Dual role for protein kinase C α as a regulator of ion secretion in the HT29cl.19A human colonic cell line

Nina VAN DEN BERGHE,*† Arie B. VAANDRAGER,* Alice G. M. BOT,* Peter J. PARKER† and Hugo R. DE JONGE

*Department of Biochemistry, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands, and †I.C.R.F., P.O. Box 123, Lincoln's Inn Fields, London WC2A 3PX, U.K.

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 esters [PMA (phorbol 12-myristate 13-acetate) and PDB (phorbol 12,13-dibutyrate)] and 8-bromo cyclic AMP (8-Br-cAMP) induced Cl^- 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 (0.5 μM) for 2 h, a time period in which PKC α , $\beta 1$ and γ levels were not changed. As shown by $^{86}\text{Rb}^+$ and $^{125}\text{I}^-$ 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-isoenzyme-specific antisera, the recovery of the 8-Br-cAMP response coincided with the down-regulation of PKC α , whereas the levels of PKC $\beta 1$ and γ were unmodified. These results suggest that PKC α , but not PKC $\beta 1$ 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 isoenzymes encoded by a family of genes (Nishizuka, 1989; Rao & De Jonge, 1990). PKC α , $\beta 1$, $\beta 2$ and γ are Ca^{2+} - and phospholipid-dependent kinases, with only minor differences in their dependence on diacylglycerol and their substrate specificity (Huang *et al.*, 1988; Marais & Parker, 1989). PKC ϵ is a Ca^{2+} -independent but phospholipid-dependent kinase with a different substrate specificity (Schaap & Parker, 1990). The more recently cloned PKC δ and ζ are not yet fully characterized (Ono *et al.*, 1988). The physiological role of PKC isoenzymes in general and in epithelial ion transport in particular is still unknown. Since PKC α , β and γ are described to be differentially down-regulated by phorbol ester, this phenomenon could possibly be used as a tool to study isoenzyme-specific effects (Isakov *et al.*, 1990).

So far, 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 pseudo-substrates) and PKC activators (phorbol esters, diacylglycerol analogues), which are unable to discriminate between distinct PKC isoenzymes (Rao & De Jonge, 1990). In membrane patches of tracheal cells, PKC provoked Cl^- -channel activation at low Ca^{2+} levels and Cl^- -channel inactivation at high Ca^{2+} levels, suggesting a model in which ion secretion in epithelial cells is controlled in an opposite direction by Ca^{2+} -dependent and Ca^{2+} -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 phorbol 12-myristate 13-acetate (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). However, recent studies in a Cl^- -secreting subclone of the

human colonic cell line, HT29cl.19A, show that phorbol esters and cyclic AMP (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 Cl^- secretion, i.e. apical Cl^- channels and basolateral-localized K^+ channels, $\text{Na}^+/\text{K}^+/\text{Cl}^-$ co-transporters and Na^+/K^+ -ATPase (Vaandrager *et al.*, 1992; Bajnath *et al.*, 1991). Hence the major difference between the stimulus–secretion pathways activated by phorbol esters and by cAMP appears to reside in the exploitation of different protein kinases, rather than different transport systems.

In the present study PKC-isoenzyme-specific antisera were used to examine the effects of phorbol ester treatment on the levels and possible translocation of each isoenzyme in HT29cl.19A cells. The effects of PKC activation and down-regulation on intestinal ion-transport pathways were monitored in parallel by a combination of short-circuit current (I_{sc}) and radioisotope efflux measurements ($^{125}\text{I}^-$ and $^{86}\text{Rb}^+$). The results suggest a key role for PKC α , but not PKC $\beta 1$ 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 phorbol 12,13-dibutyrate (PDB) were from Sigma (St. Louis, MO, U.S.A.), and A23187 and 8-bromo cAMP (8-Br-cAMP) were from Boehringer (Mannheim, Germany). Na^{125}I (14 Ci/mg), $^{86}\text{RbCl}$ (5 mCi/mg) and ^{14}C -labelled Rainbow protein molecular-mass markers were from Amersham International (Amersham, Bucks., U.K.). *myo*-[2- ^3H]-Inositol was obtained from NEN–Dupont, Frankfurt, Germany. The rabbit polyclonal anti-peptide antibodies used in this study were raised and characterized previously in the laboratory of Dr.

Abbreviations used: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PDB, phorbol 12,13-dibutyrate; cAMP, cyclic AMP; 8-Br-cAMP, 8-bromo cAMP; I_{sc} , short-circuit current.

† To whom correspondence and requests for reprints should be addressed.

P. J. Parker (Marais & Parker, 1989). These antibodies were found capable of specifically recognizing PKC isoenzymes and did not show cross-reactivity on immunoblots with other isoforms of PKC (see Fig. 5 in Marais & Parker, 1989).

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 atmosphere of air/CO₂ (19:1) at 37 °C in Dulbecco's modified Eagle's medium, containing 10% (v/v) fetal-calf serum, 40 mg of penicillin/l and 90 mg of streptomycin/l. Cells were subcultured at a surface ratio of 1:5 after trypsin treatment 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 (I_{sc}) and trans-epithelial 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 compartments of an Ussing chamber. The bathing solution was a modified Meyler buffer (108 mM-NaCl, 4.7 mM-KCl, 1.3 mM-CaCl₂, 1.0 mM-MgCl₂, 20 mM-NaHCO₃, 0.8 mM-Na₂HPO₄, 0.4 mM-NaH₂PO₄, 20 mM-Na-Hepes, 10 mM-glucose, pH 7.4), which was aerated by a gas lift system with O₂/CO₂ (19:1). 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 trans-epithelial 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 s, was $108 \pm 27 \Omega \cdot \text{cm}^2$ (mean $\pm$ S.D.; $n = 24$) in the absence of secretagogues.

SDS/PAGE and immunoblotting

SDS/PAGE was performed as described by Laemmli (1970). Samples were dissolved in SDS sample buffer (0.1 M-Tris, 1.7% SDS, 6.7% glycerol, 0.7 M- β -mercaptoethanol, 0.1% Bromophenol Blue, pH 7), boiled for 3 min 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 on to nitrocellulose paper (0.1 μm pore size; Schleicher and Schuell) in 25 mM-Tris/192 mM-glycine/20% (v/v) 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 two washes with TTBS (0.05% Tween in TBS; 5 min/wash), the blots were incubated with antisera (1:500 in TTBS) for 60 min at room temperature. The blots were washed again in TTBS (three times, 10 min/wash) and incubated with goat anti-rabbit antiserum conjugated to alkaline phosphatase for 60 min at room temperature (1:1000 in TTBS). After five washes with TTBS and two with TBS (10 min/wash), 0.8 mg of 5-bromo-4-chloro-3-indolyl phosphate and 1.65 mg of Nitro Blue Tetrazolium in 5 ml of 0.2 M-Tris/10 mM-MgCl₂, pH 9.1, was added for colour development. The reaction was stopped in water.

PKC translocation assay

HT29cl.19A cells were grown to 90–100% confluency in six-well cell-culture plates. At 1 h 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 buffer. The reaction was stopped in ice, and the cells were immediately washed twice with ice-cold buffer A (25 mM-Tris/HCl, pH 7.4, 0.25 M-sucrose, 2.5 mM-magnesium acetate, 2 mM-dithiothreitol), 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 proteinase inhibitors (leupeptin 10 $\mu\text{g}/\text{ml}$, phenylmethanesulphonyl fluoride 100 μM), sonicated (3 $\times$ 5 s), 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 proteinase inhibitors and sonicated (3 $\times$ 5 s). After protein determination, the fractions were solubilized in SDS sample buffer to a final concentration of 1 mg/ml or if necessary, concentrated by precipitation with trichloroacetic acid (10%; 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 , - β 1 and - γ specific antisera.

Isotope efflux experiments

HT29cl.19A cells were grown to 70–80% confluence in six-well cell-culture plates. Cells were loaded for 1.5 h under a humidified atmosphere of air/CO₂ (19:1) at 37 °C in 0.5 ml of a modified Meyler solution containing the radioisotopes (3 μCi of ¹²⁵I⁻/ml or 1 μCi of ⁸⁶Rb⁺/ml). Extracellular radioisotope was removed within 1 min by washing with 3 $\times$ 3 ml of modified Meyler solution at room temperature. The cells retained $0.31 \pm 0.4\%$ of the added ¹²⁵I⁻ and $23 \pm 4\%$ of the added ⁸⁶Rb⁺ ($n = 20$). The radioisotope efflux was determined at 37 °C by addition and consecutive replacements of 1 ml of a modified Meyler solution at 1–2 min intervals. The cells were kept under O₂/CO₂ (19:1), except during the replacements. The residual radioisotope at the end of the experiment was determined by addition of 1 ml of 1 M-NaOH. Owing to its relatively high uptake, ⁸⁶Rb⁺ could be determined together with ¹²⁵I⁻ in a γ -radiation counter. To compare the radioisotope efflux at the various time points, we expressed the data as the fractional efflux per minute, i.e. the amount of released radioisotope in an interval as percentage of the amount of cell-associated radioisotope present at the beginning of that interval, divided by the length of that interval (cf. Clancy *et al.*, 1990).

Determination of total inositol phosphates

Cells grown to 70–80% confluency on six-well cell-culture plates were labelled for 48 h with [³H]inositol (2 $\mu\text{Ci}/\text{ml}$) in inositol-free medium, supplemented with 5% fetal-calf serum under an atmosphere of air/CO₂ (19:1) at 37 °C. At 1 h before stimulation, the cells were shifted to serum-free Dulbecco's modified Eagle's medium containing 10 mM-Hepes (pH 7.4). Control and PMA (27 h, 0.5 μM)-pretreated cells were stimulated with 250 μM -carbachol for 30 min in the presence of 10 mM-LiCl. Incubations were terminated by replacing the medium by 1 ml of ice-cold 10% trichloroacetic acid. After 10 min, extracts were collected and trichloroacetic acid was removed by washing with diethyl ether. The samples were neutralized with 1 M-Tris, pH 8.5, and processed for analysis of the total [³H]inositol phosphate-containing fraction by anion-exchange chromatography 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 [³H]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 with the protein assay kit from Pierce (Rockford, IL, U.S.A.).

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 previously by transepithelial- and intracellular-potential measurements (Bajnath *et al.*, 1991) and radio-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 1) and PMA (results not shown) induced a sustained response (EC_{50} 50 nM), in either the presence or the absence of Ca^{2+} ionophore (Fig. 1b, Table 1). Ca^{2+} ionophore A23187 did not induce an electrical response itself (Figs. 1b and 1c; 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 & 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 h (Table 1; IC_{50} 40 nM). 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 min (Fig. 1c). Prolonged exposure of the colonocytes to PMA (2–5 h) resulted also in a strong inhibition of the response to carbachol and PDB (Figs. 1a and 1b; 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. 1; Table 1).

Both the stimulatory and the inhibitory effects of PMA on the I_{sc} were not seen in response to the inactive phorbol ester analogue 4 α -phorbol 12,13-didecanoate (0.5 μM). 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 PKC in acute stimulation (Figs. 1a and 1b; Table 1) and chronic inhibition (PMA for 2–5 h; Fig. 1; Table 1) of ion secretion in HT29cl.19A cells.

Measurements of efflux $^{125}\text{I}^-$ and $^{86}\text{Rb}^+$

Previous studies have shown that the transport system responsible for PMA-induced activation of the I_{sc} is a Cl^- 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 $^{125}\text{I}^-$ and $^{86}\text{Rb}^+$ efflux rates, mainly reflecting the activity of apical Cl^- channels and basolateral K^+ -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-increasing agent, since the response to 8-Br-cAMP was relatively slow and therefore difficult to measure accurately in the efflux assay.

Fig. 1. Stimulatory and inhibitory effects of phorbol esters on net ion secretion in HT29cl.19A colonocytes

Cells were grown as monolayers on filters and I_{sc} measurements were performed in a modified Ussing chamber. (a) Changes in the I_{sc} provoked by carbachol (250 μM ; —; control) and its inhibition after 2–5 h (---) or 24 h (·····) pretreatment with 0.5 μM -PMA. (b) Changes in I_{sc} provoked by addition of calcium ionophore (10 μM) and PDB (0.5 μM ; —; control) and its inhibition after 2–5 h (---) and 24 h (·····) pretreatment of the cells with 0.5 μM -PMA. (c) Changes in I_{sc} provoked by sequential addition of calcium ionophore (10 μM), 8-Br-cAMP (0.7 mM) and PDB (0.5 μM) in control (—) and PMA-pretreated (---, 2–5 h; ·····, 24 h) HT29cl.19A monolayers. The tracings shown are representative of at least three experiments (see Table 1) and are expressed as I_{sc} per filter (0.33 cm^2). Regarding 2–5 h PMA: full inhibition was already manifest at 2 h after adding PMA and persisted for the following 3 h.

As shown in Fig. 2(a), PMA treatment of the cells for 3 h did not inhibit the forskolin-induced $^{125}\text{I}^-$ efflux, whereas only a slight inhibition was found after 24 h of PMA exposure. In contrast, the forskolin-induced $^{86}\text{Rb}^+$ efflux was strongly inhibited after 3 h of PMA exposure and showed a partial recovery (30%) after 24 h of PMA pretreatment (Fig. 2b). Since the K^+ -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 h of PMA pretreatment apparently results from the inhibition of a K^+ -transport mechanism rather than a Cl^- channel.

Effects of PMA on PKC isoenzyme levels

In order to identify the type of isoenzyme(s) of PKC involved,

Table 1. Short-circuit current (I_{sc}) measurements

HT29cl.19A cells were grown to confluency on filters, and the 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 $\pm$ s.d.) and represent maximal values. The concentrations used are: carbachol, 100 μM ; Ca^{2+} ionophore, 10 μM ; PDB/PMA, 0.5 μM ; 8-Br-cAMP, 0.7 mM. The numbers of experiments are indicated in parentheses. Values of transepithelial electrical resistance (mean $\pm$ s.d.; $\Omega \cdot cm^2$) 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; n.d., not determined.

	I_{sc} ($\mu A/cm^2$)		
	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)	n.d.	n.d.
Ca^{2+} ionophore + PDB	21.6 ± 4.5 (6)	0.6 ± 0.6 (3)	3.6 ± 0.6 (3)
8-Br-cAMP	94.2 ± 31.8 (5)	6.6 ± 4.2 (3)	50 ± 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 ± 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)

* Full inhibition was already manifest at 2 h and persisted for the following 3 h.

Fig. 2. Effects of PMA on forskolin-induced ^{86}Rb and ^{125}I efflux

The ^{125}I (a) and ^{86}Rb (b) efflux in response to forskolin (5 μM) was studied in HT29cl.19A cells with and without PMA pretreatment for 3 or 24 h. Data are expressed as maximal values (mean $\pm$ s.d.) and were obtained after 5 min of forskolin stimulation. The data are representative of at least two other experiments. The Rb^+ efflux after 24 h PMA pretreatment was significantly different from the values obtained after 3 h PMA exposure in both control and forskolin-treated cells (each $P < 0.05$, Student's t test).

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-isoenzyme-specific antisera showed a complete down-regulation of PKC α after 24 h exposure to 0.5 μM -PMA (Fig. 3a). No down-regulation was detectable at early time points up to 5 h (Fig. 3a). In contrast, the levels of PKC $\beta 1$ and PKC γ remained unaffected after PMA treatment for up to 24 h (Figs. 3b and 3c). No significant levels of PKC $\beta 2$ or PKC ϵ could be detected by the immunoblotting technique (results not shown). Reactivity with PKC α , $\beta 1$ and γ (both bands) could be blocked by presaturating the antisera with the antigenic peptide (results not shown). As in leukaemic 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 mixture (Fig. 3). However, a single band is found in most other studies (see Marais & Parker, 1989). Whether the doublet reflects a covalent modification or different subtypes of PKC γ is at present unknown. The selective down-regulation of PKC α after 24 h 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 after 2 h of 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 might act as a mediator of carbachol- and PDB-induced ion secretion. To test this possibility, PKC translocation assays were performed with PKC-isoenzyme-specific antisera. Plastic-grown monolayers of HT29cl.19A colonocytes were exposed for 0–10 min to carbachol or for 10 min to PMA or Ca^{2+} ionophore, and the particulate and the soluble fractions were separated. Immunoblotting with PKC-isoenzyme-specific antisera identified a transient PKC α redistribution in response to carbachol: PKC α appeared in the particulate fraction at 2–5 min after carbachol addition and disappeared again after 10 min of carbachol exposure (Fig. 4). The fraction of PKC α translocated in response to carbachol was apparently too small to reveal a significant fall in 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 apparently sufficient to reach the I_{sc}

possible effects of PMA pretreatment on the cellular content of PKC isoenzymes were tested by immunoblotting with PKC-isoenzyme-specific antisera. Filter-grown monolayers of

Fig. 3. Effects of PMA pretreatment on levels of PKC α , β_1 and γ

HT29cl.19A cells were pretreated for several hours with $0.5 \mu\text{M}$ -PMA. Samples of cell homogenates ($15 \mu\text{g}$ of protein) were separated by SDS/PAGE, transferred to nitrocellulose paper and incubated with PKC-isoenzyme-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 mixture ($0.5 \mu\text{g}$) from human brain together with

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 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 dephospho-forms of the enzyme (Parker *et al.*, 1987).

Since PKC β_1 and PKC γ were located predominantly in the particulate fraction, translocation of these isoenzymes 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 by a down-regulation-resistant form of PKC. To test this possibility, we measured the formation of total inositol phosphates in the absence and presence of carbachol in control and PMA-pretreated cells (27 h). As shown in Table 2, chronic PMA pretreatment did not lead to a significant decrease in the carbachol-induced formation of inositol phosphates and thus does not interfere with carbachol-provoked phospholipase C activation. This result strongly suggests that (i) the diacylglycerol produced, in spite of its possible action as activator of PKC β_1 or γ , is apparently unable to provoke active ion secretion, and (ii) the loss of carbachol-induced ion secretion in PKC- α -depleted cells most probably results from PKC α down-regulation rather than from a lack of activating signal, i.e. diacylglycerol.

DISCUSSION

Expression pattern of PKC isoenzymes in HT29cl.19A colonocytes

In the present work, using PKC-isoenzyme-specific antisera, the HT29cl.19A cells were shown to express three PKC isoenzymes, α , β_1 and γ . Two other PKC isotypes, β_2 and ϵ , could not be detected immunologically. To our knowledge, the expression of PKC γ in non-neuronal tissue has not been described previously. 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 β_1 or γ , as a regulator of intestinal ion transport. Although PKC β_1 and γ are also expressed in significant quantities, no clue was provided as to 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 I^- and Rb^+ efflux rates respectively. Surprisingly, no evidence was found for an

Rainbow molecular-mass (kDa) marker proteins. Similar results were obtained in two other experiments.

Fig. 4. Carbachol-induced PKC α translocation

HT29cl.19A cells were exposed for several time periods to carbachol (100 μ M), Ca^{2+} ionophore (10 μ M) or PMA (0.5 μ M). Soluble (S) and particulate (P) fractions were separated by centrifugation, and samples of each fraction (20 μ g of protein) were subjected to SDS/PAGE, transferred to nitrocellulose and incubated with PKC- α -specific antiserum (1:500). Lanes: 1, control; 2–6, carbachol for 1, 1.5, 2, 5 and 10 min respectively; 7, PMA for 10 min; 8, Ca^{2+} ionophore for 10 min. Similar results were obtained in two other experiments. Migration of the Rainbow molecular-mass (kDa) marker proteins was as indicated.

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

[^3H]Inositol-labelled HT29cl.19A cells were incubated for 30 min in the presence of 10 mM-LiCl alone (a) or LiCl and 250 μ M-carbachol (b), or were pretreated for 27 h with 0.5 μ M-PMA, before addition of carbachol and LiCl (c). The reaction was stopped by adding trichloroacetic acid. Inositol phosphates were analysed on Dowex columns as described in the Methods section. Data are expressed as means $\pm$ S.D. and are representative of three experiments done in triplicate.

Addition	Inositol phosphates (d.p.m.)
(a) None	24872 $\pm$ 923
(b) Carbachol	325989 $\pm$ 23090
(c) Carbachol after 27 h PMA	324955 $\pm$ 8928

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 I^- 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 at present unknown, but may reside in possible differences in PKC isoenzyme pattern/localization, in Cl^- -channel subtypes or in other regulatory factors.

Instead, 3 h exposure of the HT29cl.19A colonocytes to PMA led to an almost complete inhibition of the forskolin-induced Rb^+ 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 $\text{Na}^+/\text{K}^+/\text{Cl}^-$ co-transport activity with a similar time course (Franklin *et al.*, 1989). Since the PMA-induced inhibition is relatively slow, the mechanism most likely involves a decrease in the number of K^+ transporters, e.g. by endocytosis or proteolytic breakdown, as has been described for the $\text{Na}^+/\text{K}^+/\text{Cl}^-$ co-transporter (Franklin *et al.*, 1989). This process may be triggered by PKC-catalysed

phosphorylation of the K^+ transporters themselves or may possibly involve different (regulatory) phosphoproteins.

Our conclusion that PKC α rather than any of the other isoenzymes expressed in this cell line (i.e. PKC β 1 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 after 24 h of PMA treatment. At this point, the cellular level of PKC α was completely down-regulated, whereas PKC β 1 and γ remained resistant to down-regulation (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 PKA α . This possibility has gained interest in view of the recent finding that forskolin is able to elevate diacylglycerol levels in tracheal cells (Anderson & Welsh, 1990). It is also possible that expression of the cAMP-activated apical Cl^- channel is impaired after chronic PMA treatment. The recent findings that (i) the cystic-fibrosis-gene-encoded protein, CFTR, is most likely identical with the cAMP-activated Cl^- channel itself (Anderson *et al.*, 1991; Kartner *et al.*, 1991) and (ii) expression of the cystic-fibrosis gene is indeed strongly inhibited by phorbol ester treatment (Trapnell *et al.*, 1990) lend some support to the latter explanation.

Role of PKC α as a mediator of ion secretion

The carbachol- and PDB-provoked rise in I_{sc} shown in Fig. 1 and the rapid increase in fractional effluxes 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 Ca^{2+} 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 min), whereas phorbol-ester-provoked changes in I_{sc} (Fig. 1) and PKC α translocation (Fig. 4) were sustained for at least 10 min. Thirdly, our finding of a selective recovery of cAMP-provoked, but not of carbachol/PDB-provoked, ion secretion in PKC- α -down-regulated cells, under conditions in which all other PKC isoenzymes 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 down-regulation 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 27 h 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 isoenzymes detected immunologically in the HT29cl.19A colonocytes (α , β 1, γ), only the α form showed rapid translocation in response to PKC activators (PMA, carbachol) and became down-regulated by chronic exposure to PMA (24 h; 0.5 μ M). [Preliminary results, using antisera specific for PKC δ , ζ and η , indicate that PKC ζ and η (but not PKC δ) are also expressed in the HT29cl.19A cells, but are not down-regulated in response to PMA.] 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. $^{86}\text{Rb}^+$ and $^{125}\text{I}^-$ efflux studies showed that inhibition was relatively slow and targeted on basolateral K^+ transport systems, whereas the stimulation was acute and exerted at the level of an apical Cl^- 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 < 5 h) and would bring about a state of resistance to other secretagogues, including microbial toxins (cholera toxin, heat-stable *Escherichia coli* toxin) acting through different intracellular signals (cAMP, cyclic GMP). Experiments to substantiate this prediction in an animal model are needed.

We thank Dr. J. A. Groot and Dr. R. B. Bajnath (University of Amsterdam, The Netherlands) for critically reading the manuscript. This study was supported by the Netherlands Organization for the Advancement of Scientific Research (NWO).

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Received 11 September 1991/27 January 1992; accepted 11 February 1992