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European Journal of Pharmacology 518 (2005) 1 – 9

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# Effect of protein kinase C activation on intracellular $Ca^{2+}$ signaling and integrity of intestinal epithelial cells

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Received 3 June 2005; accepted 7 June 2005

**Abstract**

Protein kinase C (PKC) activation and increases in cytosolic $Ca^{2+}$ cause intestinal injury. Since PKC activation can alter $Ca^{2+}$ homeostasis and increase $Ca^{2+}$ levels, we examined the effects of PKC activation on intestinal cellular integrity and the role of $Ca^{2+}$ signaling in this response. The epithelial cell line, IEC-18 was incubated with the PKC activator phorbol myristate acetate (PMA; 0.1–1.0 μM). In some experiments, cells were incubated in $Ca^{2+}$-free medium. PMA treatment produced a concentration-dependent increase in cell injury and PKC activity. This response was attenuated by addition of the pan-specific PKC inhibitor, GF 109203X. Furthermore, cell viability was maintained in cells preincubated with PKC isoform-specific inhibitors to PKCα, PKCδ and PKCε. Cell injury was also reduced if cells were incubated in $Ca^{2+}$-free medium or in the presence of the $Ca^{2+}$ channel antagonist, verapamil or the intracellular chelator BAPTA-AM. PMA, but not the inactive phorbol ester, 4αPMA, induced a dose-dependent increase in cellular $Ca^{2+}$ that was characterized by a rapid, transient spike followed by a tonic plateau phase which approximated control levels. These responses were eliminated by the addition of BAPTA-AM. Furthermore the increase in the $Ca^{2+}$ spike was reduced or eliminated by co-incubation with the PKCδ antagonist, rottlerin. Inhibition of PKCα or PKCε was less effective or ineffective in this regard.

These data suggest that PKC activation via PMA challenge affects the integrity of rat intestinal epithelial cells. PKCδ, but not PKCε or PKCα activation appears to mediate this effect via an increase in cellular $Ca^{2+}$.


*Keywords:* Protein kinase C; Calcium; Intestine; Phorbol ester; Cell damage

## 1. Introduction

Protein kinase C (PKC) is a family of at least 12 serine–threonine protein kinase isoforms which have been implicated in a number of cellular signaling pathways including changes in cellular integrity (Dempsey et al., 2000; Tepperman et al., 2000b). Increases in PKC activity has been shown to reduce the viability of a number of cell types including hepatocytes, thymocytes and neural cells (Jones et al., 1997; Pavlakovic et al., 1995; Ye et al., 1993). We have previously demonstrated that PKC activation mediates experimentally-induced colitis in rats (Brown et al., 1999) and could reduce the integrity of cultured rat and human intestinal cells (Chang and Tepperman, 2001, 2003).

Several lines of evidence suggest that individual PKC isoforms play distinct regulatory roles in maintenance of the integrity of various cell types. In HeLa and NIH3T3 cell lines, overexposure of constitutively active catalytic fragments of PKCδ causes apoptosis (Ghayuar et al., 1996). In contrast, an enhancement in the death of cardiac cells appear to be directly related to overexpression of PKCδ (Murriel and Mochly-Rosen, 2003) while hepatic cell integrity has been linked to changes in PKCα (Jao et al., 2001). In intestinal epithelial cells, pro-inflammatory mediators such as the cytokine, TNFα has been shown to mediate its necrotic and apoptotic effects via activation of specific PKC isozymes, particularly PKC δ and PKCε (Chang and Tepperman, 2003).

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doi:10.1016/j.ejphar.2005.06.008

It has also been demonstrated that the PKC activator, phorbol myristate acetate (PMA) will result in colonic cell damage as evidenced by an increase in necrosis (Tepperman et al., 2000b). Similarly, PKC activation has also been associated with an increase in colonic cellular apoptosis (O'Connell et al., 1997). In these experiments the effects of PMA appeared to be manifested primarily via activation of PKCδ and PKCε.

In addition to PKC, disruption of cellular calcium homeostasis has been shown to affect gastrointestinal epithelial cell integrity (Tepperman et al., 1991; Banan et al., 1999). An increase in intracellular $Ca^{2+}$ has been associated with an increase in the extent of cell death and disruption of the intestinal epithelial barrier. Furthermore, agents which protect the intestinal epithelium from oxidant-induced damage have been shown to do so as a result of an action of stabilization of the intracellular levels of $Ca^{2+}$ (Banan et al., 2001).

Finally, an association between PKC activation and cellular $Ca^{2+}$ has been established for a variety of cells. In these studies, PKC activation via treatment with PMA has been shown to increase intracellular $Ca^{2+}$ in red blood cells, keratinocytes and RBL-2H3 mucosal mast cells (Gönczi et al., 2002; Andrews et al., 2002; Lin and Gilfillan, 1992). In isolated intestinal cells and intestinal epithelial monolayers, PKC activation has been shown to affect cellular $Ca^{2+}$ and regulate intracellular levels of $Ca^{2+}$ (Banan et al., 1999, 2001, 2002). It is possible that such changes in intracellular $Ca^{2+}$ are part of the signaling cascade leading the physiological or pathophysiological action of PKC in those cells.

Therefore, in the present study, we have examined whether PKC activation mediates its effects on intestinal epithelial cell viability via changes in intracellular $Ca^{2+}$. We have also characterized the $Ca^{2+}$ response to PKC activation and determined which PKC isoform is most closely related to this phenomenon.

## 2. Materials and methods

### 2.1. Cell culture

IEC-18 cells were obtained from American Type Culture Collection (Rockville, MD) and were maintained in Dulbecco's modified Eagle's medium (DMEM) with 4 mM glutamine, 0.01 mg/ml insulin, 5% heat-inactivated fetal bovine serum, 50 U/ml penicillin G, and 50 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% $CO_2$. The medium was replaced every 3 days and cultures were passaged before confluency. The IEC-18 cells used for these studies are derived from rat ileal crypt epithelium (Quaroni and Isselbacher, 1981) and maintain many of the characteristics of proliferating crypt cells.

### 2.2. Treatments

Cells were treated with PMA in the concentration range 0.1–1.0 μM for 60 min. As a control, some groups of cells were incubated in the presence of the inactive phorbol ester, 4αPMA (10 μM). In some experiments, cells were incubated in serum-free DMEM medium in the absence of $Ca^{2+}$. In experiments to assess viability, cells were also pre-incubated (30 min) with the following; the pan specific PKC antagonist, GF 109203X (10 μM), the PKCα selective inhibitor, Gö 6976 (1.0 μM), the PKCδ selective antagonist, rottlerin (10 μM) or the PKCε translocation antagonist, εV1–2 (4.0 μM). The concentrations used here were obtained from previously published studies from this laboratory (Chang and Tepperman, 2001, 2003). These inhibitors have previously been shown to be selective for their respective isoforms (Fujii et al., 2000; Martiny-Baron et al., 1993; Yedovitzky et al., 1997) In experiments to test the role of $Ca^{2+}$ in PMA-induced responses, the $Ca^{2+}$ channel antagonist, verapamil (1.0 μM), the intracellular $Ca^{2+}$ chelator, acetoxymethyl-1,2-bis(0-aminophenoxy)ethane *N*,*N*,*N'*,*N'*-tetraacetic acid (BAPTA-AM; 10 μM) or the $Ca^{2+}$ entry blocker, lanthanum chloride (10 μM) were used. These agents were purchased from Biomol Research, Canada. In studies in which the intracellular concentrations of $Ca^{2+}$ were assessed, nifedipine (Sigma, St. Louis) was used in the place of verapamil, since verapamil displayed a high level of background fluorescence.

### 2.3. Cell viability assessment

Cells were examined for viability as determined by Trypan blue dye (Sigma, St. Louis) exclusion (0.4%, 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) In addition, the appearance of lysosomal enzymes, acid phosphatase and *N*-acetyl-β-glucosaminidase into the incubation medium was measured as indicators of cell lysis. Briefly, the levels of acid phosphatase were determined spectrophotometrically by the appearance of p-nitrophenol from the substrate p-nitrophenol phosphate (4 mg/ml; 100 μl) incubated at pH 4.8 in citrate buffer (0.1 M; 200 μl) at 37 °C for 10 min. The reaction was terminated by the addition of 0.5 ml of 1 M NaOH. Absorbance was measured at 400 nm. The degree of cell lysis was assessed by comparing levels of acid phosphatase in the supernatant of treated cells to the total content of acid phosphatase determined after lysis and freeze-thawing of untreated cells.

For *N*-acetyl-β-glucosaminidase release, the same protocol was used with slight modifications. Enzyme activity was determined by incubating 100 μl of supernatant with 100 μl of 0.1 citrate-phosphate buffer (pH 4.5) containing 1.5 mg/ml of p-nitrophenol-*N*-acetyl-β-glucosaminidase for 2 h at 37 °C. The reaction was terminated with 100 μl of 1 M NaOH and absorbance measured at 405 nm. A unit of enzyme activity was defined at that activity which liberates 1 μmol of p-nitrophenol from the substrate per h at 37 °C. The total enzyme content was once again determined from a control cell lysate as described above.

### 2.4. Intracellular calcium measurement

Determinations of intracellular $Ca^{2+}$ were based on the methods described by Reimer and Dixon (1992). Briefly, cells (approximately $2.0 \times 10^7$ in 8 ml) were loaded with indo-1-acetoxymethyl ester (indo-1AM; 2 μM; Molecular Probes, Eugene Oregon) by incubation for 45 min in DMEM at 37 °C. Cells were sedimented and resuspended in 2 ml ($5.0 \times 10^6$ cells) of a solution containing (in mM) 135 NaCl, % KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose and 20 Na-HEPES (pH 7.3; 290mOsm, 37 °C) in a fluorometric cuvette.

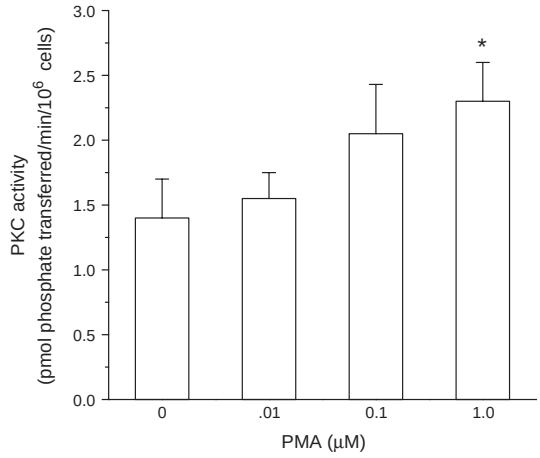


Fig. 1. The effect of phorbol myristate acetate (PMA; 0.1–1 μM) on PKC activity of IEC-18 cells. Cells were assessed 60 min after addition of PMA. Data are expressed as mean (±standard error of the mean; $N=6-7$). Asterisks (*) indicate significant differences from untreated control cells.

Intracellular $Ca^{2+}$ concentration was measured using a dual-wavelength fluorometer (excitation wavelength of 355 nm and emission wavelengths of 405 and 485 nm; Model RF-M2004, from Photon Technology International, London, Canada.) The system calculated the ratio of 405 and 485 nm fluorescence intensities and accounted for background fluorescence. Changes in $R$ reflect changes in cytsolic free calcium levels. Intracellular concentrations of $Ca^{2+}$ were derived from the equation $[Ca^{2+}]=Kd\ (R-R_{min}/R_{max}-R)$; Kd=250 nM, and results expressed as nM.

### 2.5. PKC activity

Cells were harvested and suspended in 50 mM Tris–HCl buffer (pH 7.4) containing EDTA (5 mM), EGTA (10 mM), phenyl-methlsulfonyl fluoride (50 μg/ml), benamide (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 Canada) which measures the transfer of [$\gamma-^{32}$P] ATP to a peptide specific for PKC. Results are expressed as picomoles per minute per $10^6$ cells.

### 2.6. PKC isoform translocation

Cells were separated into membrane and cytosolic components in order to estimate PKC translocation, a generally regarded index of PKC activation (Haller et al., 1998). After cells were treated with test components in DMEM, the medium was aspirated from the culture dish and cells were washed once with ice-cold phosphate buffered saline and 500 μl of homogenization buffer (50 mM Tris–HCl, 5 mM EDTA, 25 mM EGTA, 50 μg/ml phenylmethylsulfonyl fluoride, 10 mM benzamide, 25 μg/ml each of soybean trypsin inhibitor, leupeptin, aprotinin and 5% mercaptoethanol) was added. Cells were scraped into the medium to form a suspension and lysed by sonication for 10–15 s on ice. Cytosol protein was released into the medium. The resulting lysate was centrifuged at 100,000 ×$g$ for 1 h at 4 °C to pellet the membrane protein. The resulting pellet was resuspended in 500 μl of homogenization buffer to which was added Triton X-100 (final concentration 0.5%) and incubated on ice for 1 h to extract soluble membrane proteins. Samples were centrifuged again at 100,000 ×$g$ for 1 h at 4 °C to remove insoluble membrane components. The resultant supernatent was kept as the particulate fraction. Protein concentrations in both cytosolic and particulate fractions were determined before being frozen at −20 °C until further use.

### 2.7. Immunoblot analysis of PKC isoforms

Particulate and cytosol samples (10–15 μg protein) were prepared for electophoresis by boiling for 5 min in an equal

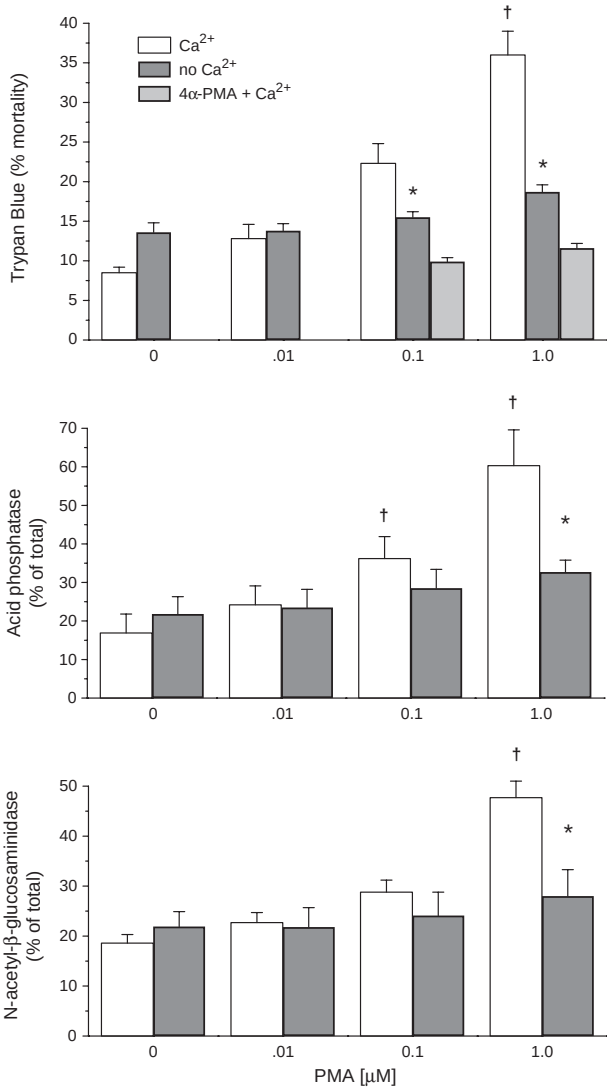


Fig. 2. The effect of phorbol myristate acetate (PMA; 0.1–1 μM) in the presence (2 mM) or absence of extracellular $Ca^{2+}$ or the inactive form of PMA (4 α PMA; 1 μM) on the integrity of IEC-18 cells as assessed by Trypan Blue uptake and the release of the cytoplasmic enzymes, acid phosphatase and $N$-acetyl-β-glucosaminidase. Cells were assessed 60 min after incubation with each concentration of PMA. Data are expressed as mean (±standard error of the mean; $N=6$). Crosses (+) indicate significant differences from PMA controls in the presence of extracellular $Ca^{2+}$. Asterisks (*) indicate significant reductions from the response seen to the corresponding concentration of PMA in the presence of extracellular $Ca^{2+}$.

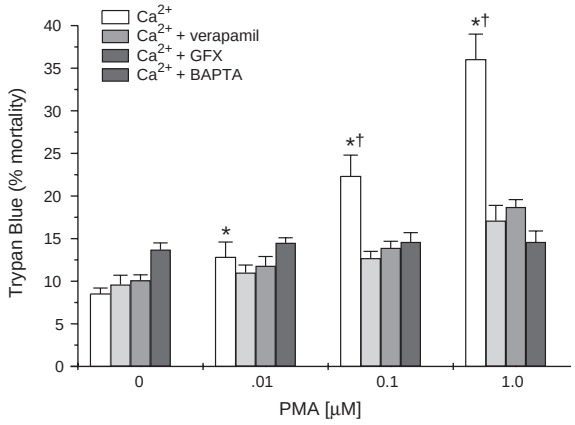


Fig. 3. The effect of PMA on cellular integrity as assessed by Trypan Blue dye uptake. In some experiments, cells were pre-incubated (30 min) with either the $Ca^{2+}$ channel antagonist, verapamil (1.0 μM), the pan-specific protein kinase C blocker, GF109203X (GFX; 10 μM), or the intracellular $Ca^{2+}$ chelator, BAPTA-AM (BAPTA; 20 μM). Trypan Blue uptake was assessed 30 min after addition of PMA addition. Data are expressed as means (±standard error of the mean; $N=5-6$). Asterisks (*) indicate significant differences from control group in the absence of PMA. Crosses (+) indicate significant differences between the Trypan blue uptake in response to PMA alone compared to the responses obtained after addition of verapamil, GFX or BAPTA.

volume of SDS sample buffer (125 mM Tris, pH 6.8, containing 20% glycerol and 10% mercaptoethanol). Samples containing equal amounts of protein were loaded in each lane of 10% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and electrophorectically transferred to nitrocellulose membranes at 100 V for 75 min. The membranes were blocked for 1 h at room temperature in PBS-Tween buffer (80 mM $Na_2HPO_4$, 10 mM NaCl and 0.05% Tween-20; pH 7.5 and containing 10% nonfat milk. Samples were then incubated for 2 h with specific PKCα antibody (1:1000) and 3 h with PKCδ, PKCε antibodies (1:800) at room temperature. This was followed by incubation with a 1:6000 dilution of horse radish peroxidase (HRP)-conjugated anti-rabbit IgG for 1 h at room temperature. All antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz California)ECL reagents were used to develop the blots. The densitometric assessment of the bands of the autoradiogram was done using Image Master VDS (Pharmacia Biotech). Band intensity was quantified by measurement of the absolute integrated optical intensity, which estimates the volume of the band in the lane profile.

### 2.8. Statistics

The statistical significance of difference within groups was determined by analysis of variance and Duncan's multiple range

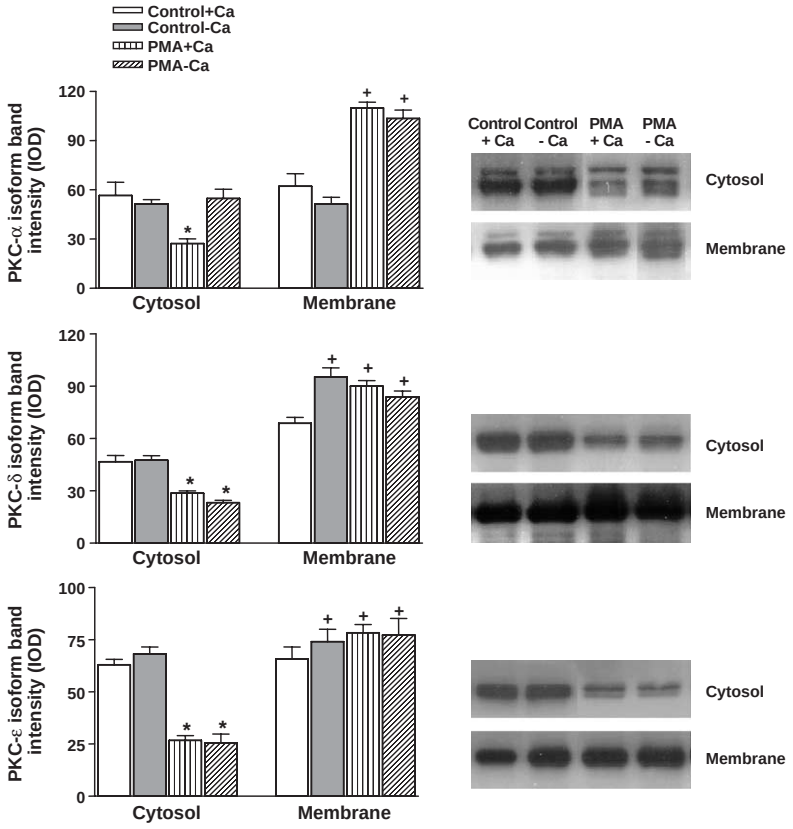


Fig. 4. Protein levels of PKCα ε and δ detected in IEC-18 cells incubated (60 min) with PMA (1 μM) either in the presence or absence of extracellular $Ca^{2+}$ (2 mM). At the end of the incubation period, cells were lysed and separated into membrane and cytosolic fractions by ultracentrifugation. PKC isoforms were assessed in each fraction by Western immunoblotting. Each histogram represents the mean (±standard error of the mean) of the densitometric analysis of 4–6 different blots obtained under each of the indicated experimental conditions. Representative blots are shown on the right-hand side of the figure. Asterisks (*) indicate significant reductions from respective controls in the presence or absence of added $Ca^{2+}$ whereas crosses (+) indicate significant increases from respective controls in the presence or absence of added $Ca^{2+}$.

test. $P<0.05$ was the accepted level of significance. Data are displayed as means (±standard error of the mean) with $N$ being the number of trials each on a different preparation of cells.

## 3. Results

PMA treatment resulted in a dose-dependent increase in PKC activity of IEC-18 cells (Fig. 1). Furthermore, the extent of cell injury as assessed by Trypan blue dye uptake or the release of acid phosphatase and *N*-acetyl-β-glucosaminidase was also increased in a dose-dependent fashion (Fig. 2). The increases were significant at 0.1 and 1.0 μM concentrations of PMA. Addition of the inactive phorbol ester, 4 αPMA, was ineffective at increasing Trypan blue dye uptake into these cells (Fig. 2).

Removal of $Ca^{2+}$ from the incubation medium significantly reduced the extent of cell injury especially at the highest dose of PMA examined. Furthermore, the extent of cell injury was reduced by the calcium channel antagonist, verapamil, and the intracellular $Ca^{2+}$ chelator, BAPTA-AM (Fig. 3). The reduction in cell viability was most marked at the 0.1 and 1.0 μM concentrations of PMA used here.

The extent of cellular injury induced by PMA was also reduced by pre-incubation with the pan-specific PKC inhibitor, GF 109203X (Fig. 3). Western immunoblotting determined that PMA treatment (1.0 μM) in the presence of extracellular $Ca^{2+}$ resulted in the activation of PKCα, PKCδ and PKCε as evidenced by a decrease in the protein level in the cytosol and an increase in the protein amount in the membrane fraction of the cell lysate (Fig. 4). Activation of each PKC isoform was not evident when $Ca^{2+}$ was removed from the medium.

Pre-incubation of cells with either pan-specific or isoform-specific inhibitors of PKC resulted in a reduction in the extent of cell damage in response to 1.0 μM PMA (Fig. 5). Inhibitors to PKCα (Gö 6976), PKCδ (rottlerin) and PKCε (Vε 1–2) each significantly reduced Trypan blue dye uptake in response to PMA.

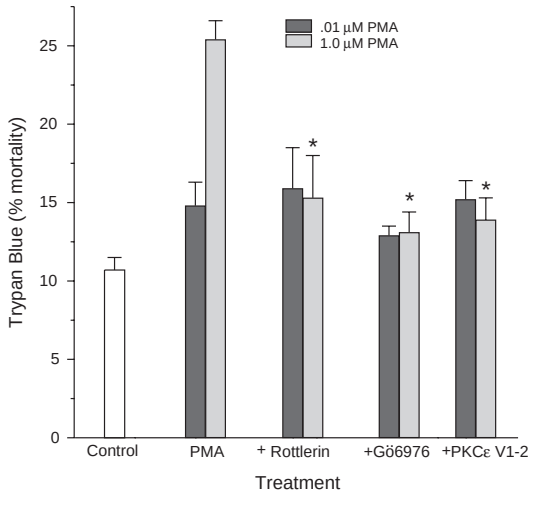


Fig. 5. The effect of PMA (0.01 and 1.0 μM) on cell integrity as assessed by Trypan Blue dye uptake. Some cells were preincubated with either the PKC δ antagonist, rottlerin (10 μM), the PKC α antagonist, Gö 6976 (1.0 μM) or the PKC ε translocation inhibitor Vε1–2 (4.0 μM). Data are expressed as means (±standard error of the mean; $N=6–7$). Asterisks (*) indicate significant reductions from the appropriate PMA control.

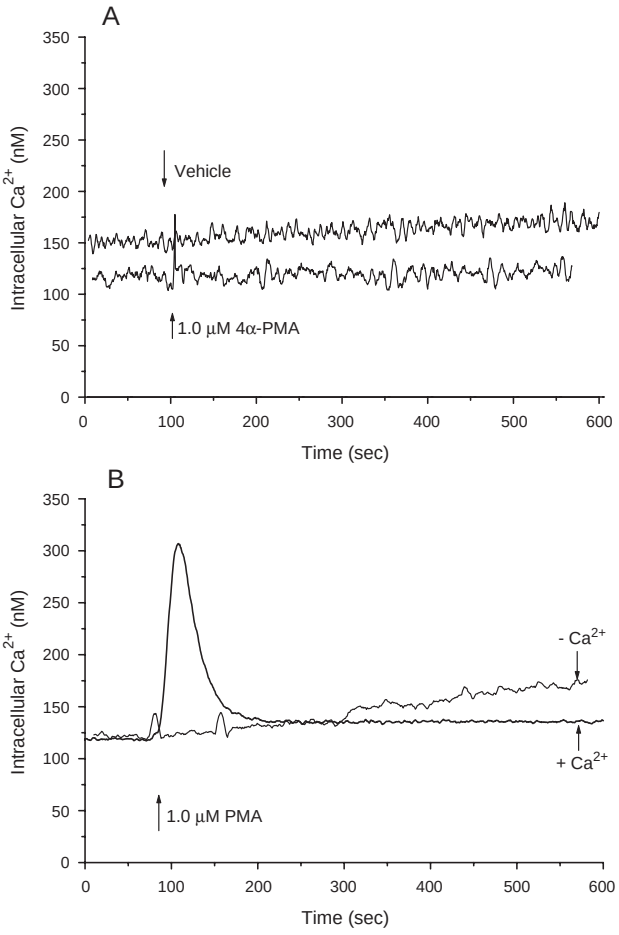


Fig. 6. (A) Intracellular $Ca^{2+}$ response to in vitro addition of vehicle (10 μl ethanol) or the negative control of PMA, 4 α PMA (1 μM). (B) Intracellular $Ca^{2+}$ response to the addition of PMA (1.0 μM) in the presence or absence of extracellular $Ca^{2+}$.

The intracellular $Ca^{2+}$ response to treatment with 1.0 μM PMA is shown in Fig. 6. Addition of PMA resulted in a rapid and transient increase in intracellular $Ca^{2+}$ levels. The levels returned to approximate pre-addition values within 100 s. This

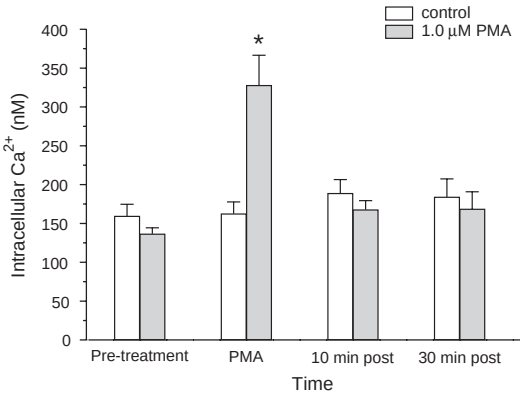


Fig. 7. The peak intracellular $Ca^{2+}$ response in control or PMA-treated (1.0 μM) IEC-18 cells. Intracellular $Ca^{2+}$ was determined fluorometrically. Cells were examined at 10 and 30 min after administration of PMA. Data are expressed as means (±standard error of the mean; $N=15$). Asterisks indicate significant increases from respective controls.

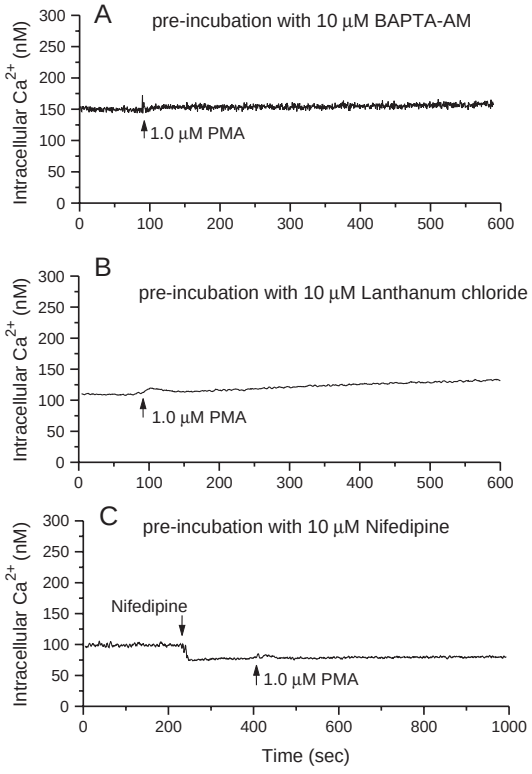


Fig. 8. Intracellular $Ca^{2+}$ response in IEC-18 cells after treatment with PMA (1.0 μM). Cells were pretreated with either the intracellular $Ca^{2+}$ chelator, BAPTA-AM (10 μM) (A), the calcium entry blocker, lanthanum chloride (10 μM ) (B), or the $Ca^{2+}$ channel antagonist, nifedipine (10 μM) (C).

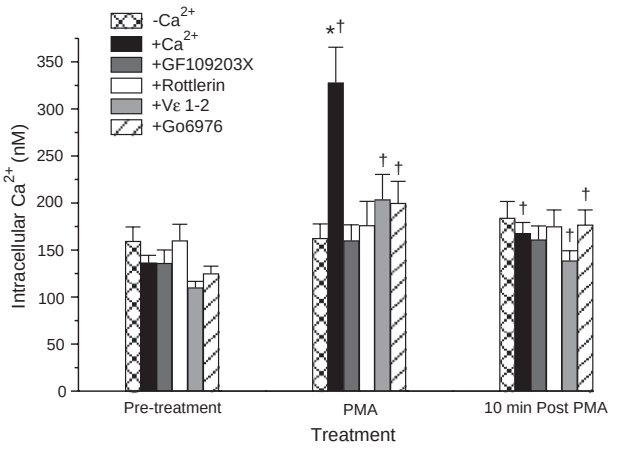


Fig. 10. Mean (±standard error of the mean; $N=7-8$) intracellular $Ca^{2+}$ in response to PMA (1.0 μM). $Ca^{2+}$ was calculated prior to, immediately upon addition of PMA and 10 min later. Asterisks (*) indicate significantly greater then the corresponding pretreatment response. All other responses were significantly smaller then the response to PMA in the presence of $Ca^{2+}$.

increase was not observed if $Ca^{2+}$ were withdrawn from the incubation medium. Treatment with 4α PMA did not affect the intracellular $Ca^{2+}$ response of the cells (Fig. 6). Post-treatment intracellular levels of $Ca^{2+}$ did not change up to 30 min after PMA addition (Fig. 7). Furthermore, the rapid, transient increase in intracellular $Ca^{2+}$ was eliminated after pre-incubation with the intracellular $Ca^{2+}$ chelator, BAPTA-AM as well as the $Ca^{2+}$ entry inhibitor, lanthanum chloride. Nifedipine treatment produced a reduction in intracellular $Ca^{2+}$ levels (Fig. 8).

The intracellular $Ca^{2+}$ response to 1.0 μM PMA was eliminated by pre-incubation with GF109203X and the PKCδ antagonist, rottlerin (Figs 9 and 10). However, pretreatment with either the PKCε translocation inhibitor, Vε 1–2 or the PKCα inhibitor, Gö 6976 reduced but did not eliminate the rapid

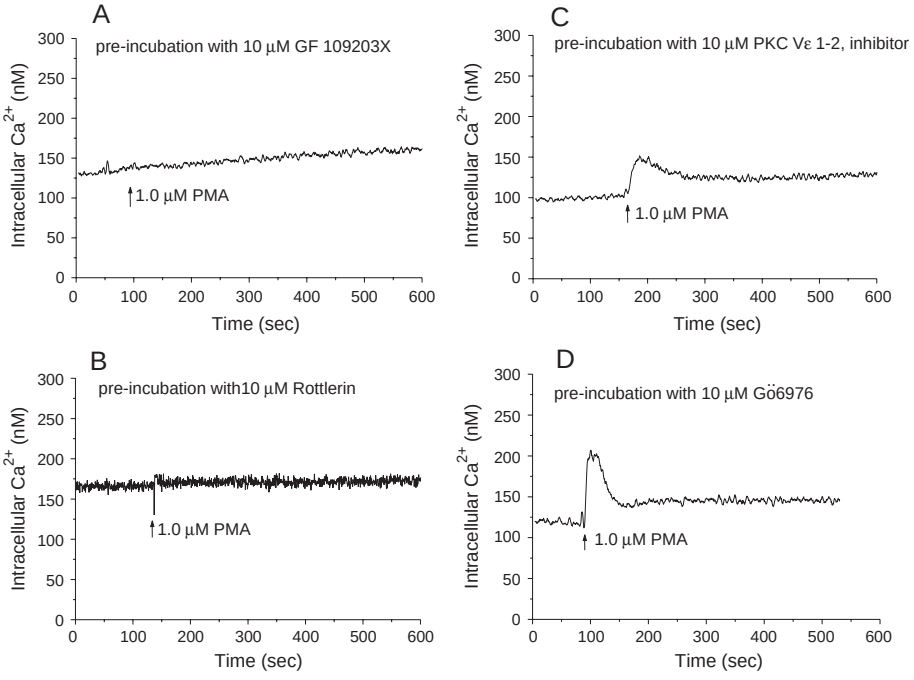


Fig. 9. Intracellular $Ca^{2+}$ response in IEC-18 cells after treatment with PMA (1.0 μM). Cells were pretreated with either the pan-specific PKC isoform inhibitor GF 109203X (A), the PKCδ inhibitor, rottlerin (B), the PKCε translocation inhibitor, Vε1–2 (D), or the PKCα antagonist, Gö 6976.

increase in cellular $Ca^{2+}$ levels. In each case the $Ca^{2+}$ levels had not returned to pretreatment levels by 10 min after addition of the inhibitor (Figs. 9 and 10).

## 4. Discussion

The results of the present study demonstrate that treatment of rat intestinal epithelial cells with the PKC activator, phorbol myristate acetate (PMA) produced an increase in the extent of damage. Previous studies from this and other laboratories have demonstrated that PMA treatment exerted damaging effects on a variety of tissues and cell types including the intestinal mucosa in vivo and on intestinal cells in vitro via the process of necrosis and apoptosis (Ye et al., 1993; Pavlakovic et al., 1995; Hetland, 1997; Jones et al., 1997; Brown et al., 1999; Tepperman et al., 2000a,b). Intraluminal instillation of phorbol ester has been found to induce ileal and colonic inflammation in experimental animals (Fretland et al., 1990; Overdahl et al., 1995). Furthermore, protein kinase C activators such as PMA have been shown to potentiate the extent of gastrointestinal cellular injury in response to hypoxia or high concentrations of nitric oxide (Carini et al., 2000; Tepperman et al., 2000a,b). While it is possible that the effect of PMA observed in this study are related to factors unrelated to PKC activation, the data presented here supports the notion that at least a portion of that response is the result of an increase in the activity of some PKC isoforms.

That the effects of PMA were mediated by PKC in the present study was proven by the demonstration that pan specific and selective PKC inhibitors would each decrease the extent of cell injury. Furthermore, PMA resulted in activation of PKC isoforms, specifically PKCα, PKCε and PKCδ as evidenced by their translocation from cytosol to membrane. Such translocation is regarded as an indicator of PKC activation (Haller et al., 1998; Miyawaki et al., 1996). Similar isoforms have been identified in intestinal tissues and cells both in animals and humans (O'Connell et al., 1997; Weller et al., 1999; Chang and Tepperman, 2001, 2003). The present data also suggest that these isoforms may play roles in maintaining cellular viability. Previous studies from this laboratory have demonstrated that PKCδ and ε were primarily associated with cytotoxicity in rat and human intestinal epithelial cells (Chang and Tepperman, 2001, 2003). Furthermore, results from other researchers confirm that in other cell types, these isoforms play similar roles in the regulation of integrity and viability (Jones et al., 1997; O'Connell et al., 1997; Sabri and Steinberg, 2003; Comalada et al., 2003; Leitges et al., 2001). In addition, we also observed that PKCα inhibition decreased the extent of cell injury. This is in contrast to what we have previously demonstrated using rat intestinal cells (Chang and Tepperman, 2001). Other studies have demonstrated that PKCα may have diverse effects on cell viability including antiapoptosis as well as cell damage depending upon the cell type and conditions of the experiment (Buchner et al., 1999; Yang et al., 2004). The reasons for the discrepancy in response to PKCα antagonism observed here and in our previous studies are not readily apparent but may be as a result of the differing experimental conditions.

The present study also demonstrated that PMA treatment of intestinal epithelial cells resulted in an increase in cellular $Ca^{2+}$. Previous work has demonstrated that $Ca^{2+}$ overload or perturbations in intracellular $Ca^{2+}$ compartmentalization can cause cytotoxicity and trigger either apoptosis or necrosis (Trump and Berezesky, 1995; Orrenius et al., 2003). Pertinent to the intestine, studies using epithelial cellular monolayers have shown that the damaging actions of certain noxious agents were mediated via changes in intracellular $Ca^{2+}$ and that the cytoprotective actions of agents such as EGF or prostaglandins occurs in part, via stabilization of cellular $Ca^{2+}$ levels (Banan et al., 1999, 2001). The present data also demonstrate that removal of $Ca^{2+}$ from the incubation medium reduced the extent of cellular damage in response to PMA and that blockade of $Ca^{2+}$ entry or buffering intracellular $Ca^{2+}$ also ameliorated the response to PMA. Together, these previous and current data support the notion that the PKC activator, PMA affects cell integrity via a change in intracellular $Ca^{2+}$.

A number of studies have provided evidence that PKC affects $Ca^{2+}$ levels in a number of cell types and does so via induction of specific $Ca^{2+}$ channels (Reader et al., 1999; Hu et al., 2000; Andrews et al., 2002; Gönczi et al., 2002; Ma et al., 2002; Qui et al., 2003). The present data suggest such a mechanism since $Ca^{2+}$ channel blockers such as nifedipine and lanthanum reduced the damaging actions of PMA. Furthermore, PMA treatment resulted in a transient but significant increase in intracellular $Ca^{2+}$, an effect which was eliminated by PKC antagonism. Other investigators have demonstrated that PKC activation resulted in an increase in cytosolic $Ca^{2+}$ in a number of tissues and cell types and that this $Ca^{2+}$ may arise from either or both extracellular or intracellular stores (Ma et al., 2002; Reader et al., 1999).

The specific PKC isoform mediating the cellular $Ca^{2+}$ and integrity responses observed here appears to reside primarily with PKCδ. Blockade of PKCδ with rottlerin eliminated the spike in cellular $Ca^{2+}$ response seen with PMA. The same dose of rottlerin also reduced the extent of cell injury. Furthermore, PKCδ was activated by PMA treatment. While PMA also resulted in the translocation of PKCα and PKCε, specific antagonism of their actions did not eliminate the transient increase in cellular $Ca^{2+}$. Thus, the cell damaging abilities of these other isoforms appear to operate via a non-$Ca^{2+}$-requiring pathway.

Previous work has suggested that prolonged increases in cellular $Ca^{2+}$ levels are necessary to invoke gastrointestinal cell damage (Tepperman et al., 1991). However, in the present study we observed a transient increase in $Ca^{2+}$ levels suggestive of a signaling response. $Ca^{2+}$ returned rapidly to near pretreatment levels although, in some cases, they

remained only slightly elevated even 30 min after PMA administration. Recent work suggests that such transient effects are crucial to a number of cellular responses including cell injury through the regulation of a number of $Ca^{2+}$-dependent enzymes such as phospholipases or may influence $Ca^{2+}$ release from intracellular stores (Orrenius et al., 2003). Furthermore, there is evidence that such $Ca^{2+}$ transients in response to a variety of challenges are directly associated with cytotoxicity in a number of cell types (Bakondi et al., 2003; Le et al., 1995). The downstream response to the transient $Ca^{2+}$ increase observed here is currently under investigation in this laboratory.

In summary, we have demonstrated that PMA damages rat intestinal epithelial cells and that this damage is mediated via PKC. The α, ε and δ isoforms appear to be involved in this response. The PKCδ isoform appears to mediate its response on intestinal cellular integrity via an event involving intracellular $Ca^{2+}$ signaling as evidenced by a transient $Ca^{2+}$ spike in response to PMA which was eliminated by inhibition of the δ isoform. Similarly, inhibition of PKCδ activity also reduced the cytotoxic actions of PMA. Thus these data suggest that PKCδ activation results in intestinal cellular damage via a transient increase in cellular $Ca^{2+}$.

## Acknowledgements

This research was funded by a grant from the Canadian Institute for Health Research, MOP 6426.

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