PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 182, 404–408 (1986)

The Effects of Phorbol Esters on Fluid Transport and Blood Flow in the Small Intestine (42359)

ANDERS SJÖQVIST, LINDA S. HENDERSON, AND JOSEPH D. FONDACARO[1]

Department of Pharmacology, Smith Kline & French Laboratories, Philadelphia, Pennsylvania 19101

Abstract. Studies were designed to examine the effects of phorbol esters on intestinal fluid transport and blood flow in the anesthetized cat and enteropooling in the conscious rat. Intraluminal administration of phorbol ester into a segment of isolated small bowel produced a copious intestinal secretion and a concomitant mesenteric hyperemia in the cat. Net fluid movement in the intestine was converted from absorption in the control state to secretion following phorbol ester administration. Intravenous atropine reduced the phorbol ester-induced secretion by 56%; clonidine abolished the remaining secretory response. In the rat, intragastric administration of phorbol ester produced enteropooling comparable to that of other potent intestinal secretagogues. Since phorbol esters are known to activate protein kinase C, these studies suggest that activation of protein kinase C in the small intestine may lead to a full secretory response. The evidence suggests that this secretion is accompanied by a metabolic hyperemia. These results suggest that protein kinase C plays an important role in the regulation of intestinal fluid transport.

Phorbol esters have been found to elicit a variety of cellular responses by binding to and activating the Ca^{2+}-activated, phospholipid-dependent protein kinase referred to as protein kinase C (1). This enzyme appears to play a pivotal role in regulatory mechanisms where the coupling of stimulus and response also involves an increase in intracellular Ca^{2+}. With respect to intestinal secretion, several secretagogues including serotonin and the cholinergic agonists have been shown to elevate cytosolic Ca^{2+} levels (2). Calcium has also been shown to be mobilized in the enterocyte in response to the cAMP analog, 8-bromo cAMP, suggesting that cAMP-mediated secretion may also have a Ca^{2+} component (3).

Recent *in vitro* studies with the rat and chicken small intestinal mucosa have demonstrated that the phorbol esters elicit a secretory response by inhibiting Na^+ and Cl^- absorption and by stimulating electrogenic Cl^- secretion (4, 5). To further our understanding of the role of protein kinase C in regulating intestinal secretion, the present studies were undertaken to characterize the *in vivo* and *in situ* effects of phorbol esters on small intestinal fluid transport and blood flow.

Materials and Methods. *Surgical procedure.* The experiments were performed on young adult cats of either sex deprived of food for at least 16 hr but with free access to water. Anesthesia was induced with ketamine (10 mg/kg, im). A catheter was inserted into the femoral vein under local anaesthesia (lidocaine) and α-chloralose was administered (25–35 mg/kg, iv). Some cats were given pentobarbital (0.3 mg/kg) to reduce their excitability. The animals breathed spontaneously through a tracheal cannula. A glucose–bicarbonate buffer solution was infused (0.1 ml/min) into the femoral vein to prevent acidosis (6). The arterial blood pressure was measured in the femoral artery with a catheter connected to a Harvard Model 360 pressure transducer. Body temperature was monitored with a thermometer in the pharynx and was maintained at 38°C using a heating pad under the animal.

The abdomen was opened with a midline incision and the greater omentum and the spleen were removed. A 10-cm-long jejunal segment with intact vascular supply was isolated and the remaining portion of the small intestine and the colon were removed. Changes in sympathetic influence on the intestines were minimized by cutting the splanchnic nerves, denervating the right ad-

[1] To whom reprint requests should be addressed: Department of Pharmacology, Smith Kline & French Laboratories (L-531), P.O. Box 7929, Philadelphia, Pa. 19101.

0037-9727/86 $1.50

renal, and by excluding the left adrenal from the circulation by ligatures. This allowed for more precise assessment of secretion, blood flow, and the effects of pharmacologic agents on these parameters.

Measurements of intestinal blood flow. Following heparinization (3–5 mg/kg, iv) the mesenteric vein of the jejunal segment was cannulated and intestinal blood flow was determined from the venous effluent of the segment, using a photoelectric drop recorder, an ordinate writer, and a Grass polygraph (7). The blood was returned to the animal via a catheter in the external jugular vein.

Measurements of intestinal fluid transport. The net intestinal fluid transport was measured with a gravimetric method described previously (8, 9). Briefly, the intestinal segment was placed on a customized plastic scale suspended from a force displacement transducer (Grass FT03). The intestinal lymph node was fixed to a ring mounted on the table to prevent respiratory movements from affecting the scale. The intestinal segment was perfused at 1.0 ml/min with a modified Krebs–Henseleit solution. A small, open, fluid basin on the scale was connected to the outflow end of the intestine and a large closed reservoir (700 ml) was part of the perfusion loop to minimize recirculation. The weight of the segment and its luminal contents were continuously recorded and fluid transport was seen as changes in weight of the system. Note that motility of the intestine might move fluid from the lumen to the open reservoir on the scale but would not change the weight of the system.

Enteropooling assay. The intestinal secretory response to phorbol ester was also examined in the rat using the enteropooling assay of Robert *et al.* (10).

Following a strict 24-hr fast, male Sprague–Dawley rats (180–210 g) were administered phorbol ester by gavage. Thirty minutes later, animals were sacrificed and the volume of fluid in the entire small intestine was determined volumetrically. The secretory response to the phorbol ester, 4β-phorbol 12-myristate 13-acetate (PMA), in this assay was compared to that elicited by 16,16-dimethylprostaglandin E_2 (PGE_2), carbachol (Carb), and cholera toxin (CT).

Materials. The modified Krebs–Henseleit solution contained 122 m*M* NaCl, 3.5 m*M* KCl, 1.2 m*M* KH_2PO_4, 25 m*M* $NaHCO_3$, 1.2 m*M* $MgCl_2$, 2 m*M* $CaCl_2$, and 30 m*M* mannitol. The following drugs were used: lidocaine (Xylocaine; Astra Pharmaceutical Products); sodium pentobarbital (Veterinary Laboratories); heparin (Eli Lilly & Co.); ketamine hydrochloride (Ketaset; Bristol Laboratories); clonidine, α-chloralose, atropine sulfate, 4β-phorbol 12-myristate 13-acetate, and 4β-phorbol 12β,13α-dibutyrate (Sigma Chemical Co.). The phorbol esters were dissolved in absolute ethanol and kept at −20°C as a stock solution 10 mg/ml).

Statistical analysis. The statistical test used was the nonparametric sign test. A probability level of 0.05 or less was considered to be significant. All data are expressed as means ± SEM.

Results. The phorbol ester, 4β-phorbol 12β,13α-dibutyrate (PDB), injected into the intestinal lumen elicited a profuse secretion within minutes in all cats studied. Net fluid transport changed from an absorption of 30 ± 45 μl min^{-1} 100 cm^{-2} of serosal surface during the control period to a secretion of 347 ± 107 μl min^{-1} 100 cm^{-2} after the intraluminal injection of PDB (Fig. 1). Net fluid transport during control conditions was observed after injecting 50 μl ethanol in 1 ml Krebs–mannitol solution into the lumen of the small intestine; this amount of ethanol did not alter basal fluid transport in the intestine. Atropine (0.5 mg/kg, iv) reduced the PDB-induced secretion by 56 ± 8%. Clonidine (50 μg/kg, iv), added after atropine, decreased the atropine resistant secretion by 84 ± 25%; thus, total secretion was reduced 97% after atropine plus clonidine and net fluid transport was not significantly different from the control level (Fig. 1).

In the rat, 30 min after intragastric administration, PMA (100 μg/kg) stimulated net fluid secretion and enteropooling. The PMA-induced enteropooling is similar to that produced by intragastric PGE_2 (50 μg/kg) and Carb (5 mg/kg) after 30 min and by CT (100 μg/kg) after 4 hr (Fig. 2). These doses have been shown to produce maximal intestinal secretion in this species (10).

Intraluminal PDB caused vasodilation in the cat small intestine concomitantly with its secretory action (Table I). The vasodilation was atropine resistant and was not altered over

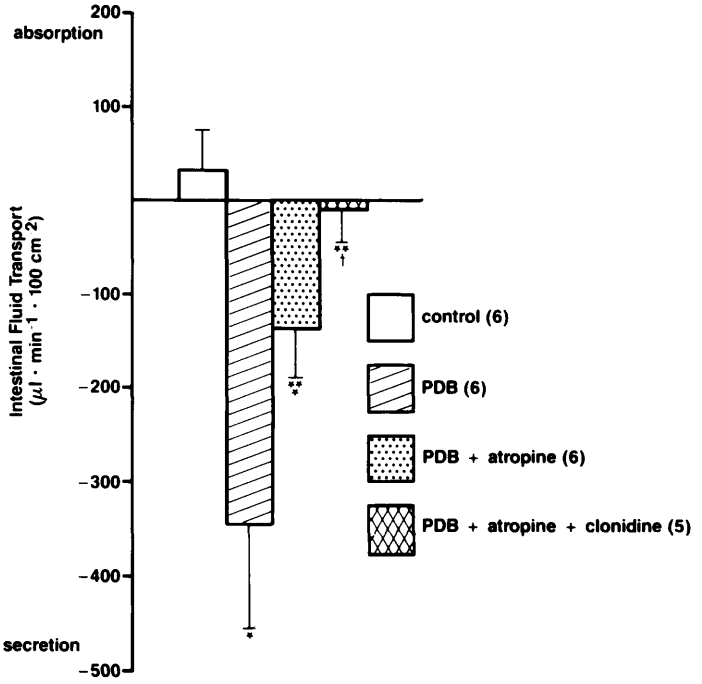

FIG. 1. The effects of intraluminal PDB (500 μg) and PDB followed by intravenous atropine (0.5 mg/kg) and clonidine (50 μg/kg) on fluid transport (mean ± SEM) in an isolated loop of cat small intestine. Number in parentheses is the number of observations for each perturbation. *Significantly different from control; **significantly different from PDB; †significantly different from PDB + atropine.

the time course of the experiments. However, intestinal blood flow returned to control level after administration of clonidine in addition to atropine as shown in Table I.

Discussion. The regulation of intestinal transport is thought to involve the complex interaction of several physiological and biochemical processes. Recently, a new regulatory pathway has been elucidated which involves the Ca^{2+}-sensitive, phospholipid-dependent protein kinase, protein kinase C. This enzyme system is normally activated by diacylglycerol, a product of phosphatidylinositol metabolism, and experimentally by phorbol esters (11). In various biological systems, activation of protein kinase C by administration of phorbol ester has been shown to promote secretory responses. For example, PMA has been reported to stimulate secretion of aldosterone from adrenal granulosa cells (12), insulin from pancreatic islet cells (13), and amylase from isolated, perfused pancreatic acini (14).

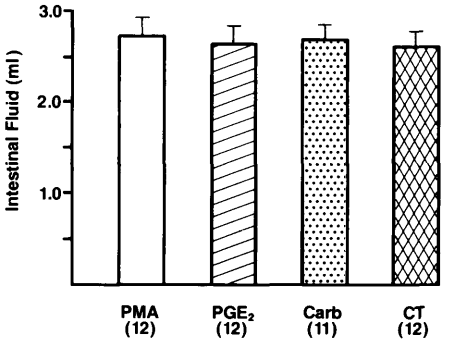

FIG. 2. A comparison of enteropooling values (mean ± SEM) resulting from intragastric administration of PMA (100 μg/kg), PGE_2 (50 μg/kg), Carb (5 mg/kg), and CT (100 μg/kg) in the rat. Values for controls (i.e., vehicle effects) have been subtracted in each case. Number in parentheses is the number of observations. Abbreviations are defined in the text.

Earlier studies in the conscious rat had shown that phorbol esters elicited a dose-dependent stimulation of fluid secretion in the small intestine and phosphorylation of specific microvillus membrane proteins in the rat (15). In the present study we have shown that after intragastric administration of the phorbol ester PMA in the rat, the level of net intestinal se-

TABLE I. HEMODYNAMIC EFFECTS OF INTRALUMINAL ADMINISTRATION OF PDB IN THE ISOLATED LOOP OF CAT SMALL INTESTINE

	Intestinal blood flow (ml min^{-1} 100 g^{-1})	Mean arterial blood pressure (mm Hg)	Peripheral resistance units (mm Hg min 100 g ml^{-1})
Control (6)	17 ± 1	113 ± 8	6.8 ± 0.8
PDB (6)	31 ± 5*	108 ± 10	4.1 ± 0.7*
PDB + atropine (6)	32 ± 5*	98 ± 11	3.5 ± 0.6*
PDB + atropine (5) + clonidine	24 ± 7	94 ± 19	4.8 ± 1.1

Note. Data are expressed as means ± SEM, with the number of experiments given in parentheses. PDB is 4β-phorbol 12β,13α-dibutyrate and is administered at a dose of 500 μg. Atropine (0.5 mg/kg) and clonidine (50 μg/kg) were administered intravenously. There was a 20- to 30-min observation period between perturbations.

* Significant change from control level at $P < 0.05$.

cretion is quantitatively similar to the enteropooling effects of other potent intestinal secretagogues, including cholera toxin. In the cat, intraluminal administration of PBD similarly induced a copious intestinal secretion; within minutes net fluid transport in the intestine was converted from absorption in the control state to secretion. The magnitude of this secretory response is comparable to that produced by cholera toxin and VIP in the same experimental model (8, 9). Atropine reduced the PDB-induced intestinal secretion in the cat by 56%. Cholinergic muscarinic receptors have been shown to mediate intestinal secretion (16) and recently Tanaka *et al.* (17) have demonstrated that phorbol ester in conjunction with the Ca^{2+} ionophore stimulated the release of acetylcholine in guinea pig ileum. Therefore, it seems possible that part of the response obtained with PDB *in vivo* in the cat is due to the local release of acetylcholine. Likewise, in the enteropooling studies in the rat, atropine reduced the PMA-induced secretion by 30–40% (15). However, in Ussing chamber studies with rat ileum, atropine had little effect on the Cl^- secretion elicited by PDB (15). Therefore, in the *in vivo* studies, the phorbol esters appear to be causing secretion directly by changing transport functions in the enterocyte and indirectly through the release of acetylcholine. One cannot rule out the possibility that part of the atropine-resistant response may involve the local release of other neurohumoral substances such as VIP and serotonin, which are known to stimulate intestinal secretion.

The present study also suggests a relationship between phorbol ester-induced secretion and intestinal blood flow. Coincident with the stimulation of net fluid secretion, intestinal blood flow nearly doubled following phorbol ester administration. Although mean arterial blood pressure remained constant, peripheral resistance in the mesenteric vasculature was reduced nearly 40%. The increase in intestinal blood flow in the cat was apparently unrelated to the release of acetylcholine since atropine did not alter the hyperemic response. Therefore, the increase in blood flow observed in these studies may be due to an increase in some oxidative step associated with phorbol ester-induced stimulation of active fluid secretion, i.e., metabolic hyperemia. However, the possibility that PDB triggers the release of a local vasodilator such as VIP must again be considered.

Clonidine is an α_2-adrenergic receptor agonist with known antisecretory activity in the small intestine (18). When clonidine was administered intravenously following atropine, intestinal secretion due to phorbol ester stimulation was further reduced and net fluid transport was not significantly different from control level. This action of clonidine may be due to stimulation of fluid and electrolyte absorption (18), thus imposing a vectorial "counter-flow" to secretion. This reduction in secretion following clonidine was also associated with a return of mesenteric blood flow to control level. The change in blood flow may be a result of a decrease in metabolic activity relating to secretion or a direct α-receptor-mediated effect of clonidine on arterioles and precapillary sphincteric smooth muscle. An α-mediated vasoconstriction of the vascular smooth muscle seems unlikely since arterial blood pressure remained the same.

Activation of protein kinase C by phorbol ester has been shown to cause contraction of

rat aortic rings *in vitro* (19). An interesting observation in our present study was the occurrence of increased intestinal contractions in the portion of the intestine which received PDB. The contractile activity began after the initiation of the secretory response and was abolished with atropine administration. These observations suggest that local release of acetylcholine was responsible for the visceral smooth muscle activity.

The present study demonstrates that intraluminal administration of phorbol esters produces a full secretory response in the cat and rat *in vivo*. This response is only partially attenuated by atropine, suggesting that the phorbol ester effect may directly involve a change in enterocyte transport function. Furthermore, our studies show that this secretion was associated with an intestinal hyperemia probably of metabolic origin. These studies corroborate earlier findings by Chang *et al.* with rabbit and chicken ileum *in vitro* (5) and by Donowitz *et al.* with the rat colon *in vitro* (20) that active phorbol esters induce electrolyte secretion. Since the effects of phorbol esters appear to be the result of protein kinase C activation, these studies provide additional evidence that protein kinase C may be an important component of the regulatory mechanisms operating in the small intestine to alter water and electrolyte transport.

The authors acknowledge the expert technical assistance of Jim Stefankiewicz.

1. Nishizuka Y. The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature (London) **308**:693–698, 1984.
2. Donowitz M. Ca^{2+} in the control of active Na and Cl transport: Involvement in neurohumoral action. Amer J Physiol **245**:G165–G177, 1983.
3. Chang EB, Semrad CE. Calcium-mediated cyclic AMP inhibition of Na/H exchange in chicken small intestine. Gastroenterology **88**:1345, 1985.
4. Fondacaro JD, McCafferty GP, Henderson LS. Further evidence for protein kinase C-mediated intestinal hypersecretion. Gastroenterology **88**:1386, 1985.
5. Chang EB, Wang NS, Rao MC. Role of protein C-kinase in intestinal anion secretion. Gastroenterology **88**:1345, 1985.
6. Haglund U, Lundgren O. Reactions with consecutive vascular sections of the small intestine of the cat during prolonged hypotension. Acta Physiol Scand **84**:580–593, 1972.
7. Sjöqvist A, Hellström PM, Jodal M, Lundgren O. Neurotransmitters involved in the colonic contraction and vasodilitation elicited by activation of the pelvic nerve in the cat. Gastroenterology **86**:1481–1487, 1984.
8. Cassuto F, Jodal M, Tuttle R, Lundgren O. 5-Hydroxytryptamine and cholera secretion: Physiological and pharmacological studies in cats and rats. Scand J Gastroenterol **17**:695–703, 1982.
9. Sjövall H, Redfors S, Biber B, Martner F, Winsö O. Evidence for cardiac volume receptor regulation of feline jejunal blood flow and fluid transport. Amer J Physiol **246**:G401–G410, 1984.
10. Robert A, Nezamis JE, Lancaster C, Hanchar AJ, Klepper MS. Enteropooling assay: A test for diarrhea produced by prostaglandins. Prostaglandins **11**:809–828, 1976.
11. Castagna M, Takai Y, Kaibuchi K, Sano K, Kikkawa U, Nishizuka Y. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J Biol Chem **257**:7847–7851, 1982.
12. Kojima I, Lippes H, Kojima K, Rasmussen H. Aldosterone secretion: Effects of phorbol esters and A23187. Biochem Biophys Res Commun **116**:555–562, 1983.
13. Zawalich W, Brown C, Rasmussen H. Insulin secretion: Combined effects of phorbol ester and A23187. Biochem Biophys Res Commun **117**:448–455, 1983.
14. Wooten MW, Wrenn RW. Phorbol ester induces intracellular translocation of phospholipid/Ca^{2+}-dependent protein kinase and stimulates amylase secretion in isolated pancreatic acini. FEBS Lett **171**:183–186, 1984.
15. Fondacaro JD, Henderson LS. Evidence for protein kinase C as a regulator of intestinal electrolyte transport. Amer J Physiol **249**(Gastrointest Liver Physiol **12**):G422–G426, 1985.
16. Cooke HJ. Influence of enteric cholinergic neurons on mucosal transport in guinea pig ileum. Amer J Physiol **246**:G263–G267, 1984.
17. Tanaka C, Taniyama K, Kusunoki M. A phorbol ester and A23187 act synergistically to release acetylcholine from the guinea pig ileum. FEBS Lett **175**:165–169, 1984.
18. Chang EB, Field M, Miller RJ. Enterocyte α_2-adrenergic receptors: Yohimbine and *p*-aminoclonidine binding relative to ion transport. Amer J Physiol **244**: G76–G82, 1983.
19. Danthuluri NR, Deth RC. Phorbol ester-induced contraction of arterial smooth muscle and inhibition of α-adrenergic response. Biochem Biophys Res Commun **125**:1103–1109, 1984.
20. Donowitz M, Cheng H, Sharp GWG. Role of protein kinase C in regulation of rat colonic active NaCl transport. Gastroenterology **88**:1367, 1985.

Received July 10, 1985. P.S.E.B.M. 1986, Vol. 182.
Accepted April 18, 1986.