) was added to cells 3 h before start of efflux determination and was also included in efflux medium. Control cells (o) received only vehicle (0.1% DMSO). Barium (2 mM; Δ) or bumetanide (100 μM; ∇) was included in efflux medium at t=0 min. Data are means ± SD (n=3 experiments).

stimulated $^{86}Rb^+$ efflux by 87 ± 6% (determined at 5.5 min after addition of forskolin, n=5) (Fig.4). A similar inhibition of basal and forskolin stimulated $^{86}Rb^+$ efflux across both the apical and basolateral membrane was observed in colonocytes grown on filters (Fig.6). The loading of $^{86}Rb^+$ into the PMA-treated cells (23.4 ± 3.3% of the isotope added; n=5) was almost identical to the loading of untreated cells (see MATERIALS AND METHODS). As shown in Fig.7, after a relatively fast initial stimulation, PMA starts to inhibit the $^{86}Rb^+$ efflux after 20 min, and reaches a maximal inhibition after 3 h. A 3 h incubation in the presence of PMA also strongly inhibited the increase in $^{86}Rb^+$ efflux induced by the Ca^{2+} ionophore A23187, whereas the A23187 stimulated $^{125}I^-$ efflux was only marginally inhibited (Fig.8).

In Figs.4 and 8 the effect of prolonged PMA treatment on $^{86}Rb^+$ efflux is compared with inhibitors of K^+ transport mechanisms. Bumetanide at a concentration that is reported to completely inhibit the Na-K-Cl_2 cotransporter in HT29 cells (100 μM; Ref.16) inhibited the basal $^{86}Rb^+$ efflux 47 ± 8 %, but started

to inhibit the forskolin- and Ca^{2+} ionophore-provoked $^{86}Rb^+$ efflux only after a lag time of 1.5-3 min after addition of the agonist. Barium (2 mM), a well-known inhibitor of epithelial K^+ channels (6,18) induced in comparison with bumetanide, a smaller reduction of basal $^{86}Rb^+$ efflux (27 ± 9%), but a faster and larger inhibition of the forskolin-stimulated $^{86}Rb^+$ efflux, and a somewhat larger inhibition of the A23187-provoked efflux. In conclusion, barium and bumetanide were found to be less inhibitory towards basal, cAMP- and Ca^{2+}-provoked $^{86}Rb^+$ efflux incomparison with prolonged PMA treatment (Figs.4 and 8). Neither barium nor bumetanide inhibited forskolin- or A23187-stimulated $^{125}I^-$ or $^{36}Cl^-$ efflux to a significant extent (results not shown; cf.Ref.8,24).

To further characterize the mechanisms of $^{86}Rb^+$ efflux from HT29cl.19A cells we also performed ion replacement studies. As shown in Fig.9, replacement of Cl^- and HCO_3^- by gluconate inhibited forskolin-stimulated $^{86}Rb^+$ efflux to a

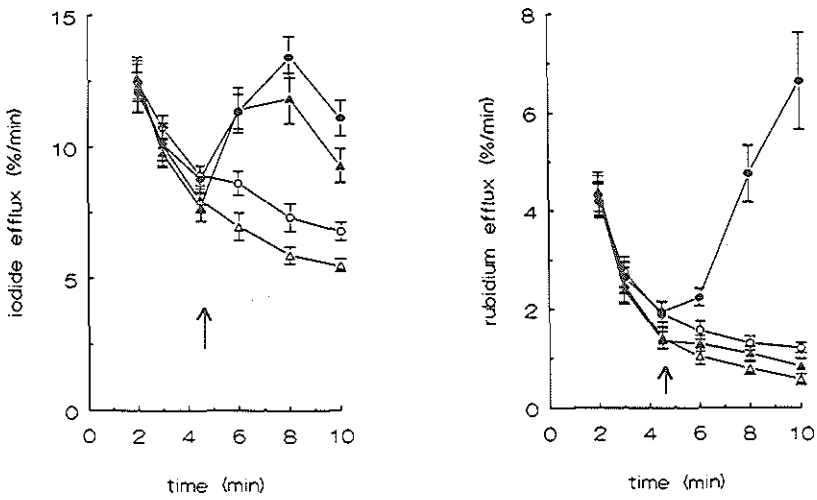

FIGURE 9. Cl^--free medium inhibits basal and forskolin-stimulated $^{86}Rb^+$ efflux but not $^{125}I^-$ efflux. HT29cl.19A cells were grown in 6-well cell culture plates to 70-80% confluence. Cells were loaded with both $^{125}I^-$ and $^{86}Rb^+$ in buffer without HCO_3^- [(in mM) 130 NaCl, 4.7 KCl, 1.3$CaCl_2$, 1.0 $MgCl_2$, 20 HEPES/Tris, pH 7.4, 10 glucose). Fractional $^{125}I^-$ (left) and $^{86}Rb^+$ (right) efflux was determined simultaneously as described in MATERIALS AND METHODS in efflux buffers similar to loading buffer (o,●) or in similar buffer in which Cl^- was replaced by gluconate (Δ,▲). Forskolin (5 μM) was included in some experiments (●,▲)in efflux medium 4.5 min after start of efflux experiment as indicated by arrow. Data are means ± SD (n=3 experiments).

similar extent as found after prolonged PMA exposure, whereas the forskolin-induced $^{125}I^-$ efflux was not inhibited. Omittance of K^+ or Na^+ was less inhibitory towards the forskolin-activated $^{86}Rb^+$ efflux, and mimicked the effect of bumetanide including a delayed onset of the inhibition (results not shown).

DISCUSSION

From both the I_{sc} and the isotope efflux measurements it is evident that phorbol esters are able to stimulate electrogenic Cl^- secretion in the HT29cl.19A human colon carcinoma cell line. In rabbit colon and chicken ileum the phorbol ester-induced Cl^- secretion was found to be mediated predominantly by prostaglandins presumably synthesized in sub-epithelial cells (7,12,19,23). However, PMA-stimulation of Cl^- transport in the HT29cl.19A cells must occur more directly, since (1) this cell line consists of a homogenous population of Cl^--secreting epithelial cells and is completely devoid of subepithelial cell types, and (2) the PMA-induced Cl^- secretion was not inhibited by the cyclooxygenase inhibitor indomethacin.

Phorbol esters added at submicromolar concentrations are thought to act as specific activators of protein kinase C (20,21). Activation of protein kinase C by PMA in HT29.cl19A cells was supported by immunolocalization studies with protein kinase C specific antibodies. These studies provided preliminary evidence for a rapid translocation of protein kinase C-α from the cytosol to the particulate fraction after addition of PMA (Van Den Berghe, unpublished data). Furthermore, OAG a membrane permeant analog of diacylglycerol (DAG), the physiological activator of protein kinase C could mimic the effect of PMA on I_{sc} in HT29cl.19A cells. Activation of protein kinase C might represent an independent route for activation of apical Cl^- channels in the HT29cl.19A cell line, distinct from the cAMP or Ca^{2+} mediated signal-transduction pathways, because 1) PMA did not change cAMP levels in HT29.cl19A cells under conditions in which it generated a clear increase in $^{36}Cl^-$ and $^{125}I^-$ efflux, and 2) several characteristics of the PMA-induced $^{36}Cl^-$ and $^{125}I^-$ efflux were different from those of the Ca^{2+} ionophore-provoked anion efflux (24).

As judged by the similarity between the PMA- and the forskolin-stimulated $^{125}I^-$ and $^{36}Cl^-$ efflux, protein kinase C and cAMP-dependent protein kinase might activate a similar or identical Cl^- channel. Such a model was suggested from patch-clamp studies with tracheal epithelial cells (15,17). In these studies both cAMP-dependent protein kinase and protein kinase C were found capable of

activating a similar outwardly rectifying Cl^- channel. However, the actual contribution of this channel to cAMP- or PMA-provoked Cl^- currents in intact epithelial cells remains to be established (9).

The main physiological activator of the protein kinase C pathway is thought to be DAG (20,21). DAG is generated by a specific phospholipase C after stimulation by a various secretagogues e.g. carbachol or bradykinin (1,20,21). This phospholipase C was found to be abundant in both the apical and the basolateral membrane of rat enterocytes (25). In T84 colonocytes carbachol and phorbol esters have been reported to provoke a similar protein phosphorylation pattern (10).

In contrast to the stimulation of Cl^- secretion observed upon short-term exposure to PMA, a prolonged activation of protein kinase C was found to result in a profound inhibition of the PMA- and cAMP-provoked I_{SC}. Most likely this inhibition was not caused by downregulation of protein kinase C, since the levels of all major protein kinase C isoenzymes remained stable after a 3 hour incubation with 0.5 µM PMA (Van den Berghe, unpublished data). The prolonged PMA treatment also induced a decrease in the basal fractional $^{125}I^-$ and $^{36}Cl^-$ efflux. However, both the basolateral anion efflux as well as the apical anion efflux became inhibited, whereas only the latter was specifically stimulated by short-term PMA exposure. This suggests that the PMA-induced decrease in $^{125}I^-$ and $^{36}Cl^-$ efflux represents an inhibition of the basal Cl^- permeability of both membranes rather than a specific inhibition of the PMA-stimulated Cl^- efflux pathway.

The cAMP-induced $^{125}I^-$ and $^{36}Cl^-$ efflux was not inhibited by a short- or long-term (3 h) exposure to PMA, arguing against a direct inhibitory effect of protein kinase C activation on the cAMP-stimulated Cl^- channels. In this respect the colonic HT29cl.19A cells clearly differ from tracheal cells, in which PMA decreased the cAMP-provoked $^{125}I^-$ efflux (17). This difference might reflect a dissimilarity in the type of Cl^- channels activated by cAMP between both cell types, as evidenced by a pronounced difference in the sensitivity of the cAMP stimulated Cl^- efflux for the Cl^- channel blocker NPPB between tracheal and HT29cl.19A cells (4,17,24). The forskolin-induced stimulation of $^{125}I^-$ and $^{36}Cl^-$ efflux was actually enhanced by the 3 h PMA treatment. This enhancement may be caused by a stimulatory effect of PMA on adenylate cyclase, since protein kinase C activation is known to increase the forskolin-provoked rise in cAMP levels in other cell types (22). We also have preliminary evidence that PMA enhances the forskolin-induced, but not the basal, cAMP formation in plastic-grown HT29.cl19A cells (unpublished observations). The Ca^{2+}-stimulated $^{125}I^-$ efflux in HT29.cl19A

cells, which presumably represents a Cl^- conductance distinct from the cAMP-activated Cl^- channel (9,24) appeared equally insensitive to a prolonged exposure to the protein kinase C activator PMA.

In contrast to the absence of clear inhibitory effects of PMA on cAMP- and Ca^{2+}-provoked anion efflux, the cAMP-stimulated $^{86}Rb^+$ efflux was dramatically inhibited by prolonged exposure to PMA. This finding indicates that the inhibition of the cAMP-induced electrogenic Cl^- secretion is caused by an inhibition of a K^+ transport system, rather than of the cAMP-stimulated apical Cl^- channel. The time course of the PMA-induced inhibition of the K^+ efflux pathway was much slower than the stimulatory effect of PMA on the Cl^- and K^+ efflux, resulting in a biphasic effect of PMA on Cl^- secretion. The difference in time course suggests that the mechanism by which PMA activates Cl^- secretion (presumably phosphorylation of a Cl^- channel or its regulatory protein) is dissimilar from that by which K^+ efflux is inhibited.

Among the different K^+ transporters known to be present in Cl^- secreting epithelial cells, the Na^+/K^+-ATPase is unlikely to serve as a major target for PMA; long-term exposure to PMA did not significantly affect the $^{86}Rb^+$ loading of the HT29cl.19A cells which apparently accumulates above its extracellular concentration due to the activity of the Na^+/K^+-ATPase.

In the parental HT29 cell line prolonged exposure to PMA has been reported to inhibit the bumetanide-sensitive Na-K-Cl_2 cotransport activity, presumably through a downregulation of the carrier itself (14). The time course of the PMA-provoked inhibition of the cotransporter in HT29 cells was comparable to the time course for the inhibition of the $^{86}Rb^+$ efflux in the subclone 19A. In our study bumetanide was also found to inhibit the $^{86}Rb^+$ efflux, although to a considerably lower extent than PMA. Only part of the PMA-induced inhibition of $^{86}Rb^+$ efflux may therefore be explained by an inhibition of the bumetanide-sensitive Na-K-Cl_2 cotransporter.

It is however unlikely that $^{86}Rb^+$ is released from the HT29cl.19A cells by direct cotransport with Cl^-, because neither basal nor cAMP-stimulated $^{36}Cl^-$ efflux was apparently inhibited by bumetanide or long-term PMA treatment. In contrast to $^{86}Rb^+$ efflux, no stimulation of $^{36}Cl^-$ efflux was observed across the basolateral membrane in response to forskolin, also arguing for different transport systems for $^{86}Rb^+$ and $^{36}Cl^-$ efflux in HT29cl.19A cells. This suggests that either $^{86}Rb^+$ efflux is (in part) mediated by a bumetanide-sensitive transport system without an obligatory coupling to Cl^- cotransport, or that $^{86}Rb^+$ efflux is indirectly affected by an inhibition of the Na-K-Cl_2 cotransporter (e.g. as a consequence of a lower Cl^-,

K^+ or Na^+ influx into the cells). Such a coupling between $^{86}Rb^+$ efflux and Cl^- influx might also explain the striking observation that replacement of extracellular Cl^- and HCO_3^- almost completely blocked the cAMP-induced $^{86}Rb^+$ efflux without affecting the anion efflux pathway.

However, because 3 h PMA pretreatment decreased the basal and forskolin-induced $^{86}Rb^+$ efflux considerably more than a maximal concentration of bumetanide (16), PMA must at least also affect additional K^+ efflux pathways besides the bumetanide sensitive transporter. As judged by the large barium-induced inhibition of the cAMP-stimulated $^{86}Rb^+$ efflux, this other PMA-inhibitable transport system might include barium-sensitive K^+ channels. These channels are known to play an important role in maintaining net electrogenic Cl^- secretion in epithelial cells (6,18).

Another intriguing possibility, raised by the similar inhibitory effects induced by extracellular Cl^- and HCO_3^- replacement and prolonged PMA exposure, is that the PMA inhibitable $^{86}Rb^+$ efflux in HT29cl.19A cells is mediated by a novel transporter which is dependent on Cl^- influx. This system would be stimulated during a state of active Cl^- secretion in order to facilitate the efflux of K^+ which accumulates by the increased activity of the Na^+/K^+-ATPase and the Na-K-Cl_2 cotransporter.

The Ca^{2+}-provoked $^{86}Rb^+$ efflux was also inhibited by PMA, barium and bumetanide, suggesting that similar K^+ transport systems are active during cAMP- and Ca^{2+}-mediated Cl^- efflux. The HT29cl.19A cell line seems to differ in this respect from the colonic cell line T84, where forskolin in contrast to Ca^{2+} ionophore induced only a very small increase in $^{86}Rb^+$ efflux (26, unpublished observations).

Clearly further research is needed to identify the PMA-sensitive $^{86}Rb^+$ efflux pathways at the molecular level and to determine the mechanism by which a tonic stimulation of protein kinase C leads to a virtually complete downregulation of these pathways in the HT29cl.19A colonocytes.

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CHAPTER 7

GENERAL DISCUSSION

This thesis describes several aspects of the regulation of intestinal chloride secretion. As a model system, freshly isolated rat enterocytes and human colon carcinoma cell lines, HT29cl.19A and T84, were used. These cell lines were not only selected for their capacity to secrete chloride, but also for their polarized morphology and their formation of tight junctions, allowing electrical measurements of ion transport in the Ussing chamber.

A major goal of our studies was to determine the relative contribution of regulatory components and ion channels localized in the apical and the basolateral membrane to transepithelial chloride secretion. In a previous study, the enterocytes were found to contain a GTP-activated phospholipase C (PLC) at the apical membrane. As all G-protein-coupled hormone receptors are localized at the basolateral membrane, GTP-activated PIP2 hydrolysis in the brush border region was unexpected. To further investigate subcellular differences in signalling pathways, we studied the distribution of several G-proteins among the apical and basolateral membrane. This study revealed an asymmetrical distribution of G-proteins: Gs and Gp were present at both the apical and the basolateral membrane, whereas Gi2 and Gi3 were predominantly localized at the basolateral membrane. Furthermore, two novel G-proteins, immunologically related to Gi, have been identified: a 39 kDa (apical) and a 78 kDa (apical and basolateral) αi-like protein [Chapter 2]. Thusfar three G-proteins have been implicated in the activation of phospholipase C: Gq, Gi2 and Go (see Chapter 1: General Introduction). In enterocytes, Gp is likely to be identical to Gq (Fig.1), as Go is not detectable by immunoblotting and Gi2 is predominantly localized at the basolateral membrane [Chapter 2]. The G-protein that couples to phospholipase C at the basolateral membrane could be identical to Gq or Gi2. However, since pertussis toxin pretreatment did not abolish carbachol-induced Cl^- secretion, the involvement of Gi2 is unlikely [De Jonge, unpublished results]. As specific antisera against the Gq family have been generated recently, a tool to study the expression of Gq in enterocytes is now available [Gutowski et al., 1991]. The specific targeting of some, but not all G-proteins to the apical membrane supports

the idea that G-proteins might have a specific function at this subcellular region.

Much attention was paid to the possibility that some of the apical G-proteins might act as regulators of apical Cl^-channels. In this study, Cl^- conductance measurements in apical membrane vesicles of rat enterocytes and HT29cl.19A colonocytes were combined with single channel analysis of Cl^- conductance in inside-out patches of HT29cl.19A membranes. In both model systems, GTPγS activated a novel type of Cl^- channel, different from CFTR, apparantly through interaction with an apical G-protein [Chapter 3]. The identity of this G-protein is presently unknown, but apical Gs and Gp (probably Gq) proteins are potential candidates. The observation, however, that choleratoxin (CT)-induced Cl^- secretion is abolished in CF patients, as concluded from short-circuit current (I_{sc}) measurements in the Ussing chamber [Bijman et al., 1990], suggests that Gs does not activate the (CF-insensitive) G-protein-gated Cl^- channel, unless: 1) the apical Gs is not activated by CT or 2) the G-channel does not contribute to the CT-provoked I_{sc}. The biophysical and regulatory properties of the G-channel are different from the cAMP-, the volume- and the Ca^{2+}-regulated Cl^- channels in T84 colonocytes as determined by the whole cell patch clamp technique [Chapter 3; Worrell and Frizzell, 1991]. Further studies defining the intracellular and tissue distribution of this G-channel and the identity of the G-protein involved might help to elucidate the function of this novel Cl^- channel. The identity of the G-protein(s) involved may be confirmed by demonstrating G-channel activation in excised membrane patches by purified G-protein α subunits.

Alternative candidates for activation of the G-channel, might be the novel 39 kDa Gi-like proteins or other yet unidentified G-proteins present at the apical membrane (Gx in Fig.1). It is tempting to speculate that the 78 kDa Gi-like protein might be a proteolytic degradation product of P100, a GTP-binding protein immunologically related to Gi [Traub et al., 1990; Traub et al., 1991]. P100 shares a putative receptor binding sequence with Gt and Gi, and is proposed to be involved in receptor trafficking. As the 78 kDa protein is localized at the basolateral membrane, the possible relationship between the 78 kDa protein and P100 deserves further study.

Furthermore, the possibility that the α subunit of Gs can be released from the apical membrane and couple to adenylate cyclase at the basolateral membrane was studied. This process might be facilitated by the lack of myristic acid on the α subunit of Gs, in contrast to most other G-protein α subunits [Buss et al., 1987]. Although several investigators have found evidence for a GTPγS-induced release of αs from plasmamembranes [Milligan and Unson, 1989],

GTPγS- or cholera toxin did not induce the release of αs from non-vesiculated intestinal brush border membranes [Chapter 2]. This finding has two important implications. First, the presence of Gs at the apical membrane and the lack of its release from this membrane, suggest that Gs in the enterocyt does not function as a "programmable messenger" [Rodbell, 1985], but may have different targets at the apical membrane (e.g. ion transport systems) and the basolateral membrane (adenylate cyclase)(Fig.1). Secondly, cholera toxin itself, rather than ADP-ribosylated Gs, must translocate from the apical membrane to the basolateral membrane in order to activate adenylate cyclase at this subcellular region (Fig.1, the A1 subunit of CT).

The identification of two G-protein-activated effectors at the apical membrane: phospholipase C and a distinct class of Cl^- channels, suggests the presence of physiological activators. The possibility that other signal transduction routes could function as activators of apical PIP2 hydrolysis was investigated, by studying the possible potentiating effects of cAMP, cGMP, Ca^{2+} and PKC on inositol phosphate production and calcium mobilization. However, PKC and cAMP inhibited rather than stimulated these processes: PKC upstream and cAMP downstream of phospholipase C activation [Chapter 4]. Negative feedback on the PI-cycle by PKC is commonly found in many cell types. In contrast, cAMP-induced inhibition of carbachol-induced calcium mobilization was rather unexpected, as in chicken enterocytes cAMP itself mobilized calcium [Semrad and Chang, 1987; Chang and Musch, 1990; Semrad et al., 1990]. The mechanism of the cAMP-induced inhibition is presently unknown, but cAMP-induced inhibition of Ins(1,4,5)P3-triggered calcium mobilization [Supattapone et al., 1988] and calcium entry [Felbel et al., 1988] have been reported for other cell types. Very recently, carbachol-stimulated calcium entry in HT29 cells was found to be inhibited by membrane depolarization [Fischer et al., 1992], which could be an alternative mechanism as cAMP has been shown to induce membrane depolarization in HT29cl.19A cells [Bajnath et al., 1991]. A possible interaction of cGMP with inositol phosphate production and diacylglycerol (DAG) formation seemed worth considering a recent report showing that ANF- and 8-Br-cGMP-induced Na^+ channel closing in renal epithelial cells was mediated by PKC [Mohrmann et al., 1987]. In enterocytes, the apical membrane contains both the cGMP pathway and a GTP-activated PI-turnover. Thus, cGMP could theoretically act through activation of this PI-cycle, leading to formation of DAG and subsequent activation of PKC in these cells as well. However, ST_A, the heat-stable E.coli toxin that raises cGMP levels did not activate phospholipase C [Chapter 4]. Since sources other than

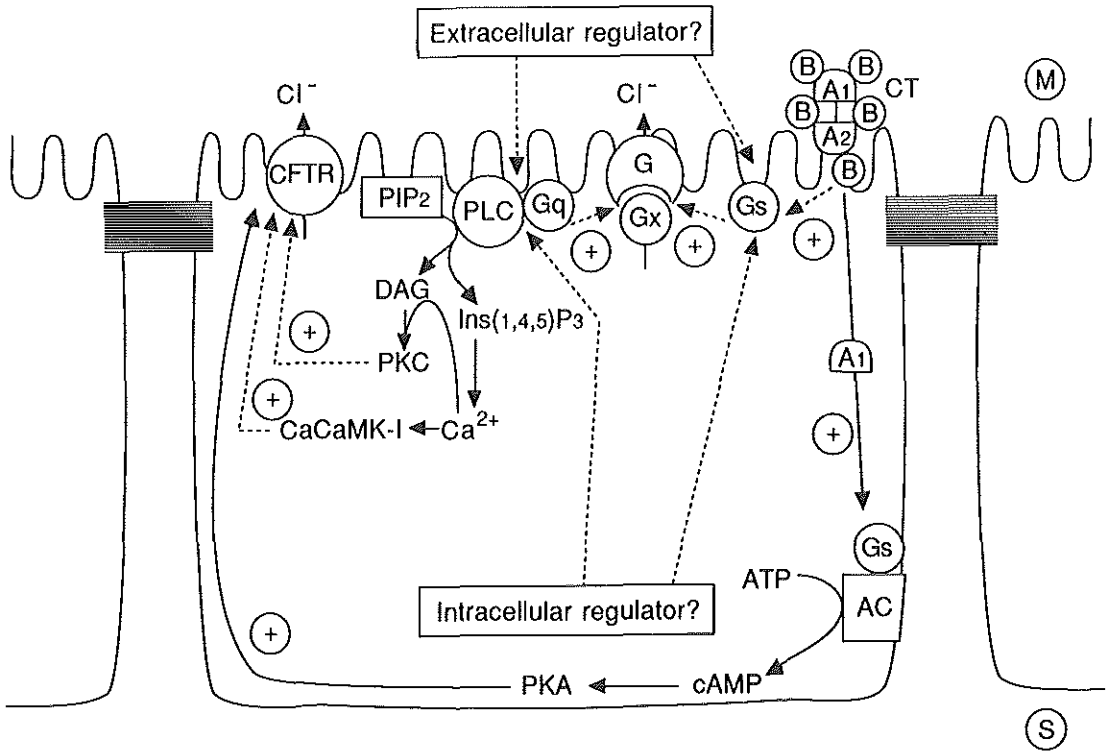

FIGURE 1. Signal transduction molecules at the apical membrane of rat enterocytes; their possible involvement in Cl^- secretion. The new findings described in Chapters 2-4 and their implications are schematically summarized (see text). Solid lines indicate established interactions, dashed lines indicate potential interactions. CFTR, cystic fibrosis transmembrane conductance regulator; G, G-protein-activated Cl^- channel; CT, cholera toxin (5 B and A subunit); Gs, Gq and Gx, different G-proteins; PLC, phospholipase C; PIP2, phospholnositide (4,5) bisphosphate; DAG, diacylglycerol; PKC, protein kinase C; Ins (1,4,5)P3, inositol (1,4,5) trisphosphate; CaCaMK-I, calcium/calmodulin-kinase-I; AC, adenylate cyclase; PKA, protein kinase A; M, mucosa; S, serosa.

PIP2 for DAG formation exist, this finding does not entirely rule out the possibility that cGMP acts through PKC. For instance, in tracheal epithelial cells, cAMP was found to raise DAG levels independently of PIP2 hydrolysis [Anderson and Welsh, 1990].

Other potential activators of the apical signalling routes may include as yet unidentified receptors at the apical membrane (Fig.1, extracellular regulator?). In addition, it is also possible that non-receptor mediated signals can activate these pathways, such as cell volume changes. Alternatively, intracellular messengers other than cAMP, cGMP, calcium and DAG could be involved (Fig.1, intracellular regulator?). Examples of such intracellular activators of G-proteins are growth cone associated protein, GAP43, which has been found to stimulate Go [Strittmatter et al., 1990], and NDP-kinase (nucleotidediphosphate kinase), which could stimulate Go, Gi and Gs by direct transfer of the γ phosphate of ATP to the GDP bound to these G-proteins [Kikkawa et al., 1990].

Stimulation of chloride efflux does not necessarily lead to net chloride secretion. This is illustrated in HT29cl.19A cells, where Ca^{2+}-ionophore induces Cl^- channel opening as measured by $^{125}I^-$ or $^{36}Cl^-$ efflux [Vaandrager et al., 1991], but does not induce an I_{sc} in the Ussing chamber, indicative for net chloride secretion [Chapter 5]. This apparant paradox is explained by the co-localization of Ca^{2+}-sensitive Cl^- and K^+ channels in both the apical and basolateral membrane (Fig.2) [Vaandrager et al., 1991]. These channels are different from CFTR by their distribution, anion selectivity and biophysical parameters [Vaandrager et al., 1991; Cliff and Frizzell, 1990].

In contrast, calcium is capable of regulating CFTR in human ileal mucosa: Ca^{2+}-ionophore-induced Cl^- secretion was absent in CF ileum [Berschneider et al., 1988; De Jonge et al., 1989]. As calcium regulation of CFTR is not observed in most other cell types [Boucher et al., 1989], a unique signalling pathway for calcium-induced CFTR activation may exist in the small intestine. The mechanism through which calcium regulates CFTR in human ileum is unknown, but direct binding of calcium to CFTR is very unlikely as EF hand structures in CFTR have not been described. Furthermore, such a regulation can not explain the difference between intestinal and other epithelial cells. Activation of CFTR by a Ca^{2+}/calmodulin kinase (CaCaMK) is more likely, since CFTR is found to be regulated by a variety of kinases, e.g. PKA, PKC [Tabcharani et al., 1991] and PKG-II [De Jonge and Bijman, in preparation]. CaCaMK-II is ubiquitously present in most epithelial and non-epithelial cell types and is linked to a class of Cl^-

channels different from CFTR [Worrell and Frizzell, 1991], making this pathway unlikely to be involved in calcium-triggered activation of CFTR. The other forms of CaCaMK, type I and III, could potentially regulate CFTR. However, CaCaMK-III is highly substrate-specific, using only elongation factor 2 as a substrate [Rao and De Jonge, 1990], suggesting that CaCaMK-I is the most plausible candidate for CFTR activation. This hypothesis is supported by the recent finding that CaCaMK-I, but not CaCaMK-II, can phosphorylate CFTR *in vitro* in the R-domain [A.C.Nairn, personal communication] and by the immunological demonstration of CaCaMK-I in intestinal epithelial cells [Rao and De Jonge, 1990]. The lack of calcium-induced CFTR activation in non-intestinal epithelial cells and in the colon carcinoma cell lines suggest that CaCaMK-I (apart from its known presence in neuronal cells) is uniquely expressed in epithelial cells of the small intestine. It is worthwile to test this hypothesis by using isoform-specific CaCaMK-directed antisera.

To establish the role of PKC in Cl^- secretion, HT29cl.19A human colon carcinoma cells were chosen as model system. In these cells, activation of PKC by phorbol esters was found to stimulate basal chloride secretion and to inhibit cAMP-induced chloride secretion [Chapter 5]. As PKC is a family of enzymes, these bimodal effects could be mediated by different forms of PKC.However, despite the variety of PKC isoforms expressed in the HT29cl.19A cells (α, β1, γ, η and ζ), only PKC α was found to play an important role in both stimulation and inhibition of Cl^- secretion (Fig.2)[Chapter 5].

As the Cl^- secretory response (I_{sc}) in the Ussing chamber depends on the proper functioning of at least four different ion transporters [Chapter 1: Fig.2], inhibition of the I_{sc} in response to chronic stimulation by PKC does not reveal which transport system is the main target for PKC-inhibition. However, by using isotope efflux studies, PKC-induced inhibition was found to be directed at the K^+ transporters (Fig.2), rather than the Cl^- channel itself [Chapter 6]. These data are different from those described in tracheal epithelial cells, in which PKC has been reported to inhibit cAMP-activated chloride efflux [Li et al., 1989]. The reason for this discrepancy is presently unknown, but could reside in differences in PKC isozyme pattern between these cell types. Even between two colon carcinoma cell lines, the T84 and HT29cl.19A cells, differences in isozyme content exist. For example, the most important PKC isoform in the regulation of chloride secretion in HT29cl.19A cells, PKC α, is not detectable in T84 cells [Van den Berghe, unpublished results]. Consequently, PMA did not induce Cl^- secretion in the T84 cells [Kachintorn et al., 1992], strengthening the idea that PKC α fulfills a key role

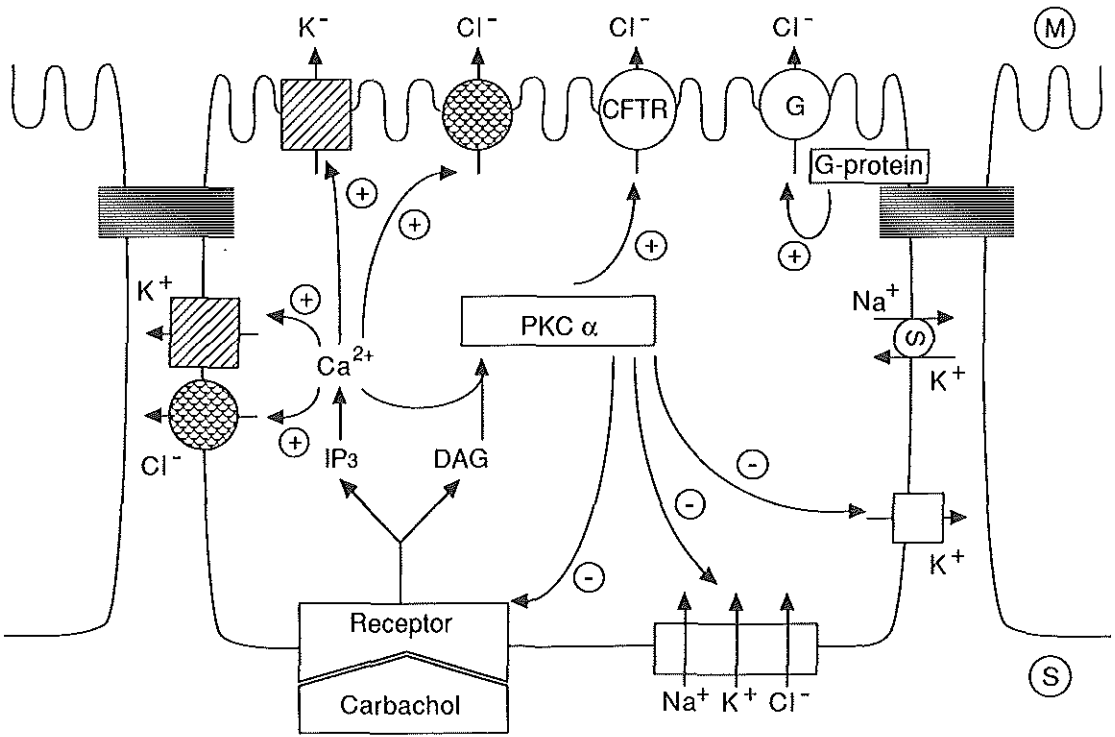

FIGURE 2. Role of PKC α in the regulation of Cl^- secretion in HT29cl.19A colonocytes. The new findings described in Chapter 5 and 6 are schematically summarized. Different types of Cl^- and K^+ channels are indicated by different symbols. CFTR, cystic fibrosis transmembrane conductance regulator; G, G-protein activated Cl^- channel; DAG, diacylglycerol; IP3, inositol (1,4,5) trisphosphate; PKC, protein kinase C; M, mucosa; S, serosa.

in the activation of intestinal Cl^- secretion. Furthermore, in human intestinal mucosa, the expression of PKC α correlates with the ability of PMA to induce Cl^- secretion [Van den Berghe, unpublished], suggesting that the HT29cl.19A cells is a better representative for studying PKC regulation than the T84 cells.

In conclusion, some novel aspects of signal transduction involved in salt and water secretion in intestinal epithelial cells are described in this thesis. The identification of signalling molecules (G-proteins, phospholipase C) at the apical border of rat enterocytes [Chapter 2] was of interest considering the localization of all known G-protein-coupled hormone receptors at the basolateral membrane. In addition, G-proteins were found to activate a novel class of Cl^- channels [Chapter 3]. However, the role of these apical signalling molecules and the G-protein-gated Cl^- channel in the regulation of net salt and water secretion remains to be established [Chapter 2 and 3]. Furthermore, despite the expression of at least five PKC isoforms in HT29d.19A cells, only PKC α was involved in the regulation of chloride secretion [Chapter 5]. The targets for PKC α induced stimulation and inhibition of Cl^- secretion were different: Cl^- channels and K^+ transporters, respectively [Chapter 6]. The identification of the K^+ transporters at the molecular level, however, awaits further research.

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SUMMARY

The major intracellular signalling molecules involved in the regulation of intestinal ion transport are: cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), calcium and diacylglycerol. An increase in the intracellular concentrations of any of these mediators results in an increase in Cl^- secretion and inhibition of Cl^- absorption, leading to a net accumulation of salt and water in the lumen. Abnormalities in the regulation of these processes are observed in two major disease states: cystic fibrosis (CF) and secretory diarrhoea (SD). CF is the most common fatal genetic disease among the Caucasian population with an incidence of about 1 in 2500 and a median life expectation of approximately 26 years. The basic defect has been characterized as an impairment of Cl^- transport in epithelial cells, leading to functional disturbances in various organs, including airways, pancreas, sweat glands and intestine. The mutated gene responsible for cystic fibrosis has recently been identified and encodes a hormonally-regulated Cl^- channel protein, designated cystic fibrosis transmembrane conductance regulator (CFTR). In CF patients, mutations in this protein result in its defective processing and/or loss of function. In secretory diarrhoea, CFTR becomes hyperactive in response to enterotoxins, such as cholera toxin, heat labile and heat stable E.coli toxin. As these enterotoxins act through the same signalling molecules (cAMP, cGMP) that are ineffective in activating CFTR in CF patients, CF heterozygotes are expected to be less sensitive to the excessive and often lethal dehydration associated with SD.

The vectorial transport of salt and water across epithelial layers is critically dependent on the structural and functional polarization of the cell types involved and requires an asymmetrical distribution of transport systems among apical (luminal) and basolateral membranes. In the intestinal crypt cells, Cl^- channels are localized almost exclusively at the apical membrane, whereas other transporters required for net Cl^- secretion (i.e. $Na^+K^+Cl_2$-cotransporters, Na^+/K^+-ATPases and K^+ channels) are localized mainly at the basolateral membrane. The receptors for hormones and neurotransmitters are likewise found predominantly at the basolateral membrane of intestinal epithelial cells. Our previous finding of a GTP-activated phospholipase C at the apical membrane of rat enterocytes was therefore rather unexpected and prompted us to investigate whether such an apical localization was unique for this intracellular signalling pathway. In a first

approach, the apical versus basolateral distribution of several G-proteins, known to act as coupling factors between hormone receptors and signalling enzymes or ion channels, was examined. This study revealed that Gs and Gp were localized both apically and basolaterally, whereas Gi2 and Gi3 were detected predominantly in the basolateral membrane [Chapter 2]. No evidence was obtained for a redistribution or solubilization of any of the G-protein subunits in response to their activation by GTPγS or following cholera toxin or pertussis toxin catalyzed ADP-ribosylation. The different distribution of Gs and Gi among the apical and the basolateral membrane strongly supports the concept that the G-proteins may have a specific function at the membrane to which they are targeted. The co-localization of several G-proteins and intestinal Cl^- channels in the apical membrane, together with recent reports documenting G-protein regulation of a variety of ion channels, prompted us to study the possible modulation of apical Cl^- channels by G-proteins. This study led to the discovery of a novel G-protein activated Cl^- channel in the apical membrane of both rat enterocytes and human colonocytes. As described in Chapter 3, this channel was clearly distinct in its biophysical and regulatory properties from the cAMP-activated Cl^- channel, CFTR. The elucidation of the physiological function of this G-protein-activated Cl^--channel and the identification of the G-protein involved awaits further research.

The presence of an apical GTP-triggered phosphoinositide (PI)-pool, suggests a local function for PI-derived second messengers (calcium and the protein kinase C (PKC) activator diacylglycerol) in this subcellular region. The lack of G-protein-coupled hormone receptors at the apical membrane, however, prompted us to search for alternative pathways possibly linked to the apical PI-cycle. Since cAMP-, cGMP- and PI-mediated pathways are all known to provoke Cl^- secretion, the possibility was considered that these second messengers could amplify their signal by activating the apical PI-pool. Effects of cAMP, cGMP, calcium and PKC on the release of inositol phosphates and calcium were studied in the absence and presence of carbachol, a potent activator of the basolateral PI-cycle [Chapter 4]. Cyclic AMP had no effect on basal levels of inositol phosphates or intracellular free Ca^{2+}, but partially inhibited the carbachol-induced calcium mobilization at a step distal from the generation of inositol phosphates. In contrast the phorbol ester PMA, a PKC activator, was found to inhibit both processes. Although the outcome of this study revealed the existence of cross-talk between the various messenger systems, no stimulatory effects on the (apical) PI-cycle were found.

Finally, the role of PKC in general and its isoforms in particular, in the

regulation of ion transport was investigated. Activators of PKC were found to exert both stimulatory and inhibitory effects on transepithelial Cl^- secretion. A possible antagonistic role of different isoforms was studied by using PKC-isotype-specific antisera. In this study, described in Chapter 5, the expression of multiple isoforms of PKC (α, ß1, γ, η, ζ) could be demonstrated immunologically in the HT29cl.19A human colonic cell line. As the prolonged exposure to PMA resulted in the selective down-regulation of PKC α, but not of the other isozymes, and was accompanied by a loss of the stimulatory effect of PMA on net chloride secretion and recovery of the cAMP response, this isoform seems to have a dual role in the regulation of intestinal chloride secretion. Further analysis of the transport systems involved, revealed that PKC α had different targets: PKC-stimulated apical Cl^- channels and PKC-inhibited basolateral K^+ transporters [Chapter 6].

SAMENVATTING

De belangrijkste intracellulaire signaalstoffen, betrokken bij de regulatie van ionen transport in darmepitheel, zijn: cyclisch adenosine monofosfaat (cAMP), cyclisch guanosine monofosfaat (cGMP), calcium and diacylglycerol. Een verhoging van de intracellulaire concentratie van elk van deze signaal moleculen leidt tot een verhoogde zout en water uitscheiding in het lumen, doordat de secretie van Cl^- gestimuleerd en de absorptie van Cl^- geremd wordt. Een pathologische stoornis op het niveau van Cl^- secretie is het kenmerk van twee veel voorkomende ziektebeelden: taai slijm ziekte of cystic fibrosis (CF) en secretoire diarree (SD). CF is de meest voorkomende lethale genetische ziekte onder het blanke ras met een incidentie van 1 op de 2500 en een levensverwachting van ± 26 jaar. De ziekte wordt gekenmerkt door een verminderd Cl^- transport in epitheelcellen van een groot aantal organen, zoals de luchtwegen, de pancreas (alvleesklier), de zweetklieren en de darm. Het gemuteerde gen dat verantwoordelijk is voor het ontstaan van CF is recent ontdekt en codeert voor een hormonaal reguleerbaar Cl^- kanaal eiwit, dat cystic fibrosis transmembrane conductance regulator (CFTR) genoemd wordt. De bij CF patienten aangetroffen mutaties in het CFTR hebben tot gevolg dat het eiwit niet in de plasma membraan terecht komt en/of functioneel gestoord is. In tegenstelling tot CF zien we bij secretoire diarree een over-aktief CFTR. De belangrijkste oorzaak van SD is een contact van het darmslijmvlies met toxines van pathogene darmbacteriën, zoals cholera toxine en het hitte labiele en hitte stabiele E.coli toxine. Deze toxines activeren CFTR door de intracellulaire concentratie van cAMP of cGMP drastisch te verhogen. Aangezien deze signaal moleculen CFTR niet kunnen activeren in CF patienten, kan verwacht worden dat CF heterozygoten (waarbij slechts één van de twee genen is gemuteerd) minder gevoelig zijn voor diarree ziekten ("heterozygoot voordeel").

Om het transport van zout en water vectorieel te laten verlopen (bijv. van serosa naar mucosa) is het noodzakelijk dat het betrokken cel type gepolariseerd is en dat de transport systemen asymmetrisch zijn verdeeld over de apicale (luminale) en de basolaterale membraan. De chloride kanalen zijn daartoe apicaal en de andere transport systemen die nodig zijn voor transepitheliale Cl^- secretie ($Na^+K^+Cl_2$-cotransporteurs, Na^+/K^+-ATPases en K^+ kanalen) basolateraal gelocaliseerd. De receptoren voor hormonen en neurotransmitters zijn ook

gepolariseerd gelocaliseerd, namelijk in de basolaterale membraan van darm epitheelcellen. Onze vroegere bevinding dat een GTP-activeerbaar fosfolipase C in de apicale membraan van rattedarm epitheelcellen aanwezig is was dan ook onverwacht. Om te testen of een apicale localisatie uniek is voor deze intracellulaire signaal route of dat ook andere signaal moleculen in de hormoon-receptor arme apicale membraan aanwezig zijn, werd de verdeling van verschillende G-eiwitten over de apicale en de basolaterale membraan bestudeerd [Hoofdstuk 2]. G-eiwitten koppelen de hormoon receptor aan intracellulaire boodschappers of aan ionen kanalen in de cel membraan. Gs and Gp bleken in zowel de apicale als de basolaterale membraan te zijn gelocaliseerd, in tegenstelling tot Gi2 en Gi3, die voornamenlijk in de basolaterale membraan werden aangetroffen. Er waren geen aanwijzingen voor een translocatie of een solubilisatie van deze G-eiwitten na activatie door GTPγS of via cholera/ pertussis toxine gekataliseerde ADP-ribosylering. Dit verschil in localisatie tussen Gs and Gi versterkt het idee dat G-eiwitten een specifieke functie hebben in die membraan waarin ze zijn gelegen. De co-localisatie van verschillende G-eiwitten en Cl^- kanalen in de apicale membraan, tesamen met recent verschenen artikelen waarin verschillende ionen kanalen rechtstreeks gereguleerd bleken door G-eiwitten, suggereerde een mogelijke modulatie van apicale Cl^- kanalen door G-eiwitten. Biochemisch en electrofysiologisch onderzoek van deze hypothese leidde tot de ontdekking van een nieuw G-eiwit geactiveerd Cl^- kanaal in de apicale membraan van zowel rattedarm epitheelcellen als humane colonocyten [Hoofdstuk 3]. Dit kanaal was duidelijk te onderscheiden van het cAMP-geactiveerde Cl^- kanaal, CFTR, op grond van zijn biofysische en regulatoire eigenschappen. Verder onderzoek is nodig om de fysiologische functie van dit G-eiwit geactiveerde Cl^- kanaal en de identiteit van de betrokken G-eiwitten te onthullen.

De localisatie van een GTP-activeerbaar fosfolipase C in de apicale membraan suggereert een locale functie in deze subcellulaire regio voor de boodschappers die ontstaan na activatie van dit enzym: calcium en diacylglycerol, de activator van protein kinase C (PKC). Aangezien de apicale membraan geen G-eiwit gekoppelde hormoon receptoren bevat, werd gezocht naar alternatieve routes voor activatie van deze apicale fosfo-inositide (PI)-cyclus. Een mogelijke interactie met basolateraal gegenereerde intracellulaire signaal moleculen (cAMP, calcium, diacylglycerol) of met de apicale cGMP route ("cross-talk") werd bestudeerd [Hoofdstuk 4]. Cyclisch AMP had geen invloed op het basale niveau van zowel de inositol fosfaten als de intracellulaire calcium concentratie, maar remde gedeeltelijk de carbachol-geïnduceerde calcium mobilisatie zonder de

productie van inositol fosfaten te beïnvloeden. Daarentegen remde de forbol ester PMA, een PKC activator, beide processen. Het resultaat van deze studie laat dus de aanwezigheid van "cross-talk" zien tussen cAMP- en PI-gemedieerde routes, maar een stimulerend effect op de (apicale) PI-cyclus kon niet worden aangetoond.

Tenslotte werd de rol van PKC in het algemeen en van zijn isoformen in het bijzonder in de regulatie van ionen transport bestudeerd. Activatoren van PKC hadden zowel een stimulerende als een remmende werking op transepitheliale Cl^- secretie. Deze antagonistische werking zou gemedieerd kunnen zijn door verschillende isoformen van PKC, hetgeen werd bestudeerd door middel van PKC isoform-specifieke antilichamen. In het model systeem voor deze studie, gepolariseerde monolagen van HT29cl.19A humane colonocyten, werd de expressie van meerdere isoformen van PKC (α, $\beta 1$, γ, η, ζ) immunologisch aangetoond. Langdurig behandelen met PMA resulteerde in de selectieve down-regulatie van PKC α, maar niet van de andere PKC isoformen, hetgeen gepaard ging met het verdwijnen van het stimulerend effect van PMA en met opheffing van de remming van de cAMP-gemedieerde netto Cl^- secretie. Deze bevindingen suggereren een rol voor PKC α in zowel de stimulering als de remming van intestinale chloride secretie [Hoofdstuk 5]. Nadere analyse van de betrokken transport systemen leidde tot de conclusie dat het stimulerend effect van PKC α verloopt via activering van apicale Cl^- kanalen, terwijl het remmend effect verklaard kan worden door inactivering van basolaterale K^+ transporteurs [Hoofdstuk 6].

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CURRICULUM VITAE

Nina van den Berghe werd op 17 april 1964 geboren te Heemskerk. Na het behalen van het VWO diploma aan het Ichthus College te IJmuiden in 1982, is zij begonnen met de studie Medische Biologie aan de Universiteit van Amsterdam. In 1983 werd de propedeuse en in 1987 het doctoraal diploma behaald, met als specialisaties medische microbiologie (o.l.v. Dr. A.J.W. Van Alphen) en oncologie (o.l.v. Dr. F.A. Ossendorp). Vanaf augustus 1987 was zij werkzaam bij de afdeling Biochemie (Prof. Dr. J.F. Koster) aan de Erasmus Universiteit te Rotterdam, alwaar het hier beschreven onderzoek onder leiding van Dr. H.R. De Jonge werd uitgevoerd. Vanaf april 1992 werkt zij bij de afdeling Endocrinologie (Prof. Dr. H.M.J. Krans) van het Academisch Ziekenhuis te Leiden aan de regulatie van glucose transport in type 2 diabetes mellitus.

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