ELSEVIER

European Journal of Pharmacology 389 (2000) 131–140

ejp

www.elsevier.nl/locate/ejphar

# The effect of protein kinase C activation on colonic epithelial cellular integrity

Barry L. Tepperman, PhD *, Brian D. Soper, Qing Chang, James F. Brown [1], Candice A. Wakulich

*Department of Physiology, University of Western Ontario, London, Ontario, Canada N6A 5C1*

Received 7 June 1999; received in revised form 15 November 1999; accepted 9 December 1999

**Abstract**

We have investigated whether activation of protein kinase C has a direct cytotoxic effect on colonic mucosal epithelial cells and whether oxidant-induced damage to colonocytes is mediated by activation of cellular protein kinase C. Incubation of freshly harvested cells from rat colon with the protein kinase C activator, phorbol 12-myristate, resulted in a concentration-dependent increase in the extent of cell injury. Phorbol 12-myristate acetate (0.1–10 μM) also increased cellular protein kinase C activity and this was reduced significantly by treating cells with the antagonists staurosporine or 2-[1-(3-dimethylaminopropyl)-indol-3-yl]3-(-indol-3-yl)maleimide (GF 109203X; 10 μM). Phorbol 12-myristate acetate treatment also resulted in increased translocation of proteins for protein kinase C isoforms α, δ and ε from cytosol to membrane particulate fractions. The antagonists reduced the extent of cell damage in response to phorbol 12-myristate acetate. Furthermore, cell injury in response to the phorbol acetate was also inhibited by the addition of the oxidant scavengers, superoxide dismutase or catalase to the cell suspension. Addition of $H_2O_2$ to the incubation medium (0.1–100 μM) resulted in an increase in cellular protein kinase C activity, an increase in the expression of the α, β and ζ isoforms and a reduction in cell integrity. The cellular damaging actions of $H_2O_2$ were significantly reduced by the protein kinase C antagonists, staurosporine or 2-[1-(3-dimethylaminopropyl)-indol-3-yl]-3-(-indol-3-yl)maleimide (GF 109203X). These findings suggest that protein kinase C activation results in colonic cellular injury and this damage is mediated, at least in part, by release of reactive oxidants. Furthermore, oxidant-mediated damage to these cells also involves protein kinase C activation. 

*Keywords:* Protein kinase C; Phorbol ester; Oxidant; Cellular injury

## 1. Introduction

Protein kinase C consists of a family of at least 12 serine–threonine protein kinases that have been implicated in many cellular signalling pathways (Nishizuka, 1992). The enzyme is activated by interaction with membranes and diacylglycerols. Activation is initiated through specific binding of ligands to cell surface receptors that are functionally linked to phospholipase C resulting in the generation of diacylglycerols from inositol lipids. In addition, phorbol esters such as phorbol 12-myristate can activate the enzyme directly.

In addition to its signal transduction role, protein kinase C has also been associated with tissue inflammation. Protein kinase C activation is associated with inflammation in vivo in a number of tissues including skin, the respiratory tract and brain (Gupta et al., 1988; St. John et al., 1991; Savithiry and Kumar, 1994; Suntres and Shek, 1995). Furthermore, protein kinase C activity has been found to be increased in colonic mucosal samples excised from patients with ulcerative colitis (Sakanoue et al., 1992). In addition, intraluminal instillation of phorbol ester that is known to activate protein kinase C also has been found to induce ileal and colonic inflammation in experimental animals. (Fretland et al., 1990; Buell and Berin, 1994; Berin and Buell, 1995; Overdahl et al., 1995). Recently we have demonstrated that trinitrobenzene sulfonic acid-induced colonic mucosal injury is mediated by increases in

* Corresponding author. Tel.: +1-519-661-3462; fax: +1-519-661-3827.

*E-mail address:* btepper@physiology.uwo.ca (B.L. Tepperman).

[1] Present address: Division of Pharmacology, School of Pharmacy, University of Portsmouth, Portsmouth PO1 2OI, UK.

0014-2999/00/$ - see front matter 
PII: S0014-2999(99)00892-4

protein kinase C activity and that this injury could be ameliorated by inhibition of protein kinase C activity (Brown et al., 1999).

Activation of the enzyme has also been shown to compromise the viability of a variety of cell types in vitro. Protein kinase C has been shown to play a role in the sensitization of endothelial cells to bacterial endotoxin challenge (Louise et al., 1997). Similarly protein kinase C activity has been shown to be elevated in a variety of cell types in response to a number of inflammatory challenges including treatment with platelet activating factor and cytokines (Kuruvilla et al., 1993; Jun et al., 1994; Phelps et al., 1995). Finally, increases in protein kinase C activity have been shown to result in reductions in the viability of hepatocytes, thymocytes, neural cells, adenocarcinoma cells and the promyelocytic cell lines HL-60 and U937 (Ye et al., 1993; Koong et al., 1994; De Vente et al., 1995; Pavlakovic et al., 1995; Hetland, 1997; Jones et al., 1997).

Evidence also suggests that protein kinase C may be one of the pathways activated within cells in response to oxidative stress and that this is linked to oxidant-mediated cell damage. In vitro studies have shown that $H_2O_2$, which induces epithelial cellular damage and lipid peroxidation (Sheridan et al., 1996), also induces protein kinase C activity in epidermal cells, endothelial cells, ventricular myocytes and astrocytes (Larsson and Cerutti, 1989; Siflinger-Birnboim et al., 1992; Brawn et al., 1995; Ward and Moffat, 1995). Furthermore, $H_2O_2$-induced damage to these cells could be reversed by pretreatment with protein kinase C inhibitors (Von Ruecker et al., 1989).

Taken together, the results of these previous studies indicate that activation of protein kinase C can mediate tissue and cellular injury and also that activation of the enzyme may be an early event in the progression to cell injury following oxidative exposure. In the present study we have investigated whether activation of protein kinase C also has a direct cytotoxic effect on colonic mucosal epithelial cells and whether oxidant-induced damage to the colonocytes is mediated by activation of cellular protein kinase C.

## 2. Materials and methods

### 2.1. Isolation of colonic epithelial cells

Nonfasted male Sprague Dawley rats (250–300 g) were sacrificed by cervical dislocation, and colonic epithelial cells were isolated from the colonic mucosa as described by Butler et al. (1988). Briefly, the colon was excised, everted, rinsed in ice-cold saline and distended with Dulbecco's phosphate buffer (pH 7.2; GIBCO, Burlington, Ont). In most studies the entire colon from caecum to rectum was used. In a few studies the colon was divided by ligation into proximal (caecal junction to the end of the herringbone musculature), transitional (the middle 2 cm) and distal segments, the transitional zone being discarded. The entire colon or the proximal and distal segments were incubated for 60 min at 37°C in 0.2% trypsin (type 1A; Sigma, St. Louis) in 50 ml Dulbecco's in a water bath that shook at 50 oscillations per minute. Epithelial cells were then removed by scraping with a glass slide and were suspended in 5 ml of Dulbecco's containing 0.5% bovine serum albumin and 0.5 mM dithiothreitol. Cells were washed twice with the phosphate buffer by centrifugation at $2000 \times g$ for 2 min, resuspended and dispersed using a Potter–Elverjheim mortar with a Teflon pestle to reduce the number of cell aggregates and crypts. The dispersed cells were filtered through 100-μm polypropylene mesh. The cells were centrifuged again and resuspended in a buffer containing 10 mM HEPES, 320 mM sucrose, 1 mM dithiothreitol and (in mg/ml) 0.01 soybean trypsin inhibitor, 0.01 leupeptin and 0.002 aprotinin (pH 7.4).

### 2.2. Treatment

Cells harvested from the rat colon were incubated (total incubation volume, 1 ml) in vitro with the protein kinase C activator phorbol 12-myristate 13-acetate (0.1–10 μM; Biomol, Plymouth Meeting, PA) for 30 min (37°C 95% $O_2$, 5% $CO_2$). These agents were added to the cells in volumes of 10 μl or less. The high concentration of phorbol ester was used to overcome potential dilution of the agent that may occur within the intact cell preparation used in these studies. In some experiments the negative control for the phorbol ester, 4α phorbol 12-myristate 13-acetate was added to the incubation medium at a final concentration of 10 μM. Control groups of cells were incubated with the vehicle for phorbol 12-myristate acetate (dimethylsulfoxide; l0 μl). Separate groups of cells were incubated with $H_2O_2$ (0.l–l00 μM; BDH Toronto) and the incubation proceeded for 15 min at 37°C (95% $O_2$, 5% $CO_2$). This time for incubation was chosen from preliminary experiments as one that produced maximal increases in protein kinase C activity as well as isozyme translocation. In some studies, the effects of the protein kinase C inhibitors, staurosporine or the highly selective antagonist 2-[1-(3-dimethylaminopropyl)-indol-3-yl]-3-(-indol-3-yl)maleimide (GF 109203X, l0 μM; Biomol, Plymouth Meeting PA) were added to the cell incubate 1 min prior to addition of phorbol ester or $H_2O_2$ and the incubation proceeded as described above. The relatively high concentration of the antagonists was chosen to effectively compete with the endogenous ATP levels of the intact cells. The effects of the oxidant scavengers, superoxide dismutase (1000 and 2000 U/ml; Sigma, St. Louis) and catalase (l000 and 2000 U/ml; Chemical, St. Louis) were also examined. These agents were added to the incubate 1 min prior to the addition of phorbol 12-myristate acetate.

### 2.3. Determination of cellular integrity

#### 2.3.1. Trypan blue dye uptake

More than 90% of the cells harvested were epithelial cells as determined by light microscopy. In all experiments an aliquot of cells was examined for viability as determined by trypan blue dye exclusion (0.5%, trypan blue in phosphate-buffered saline), which has previously been shown to be a reliable index of gastrointestinal epithelial cell injury (Tepperman et al., 1991). Cells from each fraction were counted in a randomized manner by a naive observer using a hemocytometer, and the number of non-viable cells was determined by light microscopy ($\times 200$ magnification) by counting those cells that failed to exclude the dye.

#### 2.3.2. Alamar blue dye reduction

In some studies, alamar blue was used as an indicator to determine cellular metabolism. This technique has been used previously to determine cellular viability (Pagé et al., 1993). As healthy cells metabolize, they reduce their immediate environment via the products of normal respiration. The alamar blue dye, which is non-toxic to cells, is reduced by cells and turns from blue to red in colour. Cells were placed in 250 μl aliquots in wells of a 96-well plate. Alamar blue (Immunocorp) was added in a 1:10 v/v ratio to aliquots. Cells were either preincubated with agents, resuspended and added to wells, or they were coincubated in the presence of the alamar blue dye. The colorimetric determination was done at two absorbance wavelengths on a SLT Spectra II Spectrophotometric plate reader. Absorbance of the reduced form of the dye was measured at a wavelength of 570 nm while the oxidized form was monitored at 600 nm. Values are reported as percent inhibition of metabolic activity as compared to untreated cells.

#### 2.3.3. N-acetyl-β-glucosaminidase release

The appearance of this lysosomal enzyme in the supernatant was used as an indication of cell lysis. Enzyme activity was determined by incubating 100 μl of supernatant with 100 μl of 0.1 M citrate-phosphate buffer (pH 4.5) containing 1.5 mg/ml *p*-nitrophenyl-*N*-acetyl-β-glucosaminide for 2 h at 37°C. The reaction was terminated with 100 μl of 1 M NaOH–glycine, and the absorbance measured at 405 nm. A unit of enzyme activity was defined as that activity which liberates 1 μmol of *p*-nitrophenol from the substrate per h at 37°C. The standard curve for activity of each enzyme was assessed in the presence of the vehicle, phorbol ester, or each of the test agents used in these studies.

To determine the total enzyme content, the cells in an aliquot (1 ml) of the incubation suspension were lysed by rapid freezing in dry ice followed by rewarming to 37°C (3 times). The cell debris was removed by centrifugation ($10{,}000 \times g$, 1 min), and the maximal enzyme release into the supernatant was determined as described above. Enzyme released was expressed as a percentage of the total enzyme content.

### 2.4. Measurement of protein kinase C activity

Cells were centrifuged at $2000 \times g$ for 10 min (4°C) and then were resuspended in 50 mM Tris–HC1 buffer (pH 7.4) containing EDTA (5 mM), EGTA (10 mM), phenylmethylsulfonyl fluoride (50 μg/ml), benzamide (10 mM), soybean trypsin inhibitor (10 μg/ml), leupeptin (10 μg/ml), aprotinin (10 μg/ml), β-mercaptoethanol (0.3% w/v) and okadaic acid (10 nM). The cells were lysed by sonication (10 s). A 25 μl aliquot of the sonicate was removed for determination of PKC activity using a commercially available kit (Amersham, Burlington, Ontario) which measures the transfer of [$^{32}$P]-γ-ATP to a peptide specific for protein kinase C. Results are expressed as pmol/min/$10^6$ cells.

### 2.5. Measurement of PKC protein content

#### 2.5.1. Materials

Affinity-purified rabbit polyclonal antibodies to the α, δ, ε and ζ isoforms of protein kinase C were purchased

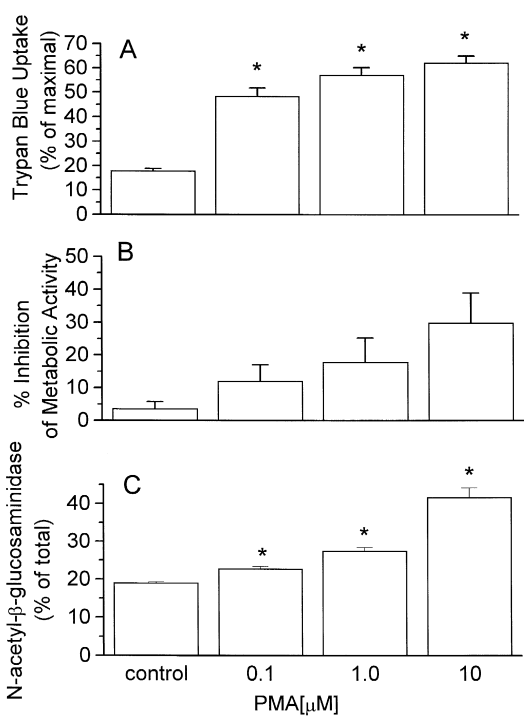


Fig. 1. The effect of increasing concentrations of phorbol 12-myristate (PMA) in the incubation medium on mean (±S.E.) cellular viability response of rat isolated colonic cells as determined by (A) trypan blue dye uptake, (B) alamar blue dye absorbance and (C) *N*-acetyl-β-glucosaminidase release. Incubations were done over a period of 15 min. Asterisks (*) indicate significant ($P < 0.05$) increases over control as determined by analysis of variance and Duncan's multiple range test. ($n = 6$–8 cell preparations, each from a different rat). There was no change in cellular viability detected when cells were incubated with 4α-phorbol ester (10 μM).

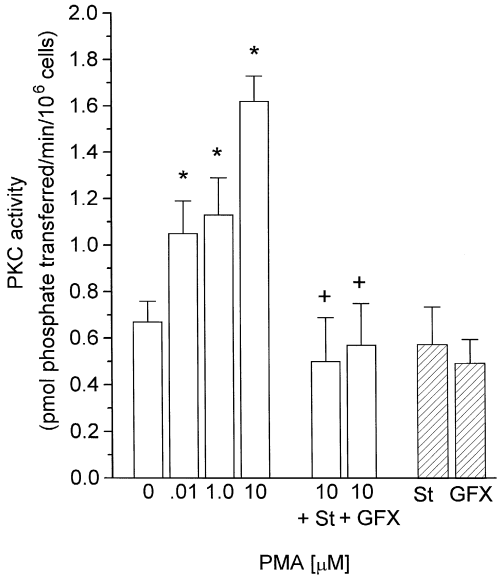


Fig. 2. The effect of increasing concentrations of PMA on mean (±S.E.) cellular protein kinase C (PKC) activity. In addition, the effect of the PKC inhibitors staurosporine (St; 10 μM) or 2-[1-(3-dimethylaminopropyl)-indol-3-yl]-3-(-indol-3-yl)maleimide (GF109203X) (GFX; 10 μM) on PKC activity in response to PMA (l0 μM) was also assessed. The effects of GFX and St alone were also assessed. Asterisks (*) indicate significant increases over vehicle treatment alone while crosses (+) indicate significant ($P < 0.05$) decreases in response to staurosporine or GFX treatment as determined by analysis of variance and Duncan's multiple range test ($n = 9–10$ cell preparations).

from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibody was a goat anti-rabbit antibody conjugated to horseradish peroxidase purchased from Amersham. Rainbow electrophoresis molecular weight marker, the enhanced chemiluminescence (ECL) kit, Hybond ECL nitrocellulose membrane, and Hyperfilm ECL were also purchased from Amersham.

### 2.5.2. Preparation of cytosolic and particulate fractions

Cell samples were resuspended in buffer and sonicated for 15 s on ice. The buffer consisted of 50 mM Tris–HCl (pH 7.5), 0.25 M sucrose, 2 mM EDTA, 1 mM EGTA, 25 μg/ml each of aprotinin, leupeptin and pepstatin, 1 μg/ml soybean trypsin inhibitor, 50 μg/ml PMSF and 10 mM β-mercaptoethanol. The samples were centrifuged at 100,000 × $g$ for 60 min. The supernatant was taken as the cytosolic fraction. The pellet was resuspended in the buffer described above to which was added 10% Triton X-100 and extracted at 4°C for 1 h before centrifugation again under the same conditions. The whole cellular sample was extracted by the same homogenization buffer containing Triton X-100 and centrifuged at 25,000 × $g$ for 20 min. The protein concentration of each sample was subsequently determined.

### 2.5.3. Immunoblotting of cellular samples

Each sample of 10–15 μg protein was boiled for 10 min in an equal volume of sample buffer (125 mM, pH 6.8, 20% glycerol and 10% mercaptoethanol) before subjecting to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After electrophoresis, the gel was soaked for 30 min in transfer buffer and electroblotted onto nitrocellulose membranes using Mini Trans-blot. The membranes were blocked for 1 h with 10% non-fat dry milk in phosphate buffered saline (80 mM $Na_2PO_4$, 20 mM $NaH_2PO_4$, 10 mM NaCl and 0.05% Tween-20 pH 7.5). The blots were then incubated for 3 h with specific protein kinase C-α antibody (1:1000), protein kinase C-δ antibody (1:800), protein kinase C-ε antibody (1:800) or protein kinase C-ζ antibody (1:800) at room temperature. Following washes with phosphate buffered saline (3 times for 10 min), a 1:5000 dilution of horseradish peroxidase-linked secondary antibody was added for 2 h at room temperature. The ECL kit was used to visualize the immunoreactive bands according to the manufacturer's protocol. The density of the immunoreactive bands on the autoradiogram was quantified by measurement of the absolute integrated optical density which estimates the volume of the band in

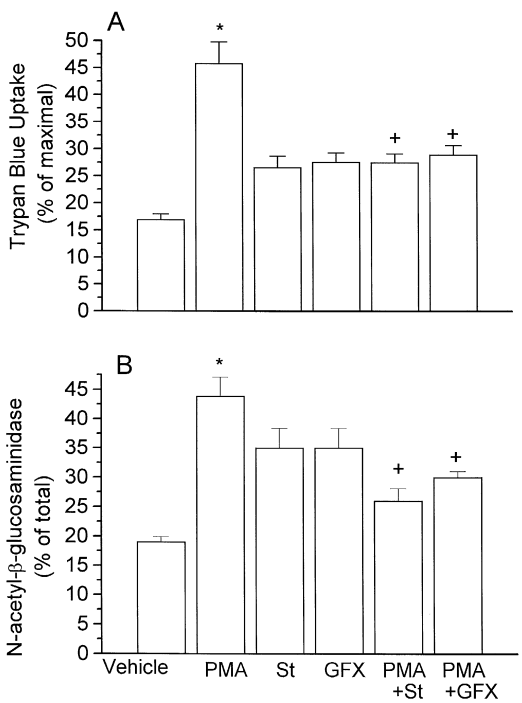


Fig. 3. The effect of protein kinase C inhibition by treatment with staurosporine (St; 10 μM) or 2-[1-(3-dimethylaminopropyl)-indol-3-yl]-3-(-indol-3-yl)maleimide (GF 109203X) (GFX; 10 μM) on the mean (±S.E.) viability response of rat isolated colonic cells to the addition of phorbol 12-myristate acetate (PMA; 10 μM) to the incubation medium. Viability was determined by trypan blue dye uptake (A) or *N*-acetyl-β-glucosaminidase release (B). Asterisks (*) indicate significant ($P < 0.05$) increases over vehicle control whereas crosses (+) indicate significant reductions from PMA treatments alone ($n = 6–8$).

the lane profile as calculated by Image Master VDS software (Pharmacia Biotech)

### 2.6. Statistical calculations

The statistical significance of differences within groups was determined using analysis of variance and Duncan's multiple range test. Statistical differences between groups treated with different agents were determined by *t*-test for paired data. $P < 0.05$ was the accepted level of significance. Data are displayed as means ± S.E., with *n* being the number of cell preparations, each from a different rat.

## 3. Results

Increasing concentration of phorbol 12-myristate acetate in the incubation medium resulted in a dose-dependent increase in the extent of colonic epithelial cellular injury. Cell death was measured by trypan blue dye uptake (Fig. 1A) *N*-acetyl-β-glucosaminidase release (Fig. 1C) and cell metabolism was assessed by alamar blue dye reduction (Fig. 1B). The negative control for the phorbol ester, 4α phorbol myristate acetate (10 μM) did not affect cell viability significantly as assessed by trypan blue dye uptake (14 ± 4% non-viable cells for control vs. 19 ± 6 for

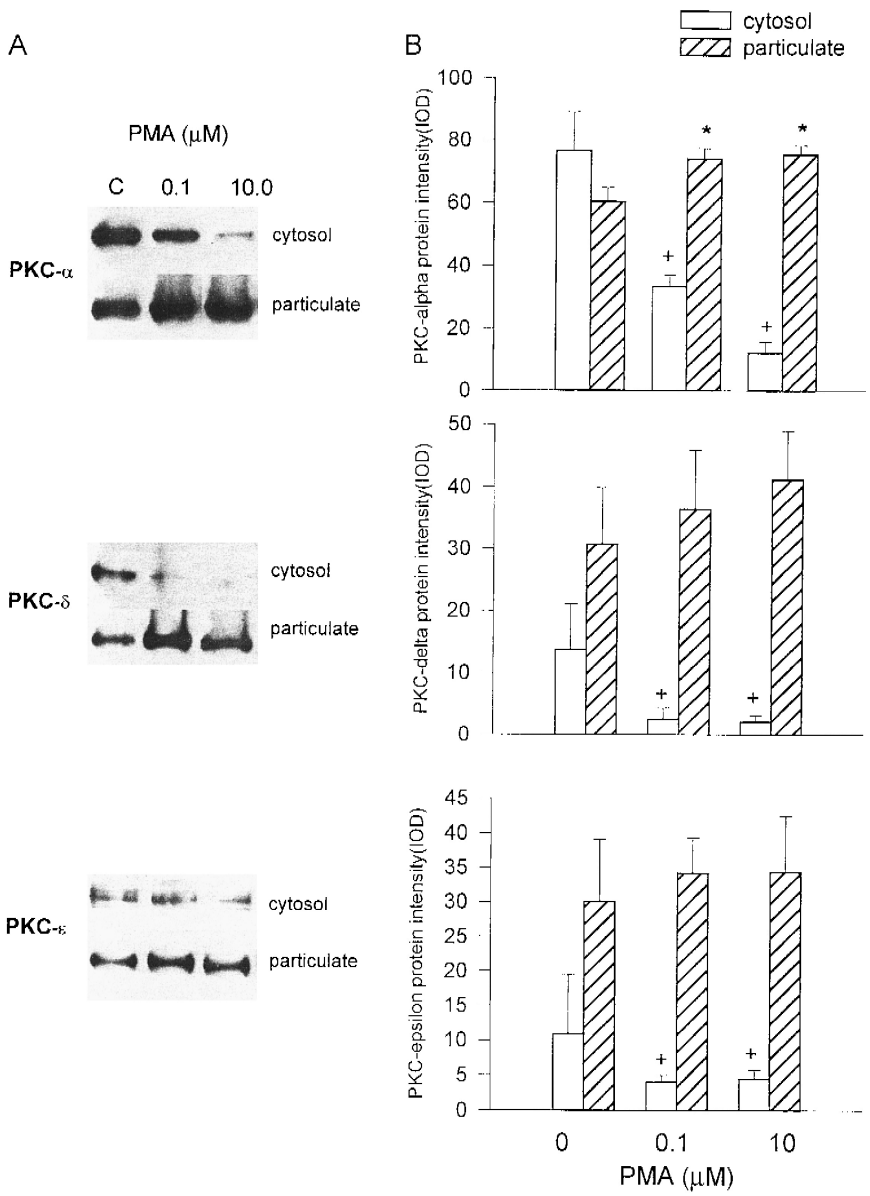


Fig. 4. The effect of phorbol 12-myristate acetate (PMA; 0.1 and 10 μM) on protein kinase C (PKC) isoform levels (PKCα, PKCδ and PKCε) in extracts of colonic epithelial cells. Representative Western blots are displayed in (A). Mean (±S.E.) densitometric analysis of six such immunoblots is displayed in (B). Crosses (+) indicate significant reductions in isoform protein from respective controls while asterisks (*) indicate significant increases over respective controls.

4α phorbol myristate acetate-treated cells; $n = 6$ for each group). There was no significant difference in the extent of phorbol ester-induced injury in cells harvested from the proximal and those harvested from the distal portions of the colon. In response to the highest concentration of phorbol acetate used in these studies (10 μM), the extent of cell injury in response to phorbol 12-myristate acetate was 62 ± 4% non-viable cells from proximal segments and 61 ± 6% non-viable cells harvested from distal segments ($n = 6$–7 per group).

Addition of phorbol ester in the concentration range of 0.1–10 μM to the cell suspension resulted in a dose-dependent increase in the protein kinase C activity of isolated colonic epithelial cells (Fig. 2). Furthermore, the increases in protein kinase C activity in response to the highest concentration of phorbol 12-myristate acetate used (10 μM) could be inhibited by coincubation of cells with the protein kinase C antagonists, staurosporine or 2-[1-(3-dimethylanimopropyl)-indol-3-yl]-3-(-indol-3-yl)maleimide (GF 109203X) (Fig. 2). Neither antagonist by themselves altered the protein kinase C activity. The damaging actions

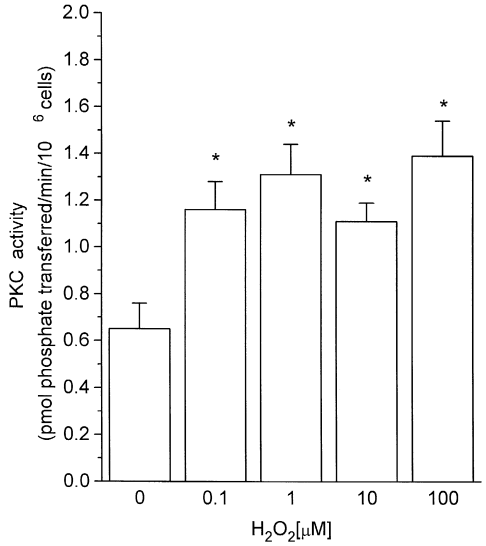


Fig. 6. The effect of increasing concentrations of $H_2O_2$ in the incubation medium on the mean (±S.E.) protein kinase C (PKC) activity response in isolated colonic cells. Asterisks (*) indicate significant increases over vehicle control alone as determined by analysis of variance and Duncan's multiple range test ($n = 7$).

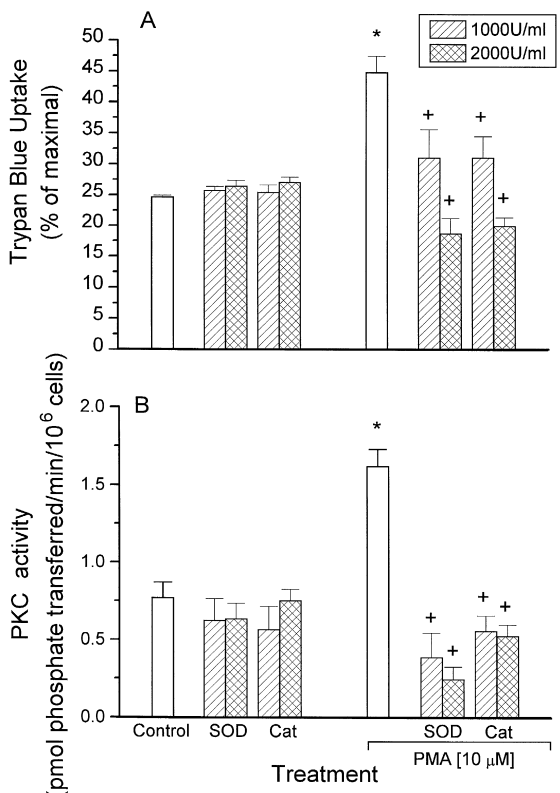


Fig. 5. The effect of addition of the oxidant scavengers catalase (Cat; 1000 and 2000 U/ml) or superoxide dismutase (SOD; 1000 and 2000 U/ml) on (A) the mean (±S.E.) extent of cellular injury as assessed by Trypan blue dye uptake and (B) protein kinase C activation in response to addition of phorbol 12-myristate acetate (PMA; 10 μM) to the incubation medium. Asterisks (*) indicate significant ($P < 0.05$) increases over control levels whereas crosses (+) indicate a significant reduction in the degree of Trypan blue uptake or protein kinase C activation as determined by analysis of variance and Duncan's multiple range test ($n = 6$–9).

of phorbol ester could be reduced significantly by coincubation with either protein kinase C antagonist (Fig. 3). This reduction in cell injury was observed whether cellular integrity was assessed by either trypan blue uptake or *N*-acetyl-β glucosaminidase release.

The proteins for the protein kinase C isoforms, α, δ and ε were detected in extracts of cells harvested from the

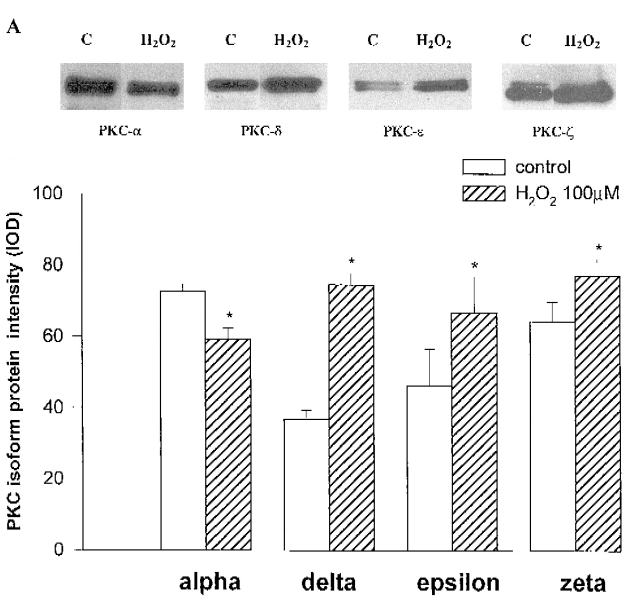


Fig. 7. The effect of $H_2O_2$ (100 μM) on protein kinase C (PKC) isoform levels in colonic epithelial cells. (A) Representative Western blots for control (C) and $H_2O_2$-treated cells. (B) The mean (±S.E.) densitometric analysis of six Western blots as displayed in (A). Asterisks (*) indicate significant differences from respective controls as determined by *t*-test for paired data.

colonic mucosa (Fig. 4A). Addition of phorbol 12-myristate acetate (0.1 and 10 μM) to the cell suspension resulted in a significant decrease in the proteins for each isoform within the cytosolic fraction of the cellular lysate (Fig. 4A,B). Protein kinase C isoform protein levels increased at the same time in the membrane fraction. However, this increase was only significant in the case of the α isoform. We were able to detect protein for protein kinase ζ in untreated colonic cells and phorbol ester treatment had no effect on its expression in these cells.

The cell injury in response to phorbol 12-myristate acetate as assessed by trypan blue uptake could be reversed by co-incubation with the antioxidants, superoxide dismutase or catalase at concentrations of 1000 and 2000 U/ml (Fig. 5A). Neither catalase nor superoxide dismutase by themselves caused any change in cell integrity. Antioxidant treatment resulted in a reduction in cellular protein kinase C activation in response to phorbol ester stimulation (Fig. 5B).

Increasing concentrations of $H_2O_2$ in the incubation medium resulted in an increase in cellular protein kinase C activity (Fig. 6). The highest concentration of $H_2O_2$ used in this study also resulted in an increase in the protein expression for δ, ε and ζ isoforms (Fig. 7A,B). However, protein kinase C α protein was significantly reduced in response to phorbol treatment (Fig. 7A,B). $H_2O_2$ treatment also increased the degree of cell injury, an effect which was reversed significantly by co-incubation with either of the protein kinase C antagonists (Fig. 8).

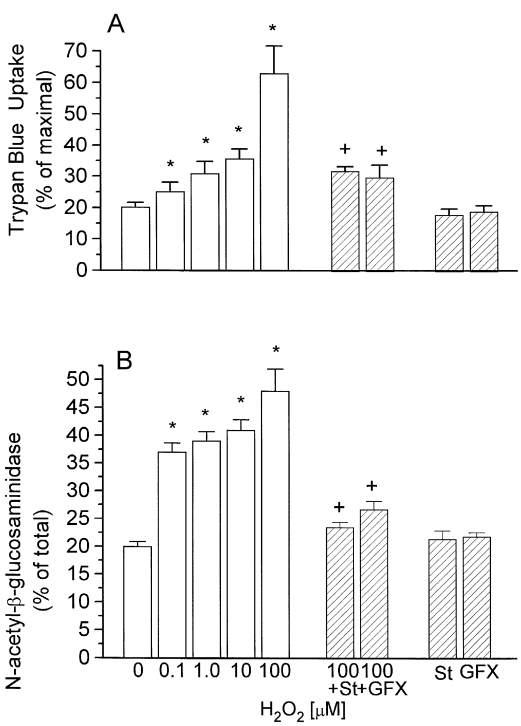


Fig. 8. The effect of increasing concentrations of $H_2O_2$ in the incubation medium on mean (±S.E.) (A) trypan blue dye uptake or (B) *N*-acetyl-β-glucosaminidase release by colonic cells. Some experiments were performed with the addition of the protein kinase C antagonists staurosporine (St; 10 μM) or 2-[1-(3-dimethylaminopropyl)-indol-3-yl]-3-(-indol-3-yl)maleimide (GF 109203X) (GFX; 10 μM). Asterisks (*) indicate significant ($P < 0.05$) increases over control while crosses (+) indicate significant decreased from the response to 100 μM $H_2O_2$ alone as determined by analysis of variance and Duncan's multiple range test ($n = 6–7$).

## 4. Discussion

The results of the present study indicate that epithelial cells isolated from the rat colon are susceptible to injury induced by treatment with the protein kinase C activating agent, phorbol 12-myristate 13-acetate. This confirms the result of previous studies which indicate that such protein kinase C activators can promote damage via necrosis or apoptosis in a variety of cell types (Ye et al., 1993; Koong et al., 1994; De Vente et al., 1995; Pavlakovic et al., 1995; Hetland, 1997; Jones et al., 1997). Furthermore, protein kinase C activators have been shown to potentiate the extent of cellular injury in response to high concentrations of nitric oxide (Jun et al., 1997) and to increase the extent of cell death in response to hypoxia (Koong et al., 1994). To our knowledge this is the first evidence for direct cytotoxicity of protein kinase C activators towards isolated colonic cells. This confirms and extends findings from in vivo experiments in which protein kinase C activation via phorbol ester treatment has been shown to mediate colonic mucosal injury (Fretland et al., 1990; Sakanoue et al., 1992; Buell and Berin, 1994; Berin and Buell, 1995). The results of the present study indicate that the extent of injury increased after 15 min of incubation with the phorbol ester. In the previous reports cited above, phorbol treatment required longer time periods to manifest a cytotoxic response. This difference in the time needed to induce cell damage could be related to different physiological and structural characteristics between cultured and freshly isolated cells.

In the present study phorbol ester-induced increases in protein kinase C activity and this increase in enzyme activity was inhibited by staurosporine or the bisindolylmaleimide, 2-[1-(3-dimethylaminopropyl)-indol-3-yl]-3-(-indol-3-yl)maleimide (GF 109203X). Increases in protein kinase C activity have also been observed in other cell types in response to such activators. (Pavlakovic et al., 1995; Jones et al., 1997). Some previous studies using cultured cells have shown that addition of phorbol ester in the nM range resulted in activation of cellular protein kinase C (Ballen et al., 1996; Balsinde et al., 1997; Ranganathan et al., 1999). In contrast, in the present study protein kinase C activation and isoform translocation in freshly harvested colonocytes occurred in the μM range. These differences may be accounted for on the basis of cell type, the technique for harvesting the cell type under investigation as well as the ability of phorbol 12-myristate

acetate to effectively distribute within the various cellular preparations.

The extent of phorbol ester-induced cell injury was reduced significantly by pretreatment with the protein kinase C antagonist staurosporine of the selective antagonist, 2-[1-(3-dimethylaminopropyl)-indol-3-yl]-3-(indol-3-yl)maleimide (GF 109203X). Similarly, protein kinase C inhibition has been shown previously to reduce the extent of phorbol acetate-induced injury in vivo (Gupta et al., 1988; Kuchera et al., 1993) and to reduce phorbol ester-mediated damage to PC12 neuronal cells and U937 cells in vitro (De Vente et al., 1995; Pavlakovic et al., 1995). In the present study both protein kinase C antagonists were used at a relatively high concentration. While this amount of the inhibitors may have accounted for the small degree of injury displayed in Fig. 3, such a concentration has been justified by Toullec et al. (1991) who has pointed out that concentrations of protein kinase C inhibitors in the micromolar range are expected to compete with ATP and to exert biological effects.

Protein kinase C activity has been identified in epithelial cells, neuronal tissue and in inflammatory cells including neutrophils and macrophages (Nishizuka, 1986; Wilson et al., 1986). However, it is likely that our present results demonstrate an epithelial source for protein kinase C. First, the cells harvested in this study have been identified as nearly exclusively epithelial cells ($>90\%$). Neuronal elements are not readily observed in these preparations. Similarly, in previous studies using identical methodology for cell isolation, inflammatory cells were not found in significant numbers in freshly harvested rat colonic tissue (Tepperman et al., 1994). Furthermore, data from experiments by Buell and Berin (1994) demonstrate that phorbol myristate acetate-induced colonic injury was observed even in neutrophenic animals. Thus, these data suggest that the colonic epithelial cells are the primary cellular types displaying the increases in protein kinase C activity observed in the present study.

Jiang et al. (1995) have demonstrated the presence of multiple protein kinase C isozymes in the colonic epithelium of the rat. In other cell types including hepatocytes and promyeloid cells, cell injury was associated with differential expression of some isozymes of protein kinase C (Pongracz et al., 1996; Jones et al., 1997). Furthermore, in the colon, protein kinase C isozyme expression can be altered by carcinogen treatment (Craven and DeRubertis, 1992). In the present study the $\alpha$, $\delta$ and $\varepsilon$ isoforms were detected in colonic epithelial cell extracts. Furthermore, the activation of these isoforms in response to phorbol 12-myristate acetate was confirmed by the observation that their respective protein levels decreased in the cytosolic fraction while the isoform protein levels increased or remained stable within the membrane fraction. Similar changes in isoform translocation in response to phorbol ester or other cytotoxic challenges have been demonstrated in a variety of cell types and tissues (Padmaperuma et al., 1996; Yoshida et al., 1996; Zhou et al., 1996; Chandrasekher et al., 1998). These different isoforms may play roles in the initiation of damage as well as during the restitution process.

The mechanisms through which protein kinase C activation induces cellular injury is unclear. However, the present results suggest that reactive oxidants are involved since the damaging effect of phorbol 12-myristate acetate was reversed by the scavengers, superoxide dismutase and catalase. Protein kinases have been shown to be involved in cellular oxidant release (Gerard et al., 1986). In contrast, the results of Berin and Buell (1995) indicate that superoxide dismutase and catalase are not effective in reducing phorbol ester-induced increases in epithelial permeability. The differences between those results and the present data may reflect differences in the measured end point as well as the many differences when comparing in vivo and in vitro studies.

In the present study, oxidant-induced injury to the colonic cells via $H_2O_2$ treatment increased protein kinase C activity in colonic epithelial cells. The increase in enzyme activity appeared to be primarily due to increases in the $\delta$, $\varepsilon$ and $\zeta$ isoforms. Protein kinase C $\alpha$ protein was downregulated by this treatment. Selective down-regulation of the $\alpha$ isoform in response to $H_2O_2$ has also been demonstrated in endothelial cells (Chen et al., 1996). $H_2O_2$ treatment increased the extent of cell injury and this damage was reduced by treatment with protein kinase C inhibitors. It has been suggested previously that $H_2O_2$ mediates its cytotoxic action via protein kinase C activation (Fiorani et al., 1995). The results of the present study also demonstrate that antioxidant treatment significantly reduces the activation of protein kinase C in response to phorbol ester treatment. Hydrogen peroxide has been shown to increase protein kinase C activity in neuronal cells, endothelial cells, cardiac myocytes, tracheal myocytes and an astrocytoma cell line, UC11MG (Ward and Moffat, 1995; Abe et al., 1998). These data implicate protein kinase C in the colonic cellular response to an oxidant challenge. The mechanisms by which protein kinase C may be involved in cellular oxidative injury is unknown. However, Ward and Moffat (1995) have demonstrated that $H_2O_2$-mediated increases in protein kinase C in cardiac myocytes is associated with increases in cellular $Ca^{2+}$. It has been proposed that $Ca^{2+}$ homeostasis is critical in maintaining cellular integrity, including cells of the gastrointestinal tract (Tepperman et al., 1991). However, it should also be pointed out that in spite of the high doses of protein kinase C inhibitors used in these studies, $H_2O_2$-induced toxicity was only partially reversed. This suggests that mechanisms other than protein kinase activation account for the cellular damaging actions of $H_2O_2$.

In conclusion, these results suggest that protein kinase C activation exerts a cytotoxic effect on rat colonocytes. The damage induced by phorbol 12-myristate acetate can be ameliorated by oxidant scavengers, suggesting that

reactive oxidant metabolites mediate this response, at least in part. Furthermore, oxidant-mediated injury to rat colonocytes is related in a causal fashion to an increase in protein kinase C activity.

## Acknowledgements

This work was supported by a grant from the Medical Research Council of Canada (MT 6426). A Canadian Association of Gastroenterology/Astra Research Initiative Award supported J.F. Brown.

## References

Abe, M.K., Kartha, S., Karpova, A.Y., Li, J., Liu, P.T., Kuo, W.L., Hershenson, M.B., 1998. Hydrogen peroxide activates extracellular signal-regulated kinase via protein kinase C, Raf-1 and MEK1. Am. J. Respir. Cell Mol. Biol. 18, 562–569.

Ballen, K.K., Ritchie, A.J., Murphy, C., Handin, R.I., Ewenstein, B.M., 1996. Expression and activation of protein kinase C isoforms in a human keratinocyte cell line. Exp. Hematol. 24, 1501–1508.

Balsinde, J., Balboa, M.A., Insel, P.A., Dennis, E.A., 1997. Differential regulation of phospholipase D and phospholipase $A_2$ by protein kinase C in P3880 macrophages. Biochem. J. 321, 805–809.

Berin, M.C., Buell, M.G., 1995. Phorbol myristate acetate ex vivo model of enhanced colonic epithelial permeability. Reactive oxygen metabolitic and protease independence. Dig. Dis. Sci. 40, 2268–2279.

Brawn, M.K., Chion, W.J., Leach, K.L., 1995. Oxidant-induced activation of protein kinase C in UC11MG cells. Free Radical Res. 22, 23–37.

Brown, J.F., Qing, C., Soper, B.D., Tepperman, B.L., 1999. Protein kinase C mediates experimental colitis in the rat. Am. J. Physiol. 276, G583–G590.

Buell, M.G., Berin, M.C., 1994. Neutrophil independence of the initiation of colonic injury. Comparison of results from three models of experimental colitis in the rat. Dig. Dis. Sci. 39, 2575–2588.

Butler, R.N., Lawson, M.J., Goland, G.J., Jarret, I.G., Roberts-Thomson, I.C., 1988. Proliferative activity in the proximal and distal colon of the rat after fasting and refeeding. Immunol. Cell Biol. 66, 193–198.

Chandrasekher, G., Bazan, N.G., Bazan, H.E.P., 1998. Selective changes in protein kinase C isoform expression in rabbit corneal epithelium during wound healing. Inhibition of corneal epithelial repair by PKC$\alpha$ antisense. Exp. Eye Res. 67, 603–610.

Chen, C.C., Liau, C.S., Lee, Y.T., 1996. Tumor necrosis factor-alpha, platelet activating factor and hydrogen peroxide activate protein kinase C subtypes alpha and epsilon in human saphenous vein endothelial cells. J. Cardiovasc. Pharmacol. 28, 240–244.

Craven, P.A., DeRubertis, F.R., 1992. Alterations in protein kinase C in 1, 2-dimethylhydrozine induced colonic carcinogenesis. Cancer Res. 52, 2216–2221.

De Vente, J., Kiley, S., Garris, T., Bryant, W., Hooker, V., Posekany, K., Parker, P., Look, P., Fletcher, D., Ways, D.K., 1995. Phorbol ester treatment of U937 cells with altered protein kinase C content and distribution induces cell death rather than differentiation. Cell Growth Differ. 6, 371–382.

Fiorani, M., Cantoni, O., Tasinato, A., Boscoboinik, D., Azzi, A., 1995. Hydrogen peroxide and fetal bovine serum-induced DNA synthesis in vascular smooth muscle cells: positive and negative regulation by protein kinase C isoforms. Biochim. Biophys. Acta 1269, 98–104.

Fretland, D.J., Widomski, D.L., Levin, S., Gaginella, T.S., 1990. Colonic inflammation in the rabbit induced by phorbol-12-myristate-13-acetate. Inflammation 14, 143–150.

Gerard, C., McPhail, L.C., Marfat, A., Stinler-Gerard, N.P., Bass, D.A., McCall, C.E., 1986. Role of protein kinases in stimulation of human polymorphonuclear leukocyte oxidative metabolism by various agonists. J. Clin. Invest. 77, 61–65.

Gupta, A.K., Fisher, G.J., Elder, T., Nickoloff, B.J., Voorhees, J.J., 1988. Sphingosine inhibits phorbol ester-induced inflammation, ornithine decarboxylase activity and activation of protein kinase C in mouse skin. J. Invest. Dermatol. 91, 486–491.

Hetland, G., 1997. Cytotoxic effect of dibutyryl cAMP, phorbol-12-myristate-13-acetate and lipopolysaccharide but not interferon $\gamma$ on promonocytic cell lines in vitro. Anti-Cancer Drugs 8, 618–622.

Jiang, Y.H., Aukema, H.M., Davidson, L.A., Lupton, J.R., Chapkin, R.S., 1995. Localization of protein kinase C isozymes in rat colon. Cell Growth Differ. 6, 1381–1386.

Jones, B.A., Rao, Y.P., Stravitz, R.T., Gores, G.J., 1997. Bile salt-induced apoptosis of hepatocytes involves activation of protein kinase C. Am. J. Physiol. 272, G1109–G1115.

Jun, C.-D., Choi, B.-M., Ryn, H., Um, J.-Y., Kwak, H.-J., Lee, B.-S., Pak, S.-G., Kim, H.-M., Chung, H.-T., 1994. Synergistic co-operation between phorbol ester and IFN for induction of nitric oxide synthesis in murine peritoneal macrophages. J. Immunol. 153, 3684–3690.

Jun, C.D., Park, S.J., Choi, B.M., Kwak, H.J., Park, Y.C., Kim, M.S., Park, R.K., Chung, H.T., 1997. Potentiation of the activity of nitric oxide by the protein kinase C activator phorbol ester in human myeloid leukemia HL-60 cells: association with enhanced fragmentation of mature genomic DNA. Cell. Immunol. 176, 41–49.

Koong, A.C., Chen, E.Y., Kim, C.Y., Giaccia, A.J., 1994. Activators of protein kinase C selectively mediate cellular cytotoxicity to hypoxic cells but not aerobic cells. Int. J. Radiat. Oncol., Biol., Phys. 29, 259–265.

Kuchera, S., Barth, H., Jacobson, P., Metz, A., Schaechtele, C., Schrier, D., 1993. Antiinflammatory properties of the protein kinase C inhibitor, 3-[1-[3-(dimethylamino) propyl]-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione monochloride (GF 109203X) in the PMA-mouse ear edema model. Agents Actions 39, C169–C173, Spec No.

Kuruvilla, A., Putcha, G., Poulos, E., Shearer, W.T., 1993. Tyrosine phosphorylation of phospholipase C concomitant with its activation by platelet-activating factor in a human B cell line. J. Immunol. 151, 637–648.

Larsson, R., Cerutti, P., 1989. Translocation and enhancement of phosphotransferase activity of protein kinase C following exposure in mouse epidermal cells to oxidants. Cancer Res. 49, 5627–5632.

Louise, C.B., Tran, M.C., Obrig, T.G., 1997. Sensitization of human umbilical vein endothelial cells to shiga toxin: involvement of protein kinase C and NF kappa B. Infect. Immun. 65, 3337–3344.

Nishizuka, Y., 1986. Studies and perspective of protein kinase C. Science 233, 305–312.

Nishizuka, Y., 1992. Intracellular signalling by hydrolysis of phospholipids and activation of protein kinase C. Science 258, 607–614.

Overdahl, M.C., Julian, M.W., Weisbrode, S.E., Dorinsky, P.M., 1995. Anti-CD 18 antibody does not block ileal injury induced by phorbol myristate acetate. Am. J. Respir. Crit. Care Med. 152, 1331–1336.

Padmaperuma, B., Mark, R., Dhillon, H.S., Mattson, M.P., Prasad, M.R., 1996. Alterations in brain protein kinase C after experimental brain injury. Brain Res. 714, 19–26.

Pagé, B., Pagé, M., Noel, C., 1993. A new fluorometric assay for cytotoxicity measurements in vitro. Int. J. Oncology 3, 473–476.

Pavlakovic, G., Kane, M.D., Eyer, C.L., Kanthasamy, A., Isom, G.E., 1995. Activation of protein kinase C by trimethylin: relevance to neurotoxicity. J. Neurochem. 65, 2338–2343.

Phelps, D.T., Ferro, T.J., Higgins, R.J., Shankar, R., Parker, D.M., Johnson, A., 1995. TNF$\alpha$ induces peroxynitrite-mediated depletion of lung endothelial glutathione via protein kinase C. Am. J. Physiol. 269, L551–L559.

Pongracz, J., Deacon, E.M., Johnson, G.D., Burnett, D., Lord, J.M., 1996. Doppa induces cell death but not differentiation of U937 cells:

evidence for the involvement of PKC-beta 1 in the regulation of apoptosis. Leuk. Res. 20, 319–326.

Ranganathan, G., Kaakaji, R., Kern, P.A., 1999. Role of protein kinase C in the translational regulation of lipoprotein lipase in adipocytes. J. Biol. Chem. 274, 9122–9127.

St. John, R.C., Mizer, L.A., Weisbrode, S.E., Dorinsky, P.M., 1991. Increased intestinal protein permeability in a model of lung injury induced by phorbol myristate acetate. Am. Rev. Respir. Dis. 144, 1171–1176.

Sakanoue, Y., Hatada, T., Horai, T., Shoji, Y., Kusumoki, M., Utsunomiya, J., 1992. Protein kinase C activity of colonic mucosa in ulcerative colitis. Scand. J. Gastroenterol. 27, 275–280.

Savithiry, S., Kumar, K., 1994. mRNA levels of $Ca^{2+}$-independent forms of protein kinase C in postischemic gerbil brain by Northern blot analysis. Mol. Chem. Neurophathol. 21, 1–11.

Sheridan, A.M., Fitzpatrick, S., Wang, C., Wheeler, D.C., Lieberthal, W., 1996. Lipid peroxidation contributes to hydrogen peroxide induced cytotoxicity in renal epithelial cells. Kidney Int. 49, 81–93.

Siflinger-Birnboim, A., Goligorsky, M.S., Del Vecchio, P.J., Malik, A.B., 1992. Activation of protein kinase C pathway contributes to hydrogen peroxide-induced increase in epithelial permeability. Lab Invest. 67, 24–30.

Suntres, Z.E., Shek, P.N., 1995. Prevention of phorbol myristate acetate-induced acute lung injury by alpha-tocapherol liposomes. J. Drug Targeting 3, 201–208.

Tepperman, B.L., Brown, J.F., Korolkiewicz, R., Whittle, B.J.R., 1994. Nitric oxide synthase activity, viability and cyclic GMP levels in rat colonic epithelial cells: effect of endotoxin challenge. J. Pharm. Exp. Ther. 271, 1477–1482.

Tepperman, B.L., Tan, S.Y., Whittle, B.J.R., 1991. Effects of calcium modifying agents on integrity of rabbit isolated gastric mucosal cells. Am. J. Physiol. 261, G119–G127.

Toullec, D., Pianetti, P., Coste, H., Belleverque, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boisson, P., Boursier, E., Loriolle, F., Duhamel, L., Charon, D., Kirilovsky, J., 1991. The bisindoyl malemide GF 109203X is a potent and selective inhibitor of protein kinase C. J. Biol. Chem. 266, 15771–15781.

Von Ruecker, A.A., Han-Joen, B.-G., Wild, M., Bidlingmaier, F., 1989. Protein kinase C involvement in lipid peroxidation and cell membrane damage induced by oxygen-based radicals in hepatocytes. Biochem. Biophys. Res. Commun. 163, 836–842.

Ward, C.A., Moffat, M.P., 1995. Role of protein kinase C in mediating effects of hydrogen peroxide in guinea pig ventricular myocytes. J. Mol. Cell. Cardiol. 27, 1089–1097.

Wilson, E., Olcoltt, M.C., Bell, R.M., Merrill, A.H. Jr., Lambeth, J.D., 1986. Inhibition of the oxidative burst in human neutrophils by sphinzoid long-chain bases. Role of protein kinase C in activation of the burst. J. Biol. Chem. 261, 12616–12623.

Ye, X., Georgoff, I., Fleisher, S., Coffman, F.D., Cohen, S., Fresa, K.L., 1993. The mechanism of epipodophyllotoxin-induced thymocyte apoptosis: possible role of a novel $Ca^{2+}$-independent protein kinase. Cell. Immunol. 151, 320–335.

Yoshida, K., Hirata, T., Akita, Y., Mizukami, Y., Yamaguchi, K., Sorimachi, Y., Ishihara, T., Kawashiama, S., 1996. Translocation of protein kinase C$\alpha$, $\delta$ and $\varepsilon$ isoforms in ischemic rat heart. Biochim. Biophys. Acta 1317, 36–44.

Zhou, L.Y., Disatnik, M., Herron, G.S., Mochly-Rosen, D., Karasek, M.A., 1996. Differential activation of protein kinase C isozymes by phorbol ester and collagen in human skin microvascular endothelial cells. J. Invest. Dermatol. 107, 248–252.