Stimulation of porcine jejunal ion secretion in vivo by protein kinase-C activators.

C S Weikel, … , J J Sando, R L Guerrant

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Research Article

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Stimulation of Porcine Jejunal Ion Secretion In Vivo by Protein Kinase-C Activators

Cynthia S. Weikel, Julianne J. Sando, and Richard L. Guerrant
Divisions of Geographic Medicine and Infectious Diseases, Departments of Medicine and Pharmacology, University of Virginia School of Medicine, Charlottesville, Virginia 22908

Abstract

Microbial toxins act through cyclic nucleotide dependent (cAMP or cGMP) or cyclic nucleotide independent pathways to cause intestinal ion secretion. To explore the calcium dependent, cyclic nucleotide independent pathway that is postulated to involve protein kinase C activation, we measured protein kinase C activity and phorbol ester binding in isolated intestinal epithelial cells and examined the effects of the C-kinase activators, phorbol myristate acetate, phorbol dibutyrate, and 4-β-phorbol-12,13-didecanoate, in weaned pig jejunum in vivo. We demonstrated both protein kinase C activity and specific phorbol ester binding in porcine jejunal epithelial cells. Phorbol myristate acetate, phorbol dibutyrate, and 4-β-phorbol-12,13-didecanoate (10^{-5} M) each caused striking secretory responses at 5 h with accumulation of Na^+, K^+, Cl^-, and HCO_3^- intraluminally. In contrast, 4-α-phorbol and 4-α-phorbol-12,13-didecanoate, which do not affect protein kinase C, allowed normal net absorption of all electrolytes from the intestinal lumen equivalent to controls with only Ringer's lactate. Time course studies revealed significant secretion within 30 min after exposure to the C-kinase activators. These data suggest an important role for protein kinase C activation in intestinal ion secretion.

Introduction

Four distinct pathways may be involved in intestinal ion secretion stimulated by bacterial toxins. The first two pathways are cyclic nucleotide-dependent: the activation of adenylate cyclase by choleratoxin and the heat-labile enterotoxin of *Escherichia coli*, and the activation of guanylate cyclase by the heat-stable enterotoxin (STa) of *E. coli* (1). Two cyclic nucleotide independent pathways have been receiving increasing attention in recent years. First is the secretion stimulated by the second heat-stable enterotoxin of *E. coli*, STb, the mechanism of action of which is unknown but does not involve activation of adenylate or guanylate cyclase (2). Second is the cyclic nucleotide independent secretion stimulated by a calcium dependent pathway. Two pools of calcium are recognized as important in intestinal secretory processes. Certain intestinal secretagogues have been shown to have a secretory effect that is dependent on extracellular calcium (calcium ionophore A23187 [3, 4], acetylcholine [3, 5], *Clostridium difficile* enterotoxin [6], and serotonin [7]). Other agents cause secretion that is dependent, in part, on intracellular pools of calcium as in secretion stimulated by the cyclic nucleotide dependent pathways (1, 8, 9).

Several authors (10, 11) have postulated a role for protein kinase C (PK-C)[1] in the stimulation of intestinal secretion, but no data showing an association between intestinal secretion and PK-C stimulation have been reported. PK-C stimulation has been shown to have a crucial role in signal transduction for a wide variety of secretory processes such as epinephrine secretion by the adrenal medulla, amylase secretion by pancreatic acinar cells, insulin secretion by pancreatic islets (12), and parathyroid hormone secretion (13). These secretory processes are initiated by the signal-dependent breakdown of inositol phospholipids that release diacylglycerol. Diacylglycerol has been shown to increase the affinity of PK-C for calcium, and, in the presence of acidic phospholipids, such as phosphatidylserine (PS), leads to full activation of this enzyme (14). Furthermore, PK-C has been shown to be widely distributed in nature (15), and recently, de Jonge (16) has reported the presence of phospholipid-stimulatable protein kinase activity (presumably PK-C) in isolated brush border membranes of rat small intestine.

The purposes of this study were to determine whether PK-C activity was detectable in intestinal epithelial cells and to evaluate whether stimulation of PK-C can cause net intestinal ion secretion in vivo. To evaluate this latter possibility we used the phorbol esters. The phorbol esters have been demonstrated to bind to PK-C and to substitute for diacylglycerol in the stimulation of PK-C without changing intracellular calcium concentrations (14, 17). We report that both PK-C activity and specific phorbol ester binding are present in isolated porcine intestinal epithelial cells. Furthermore, the beta (active) analogues of the phorbol esters stimulate ion secretion in piglet jejunum in vivo, while the alpha analogues, which have no biological activity and do not bind to PK-C, do not cause secretion. These results suggest an important role for PK-C stimulation in intestinal ion secretion.

Methods

Materials. Two beta phorbol esters, phorbol myristate acetate (PMA) and phorbol dibutyrate (PDB), were obtained from Consolidated Mid-

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1. *Abbreviations used in this paper:* 4-α (or β)-PDD, 4-α (or β)-phorbol-12,13-didecanoate; PDB, phorbol dibutyrate; PK-C, protein kinase C; PMA, phorbol myristate acetate; PS, phosphatidylserine; PSP, phenolsulfonphthalein.

Line Corp. (Brewster, New York). 4-β-phorbol-12,13-didecanoate (4-β-PDD), 4-α-phorbol-12,13-didecanoate (4-α-PDD), and 4-α-phorbol were obtained from Sigma Chemical Co. (St. Louis, MO). Stock solutions of phorbol esters (10^{-3} M) were stored in absolute ethanol at −15°C until use. For injection into ligated intestinal segments, the phorbol esters were diluted in Ringer's lactate, pH 7.4, containing 1% bovine serum albumin (BSA) (Sigma Chemical Co.). Control solutions containing Ringer's lactate, an equivalent amount of alcohol without the phorbol esters, and 1% BSA were run as paired controls in all experiments. The Ringer's lactate contained sodium (125 mM), potassium (4.7 mM), magnesium (1.2 mM), calcium (1.25 mM), chloride (127 mM), bicarbonate (25 mM), and sulfate (1.2 mM).

Cell isolation, rupture, and fractionation. Intestinal epithelial cells were isolated from the jejunum of 4–6-wk-old weaned piglets from a single supplier (Pagebrook Farm, Gordonsville, VA) using the methods of Weiser (18) or Kimmich (19). Approximately 10^8 cells or more were obtained with ≥80% viability as determined by trypan blue exclusion. Cells were ruptured in 2–10 ml of homogenization buffer (20 mM Tris, pH 7.5, 0.33 M sucrose, 2 mM EDTA, 0.5 mM EGTA, 0.5 mM benzamidine, and 25 μg/ml leupeptin) with 35 strokes in a Dounce homogenizer fitted with a tight glass pestle. Cytosol fractions were prepared by centrifuging for 45 min at 105,000 *g* and stored at −70°C until use.

Protein kinase assay. Calcium/phospholipid-dependent protein kinase activity was assayed by incubation at 30°C for 3 min in a 150 μl reaction volume containing 30 μg of histone H-1, 20 mM Tris, pH 7.5, 60 μM $CaCl_2$, 5 mM magnesium acetate, 100 μM ATP, 75–130 Ci/M [γ-^{32}P]ATP, and 60 μl of cytosol enzyme (containing 8–50 μg protein) with or without 6 μg of PS and 0.25 μg of diolein as indicated. The phospholipid and diolein were added from a stock solution prepared just before use by evaporating aliquots of PS (10 mg/ml) and diolein (1 mg/ml) under nitrogen gas and sonicating in 2 ml of 20 mM Tris, pH 7.5. Reactions were initiated by addition of the reaction mixture and terminated by pipetting 75 μl of the reaction volume onto Whatman P81 cellulose phosphate paper (Fisher Scientific Products, Springfield, NJ). Filter papers were washed three times in 50 mM NaCl, dried, and counted for radioactivity.

Phorbol ester binding assay. Phorbol ester binding was assayed on cytosolic fractions (360 μl containing 24–150 μg protein) by incubation for 2–20 h at 4°C in a 500 μl reaction volume containing 20 mM Tris, pH 7.5, 75 mM magnesium acetate, 0.5 mM $CaCl_2$, 1.2 mg/ml BSA, 96 μg/ml PS, 4×10^{-8} M [^{3}H]PDB, and either vehicle (total binding) or 3 μM unlabeled PDB or PMA (nonspecific binding). Bound [^{3}H]PDB was separated from free [^{3}H]PDB by filtering the mixture through 2.4-cm Whatman glass microfibre filters (Fisher Scientific Products) by suction. The tubes and filters were washed five times with ice-cold phosphate-buffered saline and the filters were counted for radioactivity.

Partial purification of cytosolic PK-C. PS affinity column chromatography was used to partially purify cytosolic PK-C. The column resin was prepared according to the method of Uchida and Filburn (20) as follows: Cholesterol (50 mg) and PS (10 mg in chloroform) were dried under N_2 gas and dissolved in absolute ethanol (1 ml) in a boiling water bath. A warm solution of 15% acrylamide and 5% *bis* (10 ml) was added and polymerized with the addition of 10% ammonium persulfate (0.3 ml) and *N,N,N′,N′* tetramethylethylenediamine (TEMED) (5 μl). The gel was stored in the dark, at room temperature, overnight, to ensure complete polymerization, and later stored at 4°C until used. The polymerized gel resin was then minced into small pieces and homogenized (4–6 strokes) in 20–30 ml of water using a loose-fitting Dounce homogenizer. The homogenized gel resin was defined 3–4 times, packed into a column (1.6 × 5 cm), and equilibrated in 200 mM KCl, 1 mM $CaCl_2$, 50 mM 2-mercaptoethanol, 0.5 mM benzamidine, 25 μg/ml leupeptin, and 5 mM 2[*N*-morpholino]ethane-sulfonic acid (MES), pH 6.5, at 4°C.

Cytosol was loaded in the presence of 5 mM $CaCl_2$ onto the PS affinity resin for 45 min at 4°C using a vertical rotator to maintain the suspension. The resin was loaded into the column and washed successively with three column volumes each of the 5 mM MES buffer described above with either 1 mM $CaCl_2$, 0.1 mM $CaCl_2$, or 2 mM EGTA in place of the calcium. Portions of the resulting affinity column fractions were immediately stabilized with the addition of ovalbumin (1 mg/ml). Selected column fractions were subsequently assayed for protein kinase activity and phorbol ester binding as described above. For the protein kinase assay, fractions were desalted over Sephadex G-25.

Intestinal loop assays. For these studies, 4–6-wk-old weaned piglets were used. Ligated porcine intestinal segments (~6-cm jejunal segments) were injected with 5 ml of the test substance. At 5 h the fluid was removed from the segments and assayed for electrolyte content using a sequential multiple analyzer with computer (Technician Instruments, Tarrytown, NY), and volume and length of the segment were measured. These results were compared with the electrolyte content of the preinoculation solutions measured the same way. In some experiments, the segments were exposed to the test substances for 30 min and 16 h. Portions of the experimental and control intestinal segments were fixed in 10% formalin, and subsequently the histology was evaluated by hematoxylin and eosin staining.

Time course studies. For these experiments, ligated porcine intestinal segments (~12-cm jejunal segments) were cannulated with multiperforated feeding tubes (eight French; Argyle, Div. Sherwood Medical, St. Louis, MO). Each segment was washed until clear with 15-ml vol of Ringer's lactate. Challenge solutions consisted of Ringer's lactate, pH 7.4, with 1% BSA, and the nonabsorbable volume marker phenolsulfonphthalein (PSP, 50 mg/liter) with 10^{-5} M PMA, PDB, 4-α-phorbol, or alcohol. Net water fluxes were measured during consecutive 30-min periods. Measurement of flux during a 30-min period was initiated by instillation of 15 ml of a challenge solution. After mixing, 1 ml was withdrawn as the initial sample. 1-ml samples were then taken after mixing at 30, 60, and 90 min. At 90 min, the segments were emptied, washed three times with 15 ml of Ringer's lactate, and refilled with 15 ml of Ringer's lactate with 1% BSA and PSP (50 mg/liter) without additional phorbol esters. Samples were taken as described for an additional 90 min.

The initial and final samples were analyzed for PSP concentration colorimetrically by the method of Allen (21). To determine the net water flux for each study period, initial and final volumes were calculated for each segment using the equation $V_1C_1 = V_2C_2$. V_1 and V_2 represent the initial and final volumes, respectively, for each study period. Similarly, C_1 and C_2 represent the initial and final concentrations of PSP for each study period. Net water flux = $V_2 - V_1$ (expressed as microliters per centimeter jejunum per minute).

Statistical methods. Data are expressed as mean±SEM. All statistical comparisons were done using the *t* test or, where appropriate, the paired *t* test.

Results

To investigate a potential role for PK-C in intestinal ion secretion, we first verified the existence of PK-C in jejunal epithelium. Cytosolic preparations (frozen at −70°C) from three different piglets exhibited a phospholipid-stimulatable kinase activity of 2.25±0.62 pmol phosphate incorporation into histone H-1 per milligram cytosol protein per 3 min. Additional experiments (n = 2) measuring PK-C enzymatic activity on fresh and frozen cytosols from the same piglet indicated that ~20% of the phospholipid-stimulatable kinase activity was destroyed by freezing at −70°C. PK-C also serves as the receptor protein for the tumor-promoting phorbol esters (14, 17), the most potent and widely used activators of PK-C. To further establish the identity with PK-C of the phospholipid-stimulatable kinase activity in cytosol of porcine jejunal epithelial cells, the enzyme was partially purified by PS affinity chromatography. Both enzyme and phorbol ester binding activities were monitored. Fig. 1 shows that specific PDB binding activity co-eluted with the phospholipid-stimulatable kinase activity in the presence of 2 mM EGTA as previously described (20).

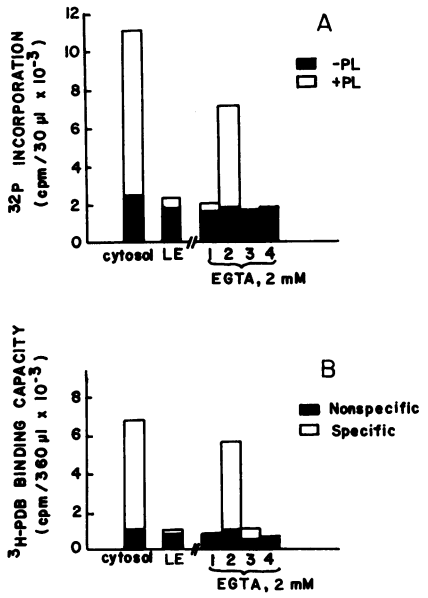

Figure 1. (*A*) PK-C enzymatic activity. ^{32}P incorporation into a histone H-1 substrate was measured on whole cytosol, and on the loading eluant (LE) and 4 cytosolic fractions eluted with 2 mM EGTA from a PS affinity column. The open bars represent the specific phospholipid-stimulatable PK-C enzymatic activity. Data are means of duplicate measurements from a representative experiment repeated once. (*B*) Phorbol ester binding capacity. PDB binding was measured in the presence of vehicle (total binding) or 3 µM unlabeled PDB or PMA (nonspecific binding) on whole cytosol, loading eluant and cytosolic fractions as for the PK-C enzymatic activity. The open bars represent the specific phorbol ester binding. Data are means of duplicate measurements from a representative experiment repeated once.

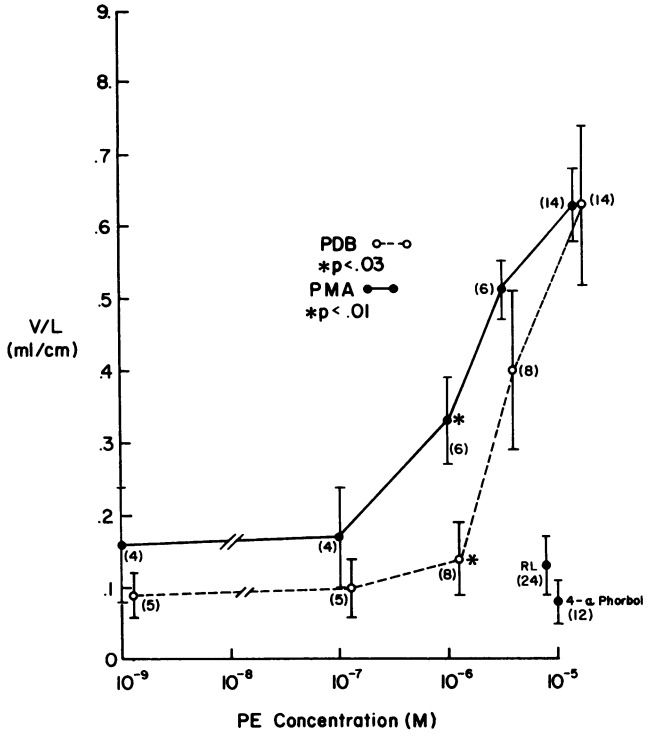

Figure 2. Phorbol ester (PE) concentration dependency for the effect of PMA and PDB on fluid secretion in porcine jejunum in vivo. Secretion was measured as the volume/length ratios (V/L, milliliters per centimeter). Segments (*n*) in 2–11 pigs. Data are expressed as mean±SEM for *n* segments.

The effect of PMA and PDB on ion secretion in porcine jejunum in vivo was then tested. Both PMA and PDB were found to cause a dose-dependent increase in the volume/length ratios measured in the intestinal segments at 5 h (Fig. 2). By paired analysis, significant secretion was first seen at a concentration of 10^{-6} M for PMA ($P \leq 0.01$) and PDB ($P \leq 0.03$), and was maximal by 10^{-5} M. Intestinal segments treated with either the Ringer's lactate control or 4-α-phorbol (10^{-5} M) did not show significant secretion. Comparison of the active and inactive analogues of phorbol-12,13-didecanoate (Fig. 3) gave volume/length ratios of 0.54±0.2 and 0.07±0.2, respectively ($P < 0.0001$).

The time course of the phorbol ester effect on the net jejunal water flux is depicted in Fig. 4. After only 30 min exposure of the jejunal mucosa to PMA or PDB, significant ($P \leq 0.03$) secretion compared with the 4-α-phorbol control was detected. Significant secretion persisted at all subsequent time points ($P \leq 0.01$ for PMA or PDB vs. controls). In contrast, the Ringer's lactate and 4-α-phorbol controls allowed normal net water absorption to occur.

Summarized in Table I are the effects of the β and α phorbol esters on the intestinal fluid electrolyte content after 5 h of incubation. Results with the 4-α-phorbol and 4-α-PDD were combined because they affected electrolyte movement in an identical fashion. In addition, the intestinal fluid electrolyte content of segments treated only with Ringer's lactate did not differ from those treated with the phorbol esters. In our system, comparison of the β phorbol esters to the α compounds revealed significant net accumulation of all electrolytes measured ($P \leq 0.004$) except for the HCO_3^- of 4-β-PDD ($P \leq 0.04$). In other systems, the phorbol esters exhibit a rigid structure activity relationship (22), with PMA being the most potent and 4-β-PDD being the least potent of the three we have examined. In our system, comparison of the effects of the individual beta analogues on the electrolyte content of the intestinal fluid revealed a differential effect on sodium, potassium, and chloride, with PDB being most potent and 4-β-PDD least potent. In contrast, bicarbonate was affected similarly by all three beta analogues. One possibility for the apparent increased potency of PDB with respect to PMA is the much greater lipophilicity of PMA. Because of intestinal nonspecific binding of PMA (23), its free concentration is probably much less than that of PDB.

Lastly, we evaluated the effect of the phorbol esters on histology. Neither the Ringer's lactate control nor the alpha analogues caused any abnormality in jejunal histology. Although a slight increase in epithelial cells was seen in the lumen at 30 min with the beta analogues, there was no inflammatory infiltrate.

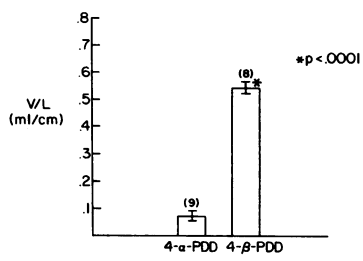

Figure 3. The effect of 4-β- and 4-α-PDD (10^{-5} M) on fluid secretion in porcine jejunum in vivo. Secretion was measured as the volume/length ratios (V/L, milliliters per centimeter) of intestinal segments (*n*) in three piglets. Data are expressed as mean±SEM for *n* segments.

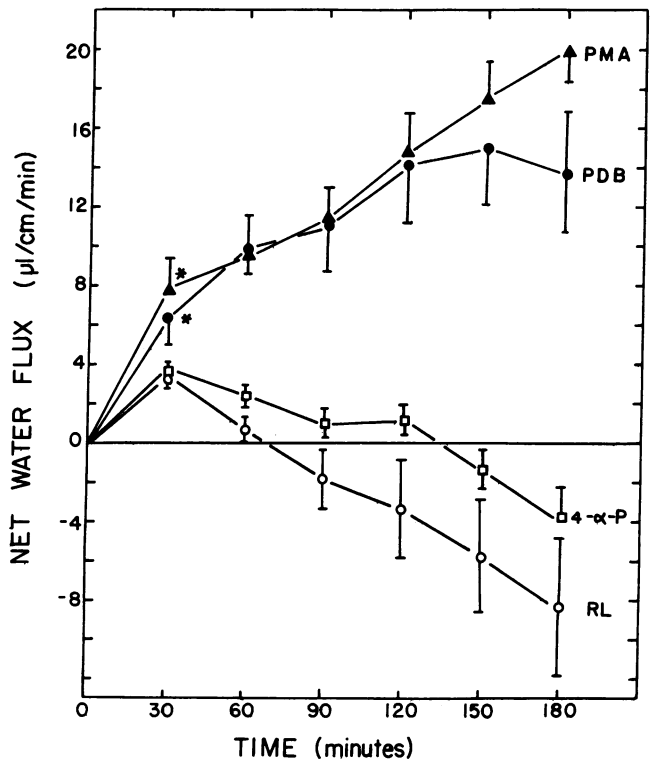

Figure 4. The time course of the effect of 4-α-phorbol, PMA, and PDB (10^{-5} M) on the net water flux in porcine jejunum in vivo. Intestinal secretion (expressed as microliters per centimeter per minute) was measured in three piglets using the nonabsorbable volume marker, PSP (50 mg/liter). Significant secretion was detected at 30 min with both PMA and PDB ($P \leq 0.03$) compared with the 4-α-phorbol control. Data are expressed as mean±SEM.

A transient, minimal increase in the number of polymorphonuclear leukocytes in the lamina propria was seen in the small bowel only at 5 h. At 16 h, secretion persisted with no histologic abnormalities. Although the phorbol esters are not chemotactic for neutrophils, these compounds do stimulate release of lysosomal enzymes, which are chemotactic and may account for the mild transient accumulation of polymorphonuclear leukocytes noted in these studies. The fact that mepacrine, a phospholipase A_2 inhibitor, did not reduce the secretory response to PMA, further argues against a role for neutrophils in the secretory response observed. The volume/length ratio in ligated intestinal segments treated intraluminally with mepacrine and PMA (10^{-4} and 10^{-5} M, respectively) was 0.89±0.04 ($n = 4$), compared with 0.81±0.07 ($n = 6$) in ligated intestinal segments treated only with PMA (10^{-5} M). The volume/length ratios in control loops treated with mepacrine alone (0.18±0.05, $n = 2$) or mepacrine and 4-α-phorbol (0.24±0.17, $n = 4$) at the same concentrations were significantly less ($P \leq 0.01$ for both).

Table I. Effect of Alpha and Beta Phorbol Esters (PE) on Net Intestinal Fluid Electrolyte Content (X±SEM)

	Na^+	K^+	Cl^-	HCO_3^-
β-PE‡				
PDB	138±3	10.2±2	115±18	12±3
PMA	93±13	6.5±1	82±12	9±1
4-β-PDD	76±8	5.3±0.3	54±4	12±6
α-PE§	27±3	3.2±0.2	26±3	1±0.5
Ringer's lactate	41±16	2±1	39±15	3±2

* Electrolytes in microequivalents per centimeter.
‡ $P \leq 0.004$ for all β-PE for each ion, compared with α-PE except for HCO_3^- of 4-β-PDD ($P \leq 0.04$).
§ Includes 4-α-phorbol and 4-α-PDD.
Electrolytes were measured on fluid removed from ligated intestinal segments after 5 h of incubation with the specified phorbol ester or Ringer's lactate.

Discussion

In this report, we present data suggesting a role for the activation of PK-C in intestinal ion secretion. PK-C has previously been identified in rat small intestine smooth muscle (23), and both de Jonge (16) and we, as reported here, have detected the presence of PK-C in isolated intestinal epithelial cell preparations. Since the enzyme co-elutes with the receptor for phorbol esters during purification to various extents (20, 24–26), and since phorbol esters directly stimulate the enzyme (14), we used these tumor promoters as probes to examine the involvement of PK-C in intestinal ion secretion. Our in vivo data indicating net intestinal secretion with micromolar concentrations of the active, but not the inactive analogues of the phorbol esters, suggest a role for the activation of PK-C in causing intestinal secretion. The concentration dependence seen in our studies for secretion is 1–2-M logs higher than in other secretory systems using isolated cell preparations (13, 27, 28), and in some systems (29, 30), stimulation of processes involving secretion has been seen with nanomolar concentrations. However, in some systems in which large numbers of cells are necessary (31), high concentrations of phorbol esters have been required, probably to overcome the large nonspecific binding due to lipid partitioning. Such nonspecific binding may similarly account for the relatively high phorbol ester concentrations needed for secretory effects in our ligated porcine intestinal segments in vivo.

Whether or not PK-C stimulation alone is sufficient to elicit measurable net secretion in vivo is uncertain at present. Intestinal secretion has been stimulated by products released during an inflammatory response. Musch et al. have shown that bradykinin added in vitro to the serosal surface of rabbit or guinea pig ileum or rabbit colon has potent secretory effects, with chloride secretion being seen at concentrations as low as 10^{-10} M (32). The effects of bradykinin were linked to the intracellular release of arachidonic acid, which may be metabolized through one of two pathways: either the cyclooxygenase pathway with the production of prostaglandins and related substances (such as prostacyclin and thromboxanes), or by the lipoxygenase pathway to produce hydroperoxyeicosatetraenoic acid or hydroxyeicosatetraenoic acid and leukotrienes. Notably, hydroperoxyeicosatetraenoic acid and hydroxyeicosatetraenoic acid have been shown to cause chloride secretion in vitro in rabbit colon but not rabbit ileum (33). In addition, prostaglandins of the E series (especially prostaglandin E_2) are known to be secretagogues (34). In our studies, there was no inflammation seen in the intestinal tissue at 30 min or at 16 h, times at which a secretory response was measured. The minimum increase in neutrophils noted histologically at 5 h is of doubtful significance, especially since mepacrine, which inhibits phospholipase A2, and thus arachidonic acid release, did not alter the secretory response we observed with PMA at 5 h. This dose of mepacrine has previously been shown to inhibit

the response to bradykinin in vitro by >80% (32). These findings suggest that products of the inflammatory reaction do not contribute to the secretory response we observed with the phorbol esters.

The effect of the phorbol esters on the electrolyte content of intestinal fluid and the rapid onset of net water accumulation suggest a direct role for PK-C activation in the transport of several ions, perhaps secondary to phosphorylation of one or more membrane ion transporters. This hypothesis is supported by the observation that treatment of certain cell types with the β analogues of the phorbol esters results in a decrease in PK-C activity in the cytosolic fraction (27, 35) and an increase in the membrane fraction (36). In our studies, the major ions present in the intestinal fluid were sodium and chloride, suggesting primarily an impact on neutral sodium chloride absorptive mechanisms. The net accumulation of potassium could reflect a change in paracellular transport secondary to a loosening of the intestinal epithelial cell tight junctions. Alternatively, the accumulation of potassium could reflect secretion by calcium-activated potassium channels (37). More detailed studies examining changes in ion transport due to phorbol esters in vitro and electron microscopic examinations of epithelium treated with phorbol esters will be necessary to evaluate these possibilities.

In summary, these results demonstrate PK-C activity and specific phorbol ester binding in porcine jejunal epithelial cells, and suggest that direct activation of PK-C by phorbol esters causes net intestinal ion secretion in piglet jejunum in vivo. Further studies evaluating the interaction of PK-C stimulation with previously described intestinal secretagogues that act by the cyclic nucleotide-dependent and independent pathways should prove fruitful in advancing our understanding of the molecular basis for alterations in ion transport.

Acknowledgments

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