J. Pharm. Pharmacol. 1993, 45: 59–62
Received March 24, 1992

Stimulation of Enterocyte Protein Kinase C by Laxatives In-vitro

ECKHARD BEUBLER AND ANDREA SCHIRGI-DEGEN

Department of Experimental and Clinical Pharmacology, Karl-Franzens-University, Graz, Austria

Abstract—To elucidate the role of protein kinase C in the mechanism of action of stimulatory laxatives, experiments were performed with preparations of rat lysed enterocytes. The phorbol ester 4-β-phorbol 12-myristate 13-acetate (PMA) concentration-dependently (2–200 μg mL^{-1}) stimulated the activity of protein kinase C in this preparation. Ricinoleic acid, the active principle of castor oil, deacetylbisacodyl, the active principle of bisacodyl, and deoxycholic acid exerted the same effect, although less efficiently. This reflects their potency for inducing intestinal fluid secretion and prostaglandin release, effects that are also induced more potently by PMA. Accordingly, the potency of the three C_{18} fatty acids, ricinoleic acid, stearic acid and oleic acid on protein kinase C activity in-vitro, on prostaglandin E_2 release and on net fluid secretion in-vivo runs in parallel. It is therefore concluded that stimulatory laxatives activate protein kinase C, leading to prostaglandin E_2 release, thus resulting in net fluid secretion.

Croton oil extracted from the seeds of *Croton tiglium* L. (Euphorbiaceae) was known for its laxative properties long before its active principles, the phorbol esters, were identified (Hecker 1962). Phorbol esters turned out to be tumour promoters and potent stimulators of protein kinase C, an intracellular Ca^{2+}-activated, phospholipid-dependent protein phosphorylase (Nishizuka 1984a).

Activation of protein kinase C stimulates intestinal ion and fluid secretion in-vivo (Fondacaro & Henderson 1985; Beubler et al 1990) and in-vitro (Chang et al 1985; Donowitz et al 1986), possibly through stimulation of arachidonic acid release, resulting in a stimulation of prostaglandin formation (Beubler et al 1990; Musch et al 1990).

Ricinoleic acid, bisacodyl and other stimulant laxatives also exert their secretory effect, at least partly, via stimulation of prostaglandin formation (Beubler & Juan 1978, 1979; Beubler & Kõllar 1985). The biochemical mechanism of this stimulation of prostaglandin formation by laxatives is still unknown.

The present study was initiated to determine whether the phorbol ester, 4-β-phorbol 12-myristate 13-acetate (PMA), stimulates protein kinase C in rat isolated enterocytes and whether the laxatives ricinoleic acid, bisacodyl and deoxycholic acid are able to mimic this effect.

In one series of experiments, the stimulatory effects of the three C_{18} fatty acids, ricinoleic acid, oleic acid and stearic acid on protein kinase C were compared with their effects on intestinal fluid secretion and prostaglandin formation.

Materials and Methods

Isolation and preparation of enterocytes

Isolated intestinal cell preparations were made according to a modification of the method of Weiser (1973). This method isolates only epithelial cells and excludes serosal and interstitial cells.

Female Sprague-Dawley rats were deprived of food 18 h before the experiments, but had free access to water. The rats were killed by a blow to the head, the abdomen was immediately opened, and the entire jejunum was removed.

The intestine was flushed with saline solution (0·9% NaCl) from a saline tower, one end was tied off and the intestine was filled with about 8 mL (enough to slightly distend the segment) of buffer I at 37°C, using a syringe with a blunt needle. Then the other end was tied off, the sac was placed in a conical flask and covered with warm buffer I. The flask was placed into a water bath at 37°C and shaken gently for 10 min. The intestine was removed, opened by a small cut at one end, and the contents were discarded. The loop was refilled with warm buffer II, closed with a new ligature, and replaced into the flask filled with buffer I. Again the flask was shaken in the water bath for 10 min, but more rigorously this time.

The sac was removed, opened and emptied by gentle squeezing. To separate mucus, the contents were filtered through nylon gauze into a plastic centrifuge tube. A sample (0·5 mL) of the filtrate was removed, added to 25 μL trypan blue (0·2%) and examined microscopically. Intact and viable enterocytes were present in the preparation, no other cell type could be seen. The number of isolated enterocytes was determined in a Bürker-Türk counting chamber.

The rest of the filtrate was centrifuged in a refrigerated centrifuge at 100 *g* for 15 min. The supernatant was discarded, and the cells were lysed with ice-cold buffer III to give a solution originating from 2×10^6 cells mL^{-1}. This solution was stored on ice and used within 4 h for the further experiments.

Protein kinase C enzyme assay system

For determination of protein kinase C activity, a protein kinase C enzyme assay system (Amersham, RPN 77) was used. This system is based upon the protein kinase C-catalysed transfer of the γ-phosphate group of adenosine-5′-triphosphate to a peptide, which is specific for protein kinase C. The assay system is determined for detecting various amounts of protein kinase C in samples.

To determine the potency of different stimuli on protein kinase C activity, the system had to be modified. The reagent

Correspondence: E. Beubler, Department of Experimental and Clinical Pharmacology, University of Graz, Universitätsplatz 4, A-8010 Graz, Austria.

containing PMA in the assay system (lipid) was replaced by a detergent-dispersed solution prepared according to Hannun et al (1985), and the substances to be tested were added to this solution in different concentrations.

These substances (PMA, ricinoleic acid, deoxycholic acid, deacetylbisacodyl, stearic acid and oleic acid) were dissolved in dimethylsulphoxide (DMSO). L-α-Phosphatidyl-L-serine was dissolved in 3% Triton X-100 in Tris-HCl, and mixed with the test substance/DMSO solution. The final lipid solution contained 8 mol% L-α-phosphatidylserine and the test-substances at three different concentrations.

Equal volumes of calcium buffer, peptide buffer, dithiothreitol buffer (all supplied with the assay system) and lipid were mixed (component mixture).

[^{32}P]ATP solution was mixed with 150 μM magnesium ATP (supplied with the assay system) to give a radioactivity of 10 μCi mL^{-1}. A sample (25 μL) of the component mixture and 25 μL of the cell suspension containing protein kinase C (prepared as described above) were mixed and the reaction was started by addition of 25 μL of magnesium [^{32}P]ATP solution and incubated at 25°C for 15 min.

The reaction was stopped by adding the stop reagent (supplied with the assay system) after 15 min. Aliquots were spotted onto binding papers, carefully washed with 75 mM orthophosphoric acid (20 mL per paper) and placed in scintillation vials containing liquid scintillation fluid (Ultima Gold). ^{32}P Activity was determined using a Beckman Liquid Scintillation Counter (LS 1801). Suitable blanks and controls were prepared according to Table 1.

Chemicals and reagents

Buffers were prepared as follows: buffer I (mM): 1·5 KCl, 96 NaCl, 27 tri-sodium citrate-2-hydrate, 8 KH_2PO_4, 5·6 Na_2HPO_4 (pH 7·3); buffer II (mM): 17 tris(hydroxymethyl)-aminomethane, 120 NaCl, 4 KCl, 1·6 $MgSO_4 \cdot 7H_2O$, 1·2 $CaCl_2 \cdot 2H_2O$, 10 glucose-monohydrate (pH 7·4); buffer III (mM): 50 Tris-HCl, 5 EDTA, 10 EGTA, 0·3% β-mercaptoethanol, 10 benzamidine, 50 μg mL^{-1} phenylmethylsulphonylfluoride (hypotonic, pH 7·5).

Protein kinase C enzyme assay system and adenosine 5′[γ-^{32}P]triphosphate were obtained from Amersham International, Amersham, UK, L-α-phosphatidyl-L-serine sodium salt, 4-β-phorbol 12-myristate 13-acetate (PMA) (identical to 12-*O*-tetradecanoyl phorbol 13-acetate (TPA)), ricinoleic acid, deoxycholic acid, stearic acid, oleic acid and Triton X-100 were from Sigma Chemical Co., St Louis, MO, USA, deacetylbisacodyl was from Dr Karl Thomae GmbH, Germany, and Ultima Gold was from Packard, USA. All other chemicals were of analytical grade (E. Merck AG, Darmstadt, Germany).

Table 1. Preparation of samples and controls.

	Sample	Blank	Solvent-control	Non-specific peptide
Cell suspension	+	−[a]	+	+
Peptide buffer	+	+	+	−[c]
Lipid	+	+	−[b]	+

[a] Buffer III. [b] DMSO/Triton X-100/L-α-phosphatidylserine. [c] Tris-HCl.

Calculation of results

The activity of protein kinase C (P) was calculated according to the method recommended by the description of the assay system and expressed as pmol phosphate transferred min^{-1}.

$$P = \frac{T \times 10^3}{I \times R} \text{ (pmol min}^{-1}\text{)}$$

where T = sample counts min^{-1} × 1·4 − blank counts min^{-1}, I = incubation time (min), and R = specific activity of [^{32}P]ATP (counts min^{-1} nmol^{-1}). Solvent controls were prepared freshly in each series of experiments. The results obtained from these controls were subtracted from the results obtained from the samples.

All the values given are significantly different from solvent controls. Phosphate transfer to unspecific peptides present in the cell preparation was negligible.

Results

Influence of PMA on protein kinase C activity

The phorbol ester PMA concentration-dependently (2–200 μg mL^{-1}) stimulated protein kinase C activity in the preparation of rat lysed enterocytes (Fig. 1).

Using the original phorbol ester solution (Kit-Standard in Fig. 1) supplied with the protein kinase C enzyme assay system, the activity of protein kinase C appeared to be slightly higher than using our own phorbol ester solution at a concentration of 2 μg mL^{-1}.

Influence of laxatives on protein kinase C activity

The active principle of castor oil, ricinoleic acid, and the bile acid deoxycholic acid were less active than PMA, but they also stimulated protein kinase C activity in a concentration dependent manner within the range 2–200 μg mL^{-1} (Fig. 2). Deacetylbisacodyl reached its maximal activity at 20 μg mL^{-1}.

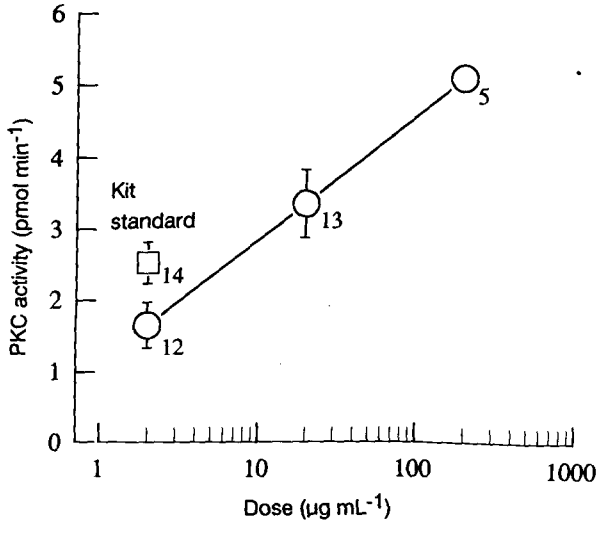

FIG. 1. Dose-response relationship for the effect of PMA (○) (2–200 μg mL^{-1}, final concentration) on protein kinase C activity in rat isolated enterocytes. □ Indicates the effect of the PMA supplied with the assay system. The activity of protein kinase C is given as pmol phosphate transferred min^{-1}. Values are the mean ± s.e.m. The numerals indicate the number of experiments.

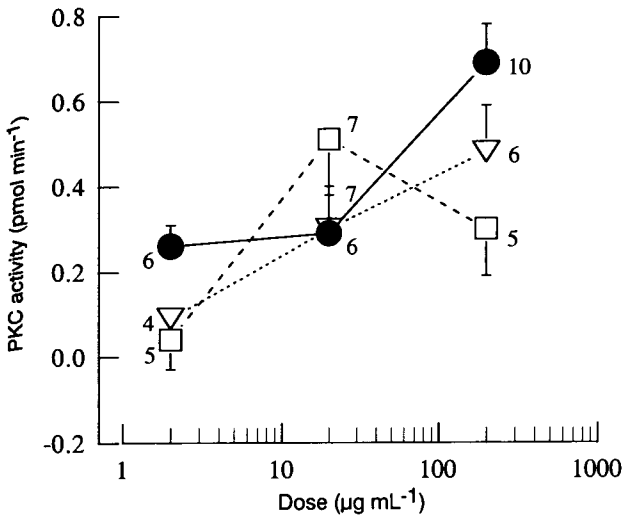

FIG. 2. Dose-response relationship for the effect of ricinoleic acid (●), deoxycholic acid (▽) and deacetylbisacodyl (□) on protein kinase C activity in rat isolated enterocytes. The activity of protein kinase C is given as pmol phosphate transferred min^{-1}. Values are mean ± s.e.m. The numerals indicate the number of experiments.

Comparison of the C_{18} fatty acids stearic acid, oleic acid and ricinoleic acid

According to their effects on intestinal fluid secretion and prostaglandin formation, oleic acid and stearic acid were less potent in stimulating protein kinase C activity compared with ricinoleic acid (Fig. 3); stearic acid may even slightly inhibit protein kinase C activity.

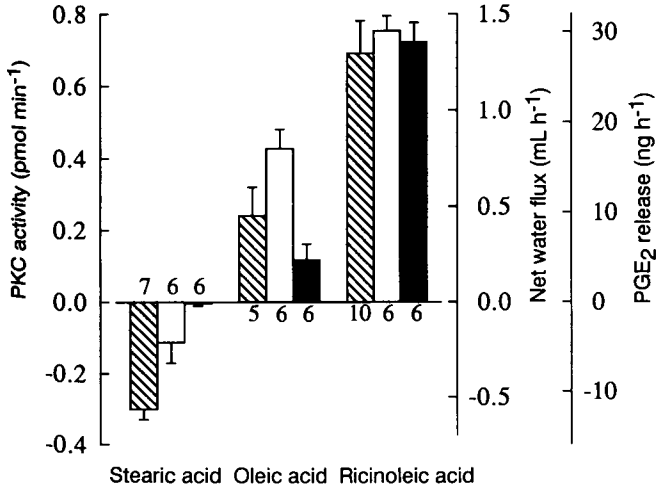

FIG. 3. Influence of stearic acid, oleic acid and ricinoleic acid on protein kinase C activity in-vitro, and on net fluid transfer and prostaglandin E_2 release in-vivo (taken from Beubler & Juan (1979)). The activity of protein kinase C is given in pmol phosphate transferred min^{-1} in the left ordinate scale (▨); net fluid transfer is given in mL h^{-1}, positive values indicate net fluid secretion and negative values indicate net fluid absorption in the first right ordinate scale (□); prostaglandin E_2 release is given in ng h^{-1} in the second right ordinate scale (■). The values represent the difference from the respective control. The concentrations used in-vitro were 200 μg mL^{-1}, those in the in-vivo experiments were 1·5 mg mL^{-1}. Values are given as mean ± s.e.m. The numerals indicate the number of experiments.

Discussion

The present experiments have demonstrated that the phorbol ester PMA concentration-dependently stimulated protein kinase C activity in an in-vitro preparation derived from rat jejunal epithelial cells. Phorbol esters, the active principles of the laxative, croton oil, stimulate intestinal fluid and electrolyte secretion through stimulation of prostaglandin formation. The active principle of castor oil, ricinoleic acid, bisacodyl, deacetylbisacodyl, and deoxycholic acid mimic this effect of PMA on protein kinase C.

It is apparent that PMA is about ten times more active in stimulation of protein kinase C than the other laxatives tested. PMA is also about ten to fifty times more potent than bisacodyl in fluid secretion and prostaglandin formation (Beubler & Juan 1978; Beubler et al 1990). In in-vivo experiments PMA stimulates intestinal fluid secretion and increases prostaglandin formation at concentrations of 1 μg mL^{-1} (Beubler et al 1990), whereas ricinoleic acid and deoxycholic acid do not show comparable effects below concentrations of about 1–2 mg mL^{-1} (Ammon & Phillips 1974; Beubler & Juan 1979).

The experiments with the three C_{18} fatty acids, stearic acid, oleic acid and ricinoleic acid convincingly demonstrate that stimulation of enterocyte protein kinase C, in-vitro, runs in parallel with stimulation of intestinal fluid secretion and prostaglandin formation in-vivo (Fig. 3).

5-Hydroxytryptamine (5-HT), another important mediator of intestinal fluid secretion is probably not involved in the secretory response to phorbol esters and laxatives (Beubler & Horina 1990; Beubler et al 1990). 5-HT stimulates phosphoinositol turnover via a 5-HT_2-receptor stimulation (Roth et al 1986). Diacylglycerol, an early product of the breakdown of phosphatidylinositol (Nishizuka 1984a), stimulates protein kinase C, followed by increasing arachidonic acid release and conversion of arachidonic acid to prostaglandins (Parker et al 1987). The phorbol ester PMA and the laxatives tested, however, are direct stimulators of protein kinase C, which is supported by the observation that PMA does not increase 5-HT release in secretory concentrations and that the effect of bisacodyl is not inhibited by the 5-HT_2-receptor blocker ketanserin (Beubler & Horina 1990).

Many stimuli that cause production of eicosanoids also activate protein kinase C including tumour promoters, growth factors, hormones and neurotransmitters (Levine & Hassid 1977; Daniel et al 1981; Nishizuka 1984b; Ohuchi et al 1985). Protein kinase C appears to activate phospholipase A_2, thus regulating arachidonic acid release and stimulating prostaglandin formation (Emilsson & Sundler 1986; Emilsson et al 1986; Bonventre & Swidler 1988; Hoffman et al 1988; Wijkander & Sundler 1989; Pfannkuche et al 1989; Balsinde et al 1990; Moore et al 1991).

It is concluded that laxatives such as ricinoleic acid, bisacodyl and deoxycholic acid activate protein kinase C in intestinal epithelial cells, thus stimulating phospholipase A_2 and resulting in prostaglandin formation, which ultimately mediates the secretory response.

Acknowledgements

This work was supported by the Austrian Scientific Research Funds (No. 7877). The authors thank B. Wascher-Hartmann and H. Hosbein for technical assistance.

References

Ammon, H. V., Phillips, S. F. (1974) Inhibition of ileal water absorption by intraluminal fatty acids: influence of chain length, hydroxylation, and conjugation of fatty acids. J. Clin. Invest. 53: 205–210

Balsinde, J., Fernandez, B., Diez, E. (1990) Regulation of arachidonic acid release in mouse peritoneal macrophages: the role of extracellular calcium and protein kinase C. J. Immunol. 144: 4298–4304

Beubler, E., Juan, H. (1978) PGE-mediated laxative effect of diphenolic laxatives. Naunyn Schmiedebergs Arch. Pharmacol. 305: 241–246

Beubler, E., Juan, H. (1979) Effect of ricinoleic acid and other laxatives on net water flux and prostaglandin E release by the rat colon. J. Pharm. Pharmacol. 31: 681–685

Beubler, E., Kollar, G. (1985) Stimulation of PGE_2 synthesis and water and electrolyte secretion by senna anthraquinones is inhibited by indomethacin. J. Pharm. Pharmacol. 37: 248–251

Beubler, E., Horina, G. (1990) 5-HT_2 and 5-HT_3 receptor subtypes mediate cholera toxin-induced intestinal fluid secretion in the rat. Gastroenterology 99: 83–89

Beubler, E., Hinterleitner, T., Horina, G. (1990) Protein kinase C and intestinal fluid secretion: involvement of prostaglandin E_2 but not of 5-hydroxytryptamine. Eur. J. Pharmacol. 182: 543–548

Bonventre, J. V., Swidler, M. (1988) Calcium dependency of prostaglandin E_2 production in rat glomerular mesangial cells: evidence that protein kinase C modulates the Ca^{2+}-dependent activation of phospholipase A_2. J. Clin. Invest. 82: 168–176

Chang, E. B., Wang, N. S., Rao, M. C. (1985) Phorbol ester stimulation of active anion secretion in intestine. Am. J. Physiol. 249: C356–C361

Daniel, L. W., King, L., Waite, M. (1981) Source of arachidonic acid for prostaglandin synthesis in Madin-Darby canine kidney cells. J. Biol. Chem. 256: 12830–12835

Donowitz, M., Cheng, H. Y., Sharp, G. W. G. (1986) Effects of phorbol esters on sodium and chloride transport in rat colon. Am. J. Physiol. 251: G509–G517

Emilsson, A., Sundler, R. (1986) Evidence for a catalytic role of phospholipase A in phorbol diester- and zymosan-induced mobilization of arachidonic acid in mouse peritoneal macrophages. Biochim. Biophys. Acta 876: 533–542

Emilsson, A., Wijkander, J., Sundler, R. (1986) Diacylglycerol induces deacylation of phosphatidylinositol and mobilization of arachidonic acid in mouse macrophages: comparison with induction by phorbol diester. Biochem. J. 239: 685–690

Fondacaro, J. D., Henderson, L. S. (1985) Evidence for protein kinase C as a regulator of intestinal electrolyte transport. Am. J. Physiol. 249: G422–G426

Hannun, Y. A., Loomis, C. R., Bell, R. M. (1985) Activation of protein kinase C by Triton X-100 mixed micelles containing diacylglycerol and phosphatidylserine. J. Biol. Chem. 260: 10039–10043

Hecker, E. (1962) Toxic, inflammatory and co-carcinogenic principles from croton oil. Angew. Chem. Int. Edn. 1: 602–603

Hoffman, T., Lizzio, E. F., Suissa, J., Rotrosen, D., Sullivan, J. A., Mandell, G. L., Bonvini, E. (1988) Dual stimulation of phospholipase activity in human monocytes: role of calcium-dependent and calcium-independent pathways in arachidonic acid release and eicosanoid formation. J. Immunol. 140: 3912–3918

Levine, L., Hassid, A. (1977) Effects of phorbol-12,13-diesters on prostaglandin production and phospholipase activity in canine kidney (MDCK) cells. Biochem. Biophys. Res. Commun. 79: 477–484

Moore, J. J., Moore, R. M., Vander Kooy, D. (1991) Protein kinase-C activation is required for oxytocin-induced prostaglandin production in human amnion cells. J. Clin. Endocrinol. Metab. 72: 1073–1080

Musch, M. W., Nahkla, A. M., Chang, E. B. (1990) Phorbol ester-stimulated secretion in chicken ileum: role of arachidonic acid metabolism. Gastroenterology 99: 393–400

Nishizuka, Y. (1984a) The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature 308: 693–698

Nishizuka, Y. (1984b) Turnover of inositol phospholipids and signal transduction. Science 225: 1365–1370

Ohuchi, K., Watanabe, M., Yoshizawa, K., Tsurufuji, S., Fujiki, H., Suganuma, M., Sugimura, T., Levine, L. (1985) Stimulation of prostaglandin E_2 production by 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-type and non-TPA-type tumor promoters in macrophages and its inhibition by cycloheximide. Biochim. Biophys. Acta 834: 42–47

Parker, J., Daniel, L. W., Waite, M. (1987) Evidence of protein kinase C involvement in phorbol diester-stimulated arachidonic acid release and prostaglandin synthesis. J. Biol. Chem. 262: 5385–5393

Pfannkuche, H. J., Kaever, V., Gemsa, D., Resch, K. (1989) Regulation of prostaglandin synthesis by protein kinase C in mouse peritoneal macrophages. Biochem. J. 260: 471–478

Roth, B. L., Nakaki, T., Chuang, D., Costa, E. (1986) 5-Hydroxytryptamine$_2$ receptors coupled to phospholipase C in rat aorta: modulation of phosphoinositide turnover by phorbol ester. J. Pharmacol. Exp. Ther. 238: 480–485

Weiser, M. M. (1973) Intestinal epithelial cell surface membrane glycoprotein synthesis. J. Biol. Chem. 248: 2536–2541

Wijkander, J., Sundler, R. (1989) A role for protein kinase C mediated phosphorylation in the mobilization of arachidonic acid in mouse macrophages. Biochim. Biophys. Acta 1010: 78–87