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Uncoupling of bradykinin-induced phosphoinositide hydrolysis and Ca^{2+} mobilization by phorbol ester in canine cultured tracheal epithelial cells

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1 Regulation of the increase in inositol phosphates (IPs) production and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$ by protein kinase C (PKC) was investigated in canine cultured tracheal epithelial cells (TECs). Stimulation of TECs by bradykinin (BK) led to IPs formation and caused an initial transient $[Ca^{2+}]_i$ peak in a concentration-dependent manner.

2 Pretreatment of TECs with phorbol 12-myristate 13-acetate (PMA, 1 μM) for 30 min attenuated the BK-induced IPs formation and Ca^{2+} mobilization. The maximal inhibition occurred after incubating the cells with PMA for 2 h.

3 The concentrations of PMA that gave half-maximal (pEC_{50}) inhibition of BK-induced IPs accumulation and an increase in $[Ca^{2+}]_i$ were 7.07 M and 7.11 M, respectively. Inactive phorbol ester, 4α-phorbol 12,13-didecanoate at 1 μM, did not inhibit these responses. Prior treatment of TECs with staurosporine (1 μM), a PKC inhibitor, inhibited the ability of PMA to attenuate BK-induced responses, suggesting that the inhibitory effect of PMA is mediated through the activation of PKC.

4 In parallel with the effect of PMA on the BK-induced IPs formation and Ca^{2+} mobilization, the translocation and down-regulation of PKC isozymes were determined. Analysis of cell extracts by Western blotting with antibodies against different PKC isozymes revealed that TECs expressed PKC-α, βI, βII, γ, δ, ε, θ and ζ. With PMA treatment of the cells for various times, translocation of PKC-α, βI, βII, γ, δ, ε and θ from cytosol to the membrane was seen after 5 min, 30 min, 2 h, and 4 h treatment. However, 6 h treatment caused a partial down-regulation of these PKC isozymes. PKC-ζ was not significantly translocated and down-regulated at any of the times tested.

5 Treatment of TECs with 1 μM PMA for either 30 min or 6 h did not significantly change the K_D and B_{max} receptor for BK binding (control: $K_D = 1.7 \pm 0.3$ nM; $B_{max} = 50.5 \pm 4.9$ fmol/mg protein), indicating that BK receptors are not a site for the inhibitory effect of PMA on BK-induced responses.

6 In conclusion, these results suggest that activation of PKC may inhibit the phosphoinositide hydrolysis and consequently attenuate the $[Ca^{2+}]_i$ increase or inhibit independently both responses to BK. The translocation of pKC-α, βI, βII, δ, ε, γ, and θ induced by PMA caused an attenuation of BK-induced IPs accumulation and Ca^{2+} mobilization in TECs.

Keywords: Phorbol ester; bradykinin; protein kinase C; inositol phosphates; Ca^{2+}; tracheal epithelial cells

Introduction

In the airways, bradykinin (BK) has been shown to cause bronchoconstriction, pulmonary and bronchial vasodilatation, mucus secretion and microvascular leakage (Barnes, 1992). Most of biological actions of BK are mediated through the activation of BK B_2 receptors. In many cell types, including the neuroblastoma-glioma hybrid NG108-15 (Osugi *et al*., 1987), glioma C6-4-2 (Reiser *et al*., 1990), human astrocytoma cell line D384 (Balmforth *et al*., 1992), and tracheal smooth muscle cells (March & Hill, 1992; Yang *et al*., 1994a), BK receptors activate phospholipase C (PLC)-mediated phosphoinositide (PI) hydrolysis in the plasma membrane. The resultant increase in IP_3 releases Ca^{2+} from internal stores in several types of cells including the NG108-15 (MacNicol & Schulman, 1992), endothelial cell (Buchan & Martin, 1991), NCB-20 cells (Garristen & Cooper, 1992), and tracheal smooth muscle cells (Marsh & Hill, 1993; Yang *et al*., 1994b). Recently, we have reported that BK induces an increase in PI hydrolysis in canine cultured tracheal epithelial cells (TECs) which appears to be mediated *via* activation of the B_2 receptors (Luo *et al*., 1996). The existence of B_2 receptor subtype in TECs was further confirmed by a direct binding assay (Luo *et al*., 1996). Thus, in terms of second messenger generation, regulation of tracheal epithelial functions may be mediated by IP_3-induced Ca^{2+} mobilization from intracellular stores.

In addition, activation of PI hydrolysis by agonists also leads to the formation of diacylglycerol (DAG), which activates the important regulatory enzyme protein kinase C (PKC) (Nishizuka, 1992). Several studies have suggested that the PI-Ca^{2+} signalling system is negatively regulated by PKC activation in different cell types, by showing a decrease in the PI hydrolysis (Daykin *et al*., 1993; Pfeilschifter *et al*., 1989; Abdel-Latif, 1989; Pearce *et al*., 1988), intracellular Ca^{2+} mobilization (McCarthy *et al*., 1989; Buchan & Martin, 1991), or both responses (Sena *et al*., 1996; Yang *et al*., 1997). These reports suggest that there are many candidates for target sites of PKC as a negative feedback regulator in the PI-Ca^{2+} signalling transduction in these cells.

PKC consists of a family of serine/threonine kinases of fundamental importance in signal transduction in diverse

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biological systems (Nishizuka, 1992). To date, 12 isozymes with distinct enzymological characteristics and differential tissue expression and intracellular localization have been identified (Dekker & Parker, 1994; Nishizuka, 1992). These differences, together with the varied consequences of PKC activation in the same cells suggest that individual isozymes have distinct and specialized functions in cell signalling. As the phorbol esters are known to activate PKC, mimicking the physiological activator DAG, PKC is thought to mediate phorbol ester-induced biological responses (Nishizuka, 1992). Translocation of PKC activity from the cytosolic to the membrane fractions of the cells is considered the first step in activation of the enzyme (Zidovetzki & Lester, 1992). This activation is eventually terminated by the subsequent proteolytic degradation (down-regulation) of PKC (Young *et al.*, 1987). Although cells generally express more than one PKC isozyme, little is known concerning their individual roles. Therefore, the translocation and down-regulation of PKC isozymes involved in the modulation of BK-induced PI hydrolysis and Ca^{2+} mobilization needed to be investigated in TECs.

Up to date, the precise molecular and biochemical mechanisms of phorbol esters related to inhibition of BK-mediated physiological responses in tracheal epithelium are not clear. To elucidate the contribution of PKC to cellular function, the purpose of the present study was to investigate the role of the activating PKC pathway in the modulation of BK receptors, PI breakdown, change in $[Ca^{2+}]_i$, and the involvement of PKC isozymes in the inhibition of BK-mediated responses to TECs by phorbol esters. These data conform well with the role of PKC as a negative feedback regulator of the inositol lipid signalling pathways in these cells.

Methods

Materials

Dulbecco's modified Eagle's medium (DMEM)/Ham's nutrient mixture F-12 (F-12) medium and foetal bovine serum (FBS) were purchased from J.R. Scientific (Woodland, CA, U.S.A.). Myo-[^{3}H]inositol (18 Ci mmol^{-1}) was from Amersham (Buckinghamshire, England). Fura-2/AM was from Molecular Probes Inc (Eugene, OR, U.S.A.). Rabbit polyclonal antibodies raised against peptide sequences unique to PKC-α, βI, βII, γ, δ, ε, η, θ, ζ, ι, λ and μ were from Santa Cruz (Santa Cruz, CA, U.S.A.). Enzymes and other chemicals were from Sigma Co (St. Louis, MO, U.S.A.).

Animals

Mongrel dogs, 10–20 kg, both male and female, were purchased from a local supplier. Dogs were housed indoors in the animal facilities under automatically controlled temperature and light cycle conditions and fed standard laboratory chow and tap water *ad libitum*. Dogs were anaesthetized with ketamine (20 mg/kg, intramuscularly) and pentobarbitone (30 mg/kg, intravenously). The tracheae were surgically removed.

Isolation and culture of tracheal epithelial cells

Cells were isolated essentially as described by Wu *et al.* (1985). The trachea was cut longitudinally through the cartilage rings, and strip epithelium was pulled off the submucosa, rinsed with phosphate buffered saline (PBS) containing 5 mM dithiothreitol, and digested with 0.05% (w/v) protease XIV in PBS at 4°C for 24 h; after vigorous shaking of the strips at room temperature, 5 ml of foetal bovine serum (FBS) was added to terminate the digestion. The released cells were collected and washed twice with 50% Dulbecco's modified Eagle's medium (DMEM) and 50% Ham's nutrient F-12 medium that contained 5% FBS, (v/v), nonessential amino acids, penicillin (100 u ml^{-1}), streptomycin (100 μg/ml), gentamicin (50 μg ml), and fungizone (2.5 μg/ml). Cell number was counted and diluted with DMEM/F-12 to 2×10^6 cells/ml. The cells were plated onto (0.5 ml/well) 24-well, (1 ml/well) 12-well or (2 ml/well) 6-well culture plates containing glass coverslips coated with collagen for receptor binding assay, IPs accumulation, and $[Ca^{2+}]_i$ measurement, respectively. The culture medium was changed after 24 h and then changed every 2 days.

In order to characterize the isolated and cultured TECs, an indirect immunofluorescent staining was performed as described by O'Guin *et al.* (1985) using AE1 and AE3 mouse monoclonal antibodies and fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG.

Accumulation of inositol phosphates

Effect of BK on the hydrolysis of PI was assayed by monitoring the accumulation of [^{3}H]IPs as described by Yang *et al.* (1994a). Cultured TECs were incubated with 5 μCi/ml of myo-[2-^{3}H]-inositol at 37°C for 24 h. TECs were washed two times with and incubated in Krebs-Henseleit buffer (KHS, pH 7.4) containing (in mM) 117 NaCl, 4.7 KCl, 1.1 $MgSO_4$, 1.2 KH_2PO_4, 20 $NaHCO_3$, 2.4 $CaCl_2$, 1 glucose, 20 HEPES and 10 LiCl at 37°C for 30 min. After BK added at the concentration indicated, incubation was continued for another 60 min in the presence of 2 μM indomethacin and 10 μM phosphoramidon. Reactions were terminated by addition of 5% perchloric acid followed by sonication and centrifugation at $3000 \times g$ for 15 min.

The perchloric acid soluble supernatants were extracted four times with ether, neutralized with potassium hydroxide, and applied to a column of AG1-X8, formate form, 100–200 mesh (Bio-Rad). The resin was washed successively with 5 ml of water and 5 ml of 60 mM ammonium formate-5 mM sodium tetraborate to eliminate free [^{3}H]-inositol and glycerophosphoinositol, respectively. Total IPs were eluted with 5 ml of 1 M ammonium formate-0.1 M formic acid. The amount of [^{3}H]-IPs was determined in a radiospectrometer (Beckman LS5000TA, Fullerton, CA, U.S.A.).

Measurement of intracellular Ca^{2+} level

$[Ca^{2+}]_i$ was measured in confluent monolayers with the calcium-sensitive dye fura-2/AM as described by Grynkiewicz *et al.* (1985). Upon confluence, the cells were cultured in DMEM/F-12 with 1% FBS one day before measurements were made. The monolayers were covered with 1 ml of DMEM/F-12 with 1% FBS containing 5 μM fura-2/AM and was incubated at 37°C for 45 min. At the end of the period, the coverslips were washed twice with the physiological buffer solution containing (mM): 125 NaCl, 5 KCl, 1.8 $CaCl_2$ 2 $MgCl_2$, 0.5 NaH_2PO_4, 5 $NaHCO_3$, 10 HEPES and 10 glucose, pH 7.4. The cells were incubated in PBS for further 30 min to complete dye de-esterification. The coverslip was inserted into a quartz cuvette at an angle of approximately 45° to the excitation beam and placed in the temperature controlled holder of a Hitachi F-4500 spectrofluorometer (Tokyo, Japan). Continuous stirring was achieved with a magnetic stirrer.

Fluorescence of Ca^{2+}-bound and unbound fura-2 were measured by rapidly alternating the dual excitation wavelengths between 340 and 380 nm and electronically separating the resultant fluorescence signals at emission wavelength 510 nm. The autofluorescence of each monolayer was subtracted from the fluorescence data. The ratios (R) of the fluorescence at the two wavelengths are computed and used to calculate changes in $[Ca^{2+}]_i$. The ratios of maximum (R_{max}) and minimum (R_{min}) fluorescence of fura-2 were determined by adding ionomycin (10 μM) in the presence of PBS containing 5 mM Ca^{2+} and by adding 5 mM EGTA at pH 8 in a Ca^{2+}-free PBS, respectively. The K_d of fura-2 for Ca^{2+} was assumed to be 224 nM (Grynkiewicz *et al*., 1985).

[^{3}H]-BK binding assay

For detecting the effect of PMA on BK receptor density or affinity of TECs, [^{3}H]-BK was used as a ligand. Binding assays were performed with confluent TECs on 24-well culture plates, with or without PMA treatment in DMEM/F-12 containing 1% FBS for 30 min or 6 h prior to the binding experiments, as described (Yang *et al*., 1995; Luo *et al*., 1996). Culture medium was removed and 1 ml of binding buffer containing (mM): 20 HEPES, pH 7.4, 17 NaCl, 5.4 KCl, 0.44 KH_2PO_4, 0.63 $CaCl_2$, 0.21 $MgSO_4$, 0.34 Na_2HPO_4, 110 N-methylglucamine, 0.1% (w/v) BSA and 2 bacitracin, was added to each well. Cells were equilibrated on ice for 10 min, after which the binding buffer was replaced with 0.25 ml of binding buffer containing the appropriate concentration of [^{3}H]-BK in the absence or presence of unlabelled BK (10 μM). After 4 h incubation at 4°C, the binding buffer was removed and cells were washed three times with 2 ml of binding buffer at 4°C. Cells were suspended in 0.25 ml of 0.1 N NaOH and counted in a radiospectrometer. The amount of specific binding was calculated as the total binding minus the binding in the presence of 10 μM unlabelled BK. Total receptor density (B_{max}) and dissociation constant (K_D) were calculated by Ligand program, as described previously (Yang *et al*., 1995). Protein concentration was measured by the method of Bradford (1976).

Preparation of cell extracts and immunoblot analysis of PKC isozymes

Cells were treated with 1 μM PMA in DMEM/F-12 medium containing 1% FBS for various periods. Dimethylsulphoxide (0.1%) was added to control cells for 6 h. The cells were then rapidly washed with ice-cold PBS, scraped and collected by centrifugation at 1000 × *g* for 10 min. The preparation of cell extracts and immunoblot analysis were performed as previously described (Yang *et al*., 1997). Briefly, the collected cells were lysed in ice-cold homogenization buffer containing (mM): 20 Tris-HCl, pH 7.4; 1 dithiothreitol, 5 EGTA, 2 EDTA, 10% glycerol, 0.5 PMSF, and 5 μg/ml leupeptin. The homogenates were centrifuged at 45,000 × *g* for 1 h at 4°C to yield the supernatants (cytosolic fractions) and pellets (membrane fractions). The protein concentration was determined by the method of Bradford (1976). Samples from these two fractions (100 μg protein) were denatured and subjected to SDS–PAGE using a 10% running gel. Proteins were transferred to nitrocellulose membrane and the membrane was incubated successively at room temperature with 0.1% dry milk in TTBS for 1 h, with rabbit antibodies specific for PKC isoforms (α, βI, βII, γ, δ, ε, θ, η, ι, λ, μ and ζ, 1 : 1000 dilution) for 1 h, and with anti-rabbit horseradish peroxidase antibody (1 : 2000) for 1 h. Following each incubation, the membrane was washed extensively with TTBS. The immunoreactive bands detected by ECL reagents were developed by Hyperfilm-ECL (Amersham International). All these antibodies could be used to detect PKC isozymes in canine cortex, used as a positive control (data not shown).

Analysis of data

Concentration-effect curves were fitted by Prizm Program (GraphPad, San Diego, CA, U.S.A.). EC_{50} values were estimated by the same program and expressed as the mean pEC_{50} (M, unless stated otherwise) ± s.e.mean. The data were expressed as the means ± s.e.mean with statistical comparisons based on a two-tailed Student's *t*-test at a $P < 0.01$ level of significance.

Results

Effect of PMA on BK-stimulated IPs accumulation

To determine whether PKC activation by phorbol ester caused a change in BK-induced IPs accumulation, the concentration-response relationship for PMA inhibition was examined in cultured TECs. As shown in Figure 1, PMA was very potent that significantly inhibited the BK-induced IPs accumulation at 3 nM, consistent with previous reports in several cell types (Daykin *et al*., 1993; Sena *et al*., 1996; Yang *et al*., 1997). The

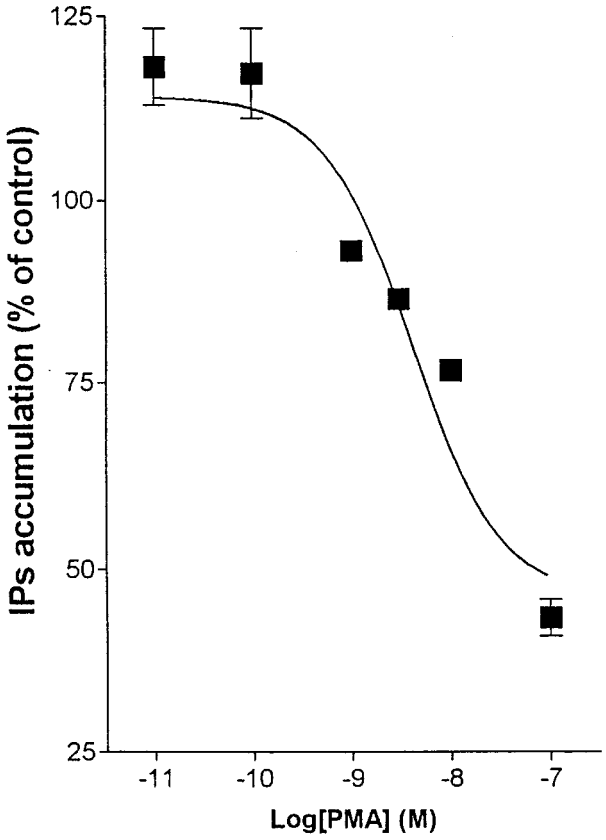

Figure 1 Concentration dependence of PMA inhibition of BK-stimulated IPs accumulation. Cells prelabelled with [^{3}H]-inositol were washed with KHS, treated with various concentrations of PMA (0.1 nM – 0.1 μM) for 30 min and then exposed to BK (10 μM) for 60 min. The accumulation of IPs was determined, as described under Methods. Values are expressed as the mean ± s.e.mean from three separate experiments determined in triplicate.

inhibitory effect of PMA was concentration dependent, PMA induced half-maximal (pEC_{50}) and maximal inhibition of BK-stimulated IPs accumulation at 7.07 M and 0.1 μM, $n=3$, respectively, but had no effect on the basal levels of IPs accumulation at any of the concentrations tested. The inhibitory action of PMA appeared to result from a decrease in the maximal response and a shift to the right in the concentration-effect curve for BK-induced IPs accumulation (Figure 2). The half maximal value (pEC_{50}) for the stimulatory effect of BK on IPs accumulation in the presence of 1 nM PMA (7.65 M) was close to that of control cells (7.73 M).

Effect of PKC inhibitor on IPs response to BK

Furthermore, TECs were treated with phorbol esters (1 μM) and then stimulated with 10 μM BK in the continuous presence of phorbol esters (Figure 3). Treatment of TECs with 1 μM PMA for 30 min led to an inhibition of BK-stimulated IPs accumulation by 45% ($P<0.001$, as compared with non-treated cells). The inactive phorbol ester, 4α-phorbol 12,13-didecanoate (4α-PDD, 1 μM), did not attenuate BK-induced IPs accumulation (Figure 3). When TECs were pretreated with staurosporine (1 μM), a potent PKC inhibitor, the inhibitory effect of PMA on BK-stimulated IPs accumulation was significantly reversed ($P<0.001$, $n=3$, as compared with PMA-treated cells). However, it should be noticed that staurosporine was not specific to PKC, especially at the concentrations used (1 μM), and may also inhibit other kinases.

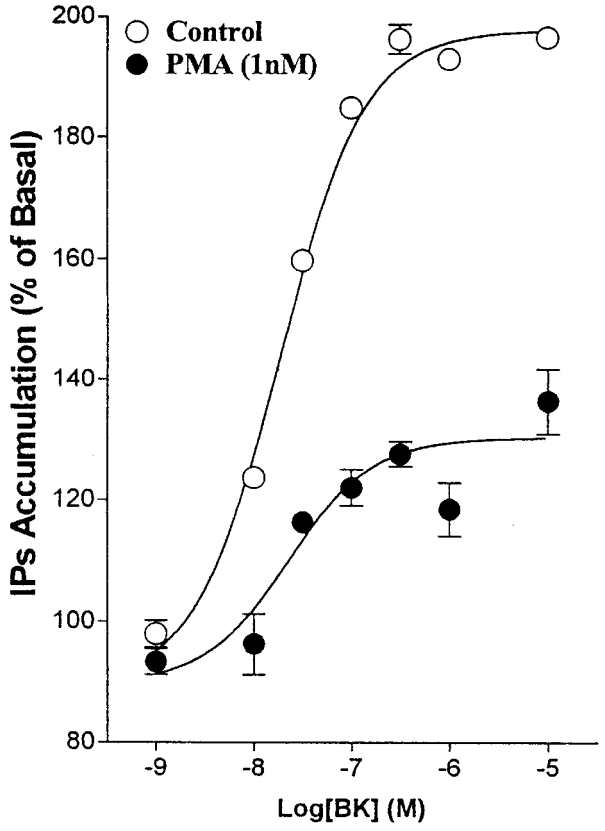

Figure 2 Effect of PMA on concentration-effect curves for BK-stimulated IPs accumulation. Cells prelabelled with [^{3}H]-inositol were washed with KHS, and incubated in the absence (control) or presence of 1 nM PMA for 30 min and then exposed to various concentrations of BK for 60 min. The accumulation of IPs was determined, as described under Methods. Values are expressed as the mean$\pm$ s.e.mean from three separate experiments determined in triplicate.

Effect of PMA on the $[Ca^{2+}]_i$ response to BK

As IP_3 is the second messenger involved in the mobilization of Ca^{2+} from intracellular stores (Yang *et al*., 1994b), one would expect that the mobilization of Ca^{2+} in response to BK may be also inhibited by activation of PKC. The effect of BK, a stimulant of epithelial tissues that operates through calcium-dependent pathway, on $[Ca^{2+}]_i$ was studied in cultured TECs. To examine a possible role of PKC in the regulation of $[Ca^{2+}]_i$ in TECs, we tested the effect of PMA on BK-induced changes in $[Ca^{2+}]_i$. Figure 4A depicts tracings of BK-induced $[Ca^{2+}]_i$ changes in TECs following treatment with various concentrations of PMA for 30 min. In control cells, the resting level of $[Ca^{2+}]_i$ was 222 ± 10 nM ($n=6$). Addition of BK (10 μM) resulted in a rapid and transient elevation of $[Ca^{2+}]_i$ to 546 ± 60 nM within 30 s. As shown in Figure 4B, prior treatment of TECs with PMA followed by subsequent exposure to 10 μM BK markedly inhibited the Ca^{2+} mobilization. PMA induced half-maximal (pEC_{50}) and maximal inhibition of BK-stimulated $[Ca^{2+}]_i$ changes at 7.11 M and 1 μM, ($n=6$), respectively (Figure 4B). The inhibitory effect of PMA resulted from a decrease in the maximal response and a shift to the right in the concentration effect curve for BK-induced $[Ca^{2+}]_i$ (Figure 5). The half-maximal value (pEC_{50}) for the stimulatory effect of BK on Ca^{2+} mobilization in the presence of 3 nM PMA (7.83 M) was close to that of control cells (7.80 M).

Effect of PKC inhibitors on $[Ca^{2+}]_i$

We then examined the effect of staurosporine, a PKC inhibitor, on PMA-induced inhibition of $[Ca^{2+}]_i$ response to BK. As shown in Figure 6, TECs were treated with phorbol esters (1 μM) and then stimulated with 10 μM BK. Treatment of TECs with 1 μM PMA for 30 min inhibited the BK-stimulated $[Ca^{2+}]_i$ response by 62% ($P<0.001$, $n=8$, as compared with the control). When TECs were preincubated with staurosporine (1 μM), the inhibitory effect of PMA on BK-stimulated Ca^{2+} mobilization was reversed, although pretreatment with these PKC inhibitors alone did not affect the $[Ca^{2+}]_i$ response to BK. It is clear that this inhibition could be prevented by the PKC inhibitors. The inactive phorbol ester

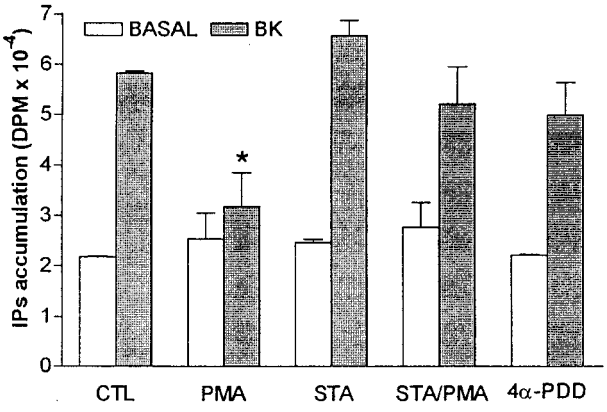

Figure 3 Effects of phorbol ester and staurosporine treatment on BK-stimulated IPs accumulation in cultured TECs. [^{3}H]-inositol-labelled TECs were pretreated with PMA (1 μM), staurosporine (STA, 1 μM), STA plus PMA, or 4α-PDD (1 μM) for 30 min and then exposed to BK (10 μM) for 60 min. The accumulation of IPs was determined, as described under Methods. Values are expressed as the mean$\pm$s.e.mean from three separate experiments determined in triplicate. CTL, without treatment with PMA or STA, $*P<0.001$, as compared with control cells stimulated with BK.

4α-PDD (1 μM) did not block BK-induced Ca^{2+} mobilization (data not shown).

Translocation of PKC isozymes from cytosol to membrane in response to PMA

Several studies show that activation of cells with PMA results in a translocation of PKC from cytosol to membrane, and that this translocation differs between the various isozymes. To determine which PKC isozyme is associated with the regulation of BK-induced responses, the expression of PKC isozymes in cultured TECs was characterized by a Western blot analysis. Immunoblot analysis of whole cell extracts by using antibodies against PKC-α, βI, βII, γ, δ, ε, η, θ, ζ, ι, λ and μ and revealed the presence of PKC-α, βI, βII, δ, ε, θ and ζ in cultured TECs (data not shown). In Figure 7A, we show the effect of PMA treatment for various periods on the translocation of PKC-βI from cytosol to membrane fractions, as revealed by immunoblots. The PKC-βI antiserum bound protein revealed as a doublet in TECs. The use of polyclonal and not monoclonal antibodies in our studies would mean that minor epitope specific differences would not affect the interpretation of our data. Furthermore, to evaluate the effect of PMA treatment on the levels of these detectable PKC isozymes in TECs, cell extracts were subjected to immunoblotting with specific antibodies to various PKC isozymes. Typical autoradiographs collected by scanning are presented in Figure 7B. A short (5 min, 30 min, and 2 h) exposure of the TECs to 1 μM PMA resulted in a rapid translocation of PKC-α, βI, βII, δ, ε, γ and θ from cytosolic to membrane fractions, but not PKC-ς and this was sustained over the levels in control for 4 h. When the cells were exposed to PMA for 6 h, all PKC isozymes translocated from the cytosol to the membrane underwent partial down-regulation. Moreover, PKC inhibitors staurosporine and GF109203X did not significantly block the translocation of these isozymes induced by PMA (data not shown).

Correlation between the effect of PMA on the BK-stimulated responses and translocation of PKC isozymes to membrane

The effect of PMA on BK-induced responses and the translocation and down-regulation of PKC isozymes after various periods of treatment is shown in Figure 8. Pretreatment of TECs with PMA for 30 min resulted in a rapid inhibition of the BK-induced IPs accumulation and Ca^{2+} mobilization by 27±5% and 58±4%, respectively (Figure 8). Following preincubation, maximal inhibition of BK-stimulated IPs accumulation and Ca^{2+} mobilization by 38±5% and 78±6%, respectively, was obtained within 2 h. After longer periods of PMA treatment, the inhibitory effect of PMA on BK responses was still remained after 6 h (Figure 8). The long-term treatment with PMA was not observed since TECs were detached from coverslips. Under similar experimental conditions, the results from scanning of autoradiographs from repetitive experiments were pooled (Table 1), the loss from the cytosolic fraction expressed as a percentage of the level found

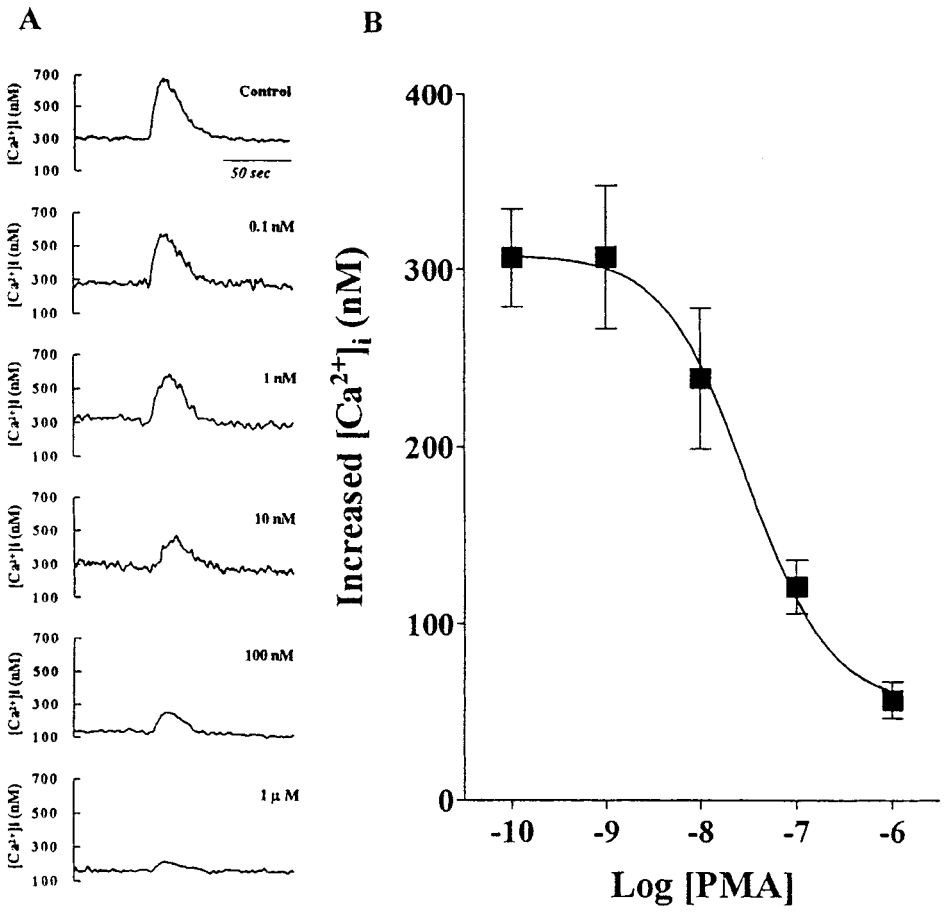

Figure 4 Concentration dependence of PMA inhibition of BK-stimulated $[Ca^{2+}]_i$ change in cultured TECs. A: Cells were incubated with various concentrations of PMA as indicated for 30 min. $[Ca^{2+}]_i$ was measured when BK (10 μM) was added. Data expressed as the mean±s.e.mean from eight separate experiments are shown in (B).

in controls (no PMA treatment), and the gain in the membrane fraction expressed as a percentage over the level seen in controls. As shown in Table 1, translocation of PKC-δ was the most profound among these PKC isoforms detected in TECs. PMA treatment for 5 min, the levels of PKC-α, βI, βII, δ, ε, γ and θ were significantly increased over the level seen in controls by 194, 221, 232, 349, 121, 113 and 171%, respectively ($P<0.01$). The maximal increases of PKC isozymes in membrane fraction were 197, 226, 268, 416, 157, 115 and 171%, respectively, when TECs were treated with PMA for various times (5 min to 2 h). A down-regulation of these PKC isoforms close to those in controls was seen after 6 h treatment with PMA. In cytosolic fraction, at 5 min of PMA treatment, the loss of PKC-α, βI, βII, δ, ε, γ and θ from this fraction was 72, 70, 89, 72, 63, 58 and 79%, respectively. The maximal loss in the cytosol was reached by 4 h, with no further depletion of cytosolic levels at later times.

Effect of PMA on the density and affinity of the BK receptors

Long-term incubation with phorbol esters has been reported to cause a down-regulation of agonist receptors in some types of cells (Leeb-Lundberg *et al*, 1985). To determine whether the observed PMA-induced inhibition of responses stimulated by BK represented an actual decrease in the number of binding sites or change in the K_D of the BK receptors for [^{3}H]-BK, complete saturation binding curves were obtained for parallel control cultures and cultures incubated with 1 μM PMA for either 30 min or 6 h (Table 2). Scatchard analysis showed that control cells had a single class of binding sites with a B_{max} of 50.5 ± 4.9 fmol/mg protein and a K_D of 1.7 ± 0.3 nM. No significant difference in the B_{max} and K_D of the BK receptors was obtained with the cells treated with PMA for 30 min or 6 h, as compared to control cells (Table 2).

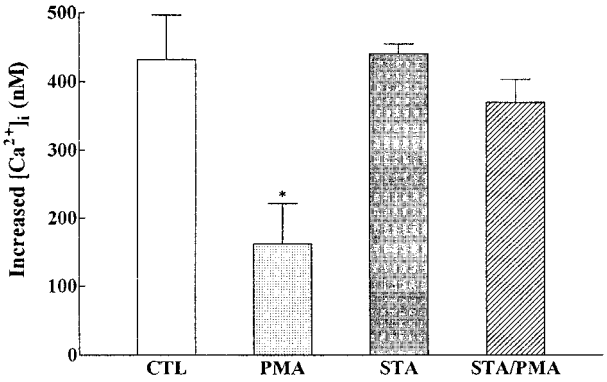

Figure 6 Effects of phorbol esters and PKC inhibitors on BK-induced $[Ca^{2+}]_i$ in cultured TECs. Cells were incubated with PMA (1 μM), staurosporine (STA, 1 μM), or STA plus PMA, at 37°C for 30 min. $[Ca^{2+}]_i$ was measured when BK (10 μM) was added. Values are expressed as the means$\pm$s.e.mean from eight separate experiments. $^*P<0.001$, as compared with control cells.

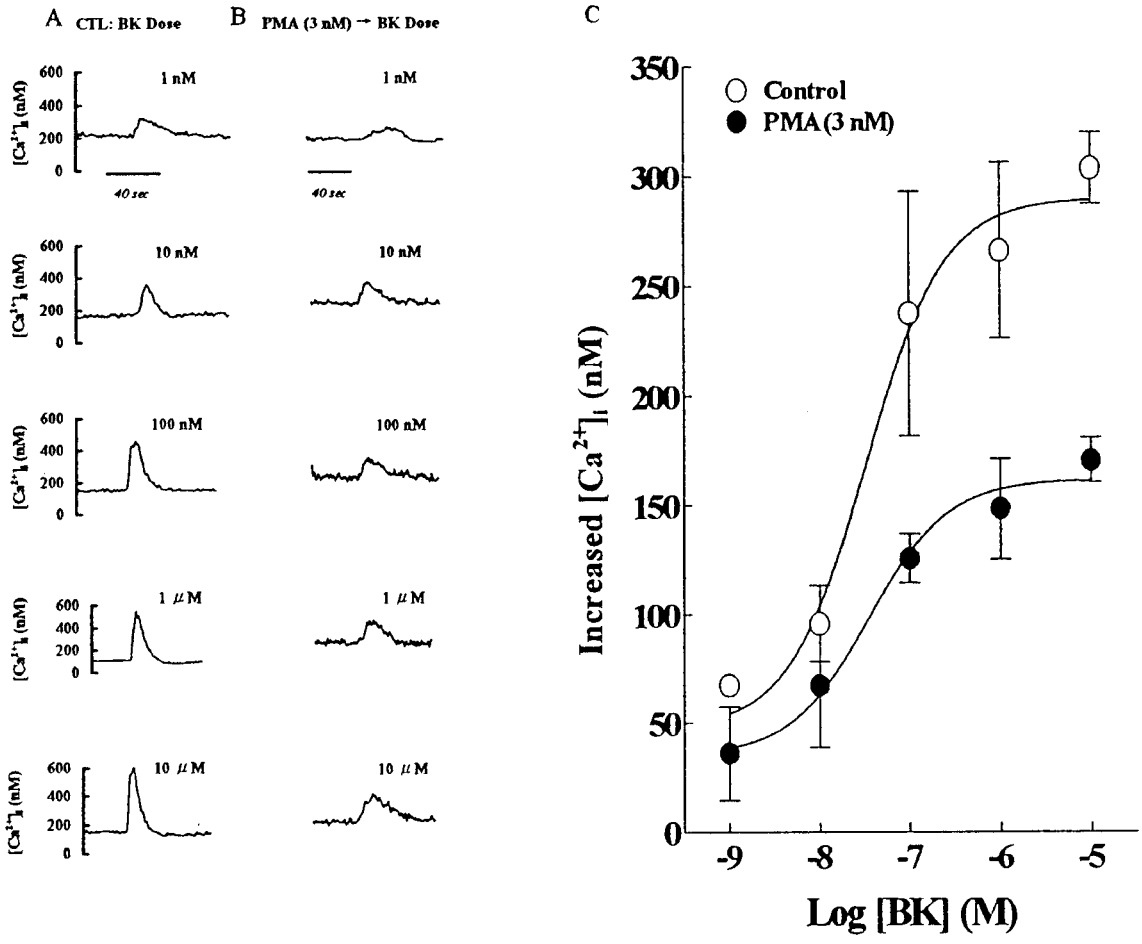

Figure 5 Effect of PMA on concentration response curves for BK-induced $[Ca^{2+}]_i$ change in cultured TECs. Cells were incubated in the absence (A) or presence of 3 nM PMA (B) at 37°C for 30 min. $[Ca^{2+}]_i$ was measured, when various concentrations of BK were added. Data expressed as the mean$\pm$s.e.mean from eight separate experiments are shown in (C).

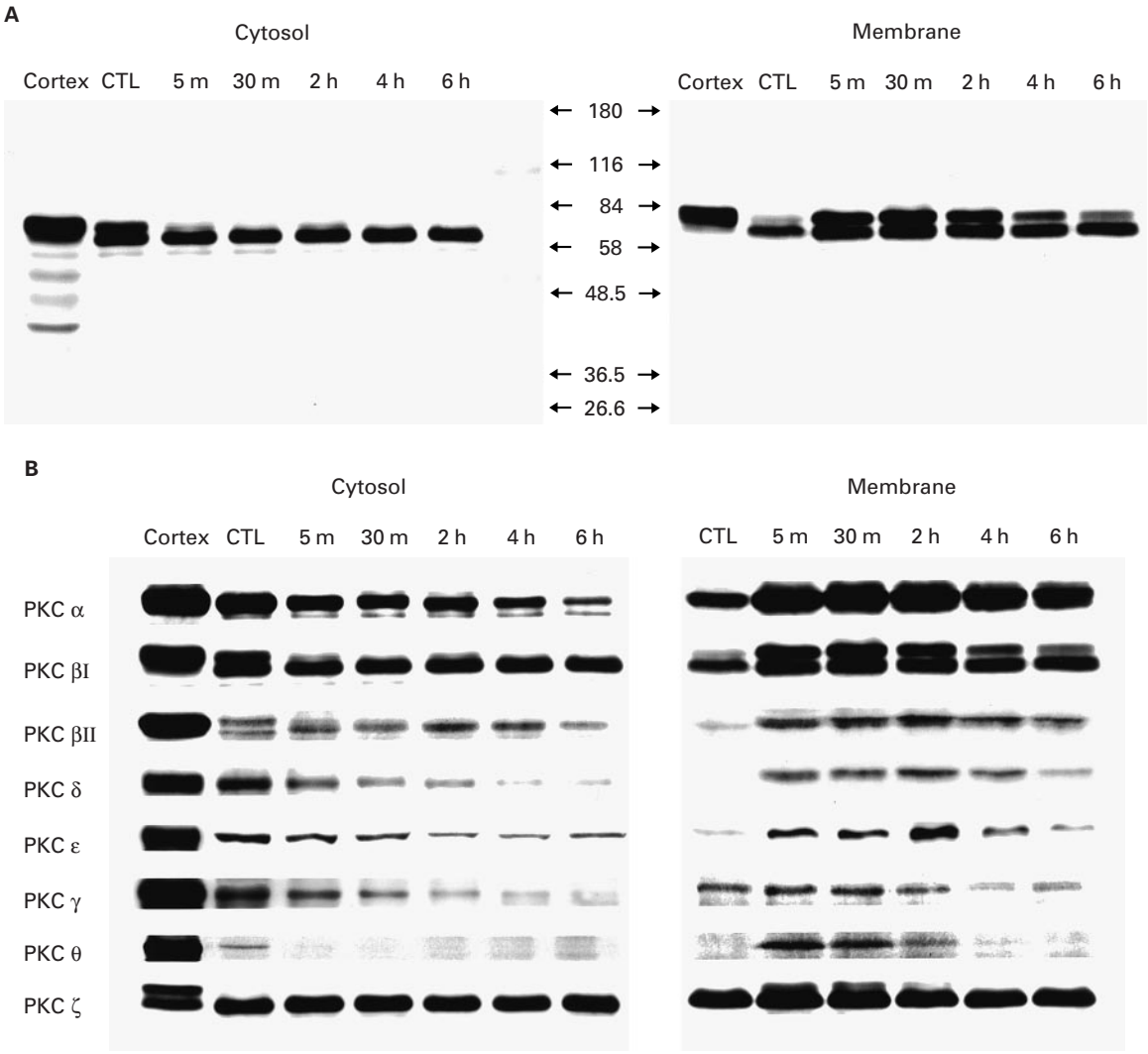

Figure 7 Time course of translocation and down-regulation of PKC isozymes in TECs. Cells were exposed to 1 μM PMA for the indicated times. Membrane and cytosolic fractions were prepared. (A) Effect of PMA treatment on the translocation of PKC-βI immunoreactivity in Western blot from TECs. The proteins were separated by 10% SDS–PAGE, transferred to nitrocellulose paper and immunodetected with PKC-βI. (B) Typical autographs collected by scanning are presented. The proteins were separated by 10% SDS–PAGE, transferred to nitrocellulose paper and immunodetected with PKC-α, βI, βII, γ, δ, ε, η, θ, ζ, ι, λ and μ specific antibodies as described in Methods. The immunoreactive bands were visualized using an ECL detection. Cortex: canine brain cortex as a positive control; CTL: non-PMA treatment; m: min; h: hour.

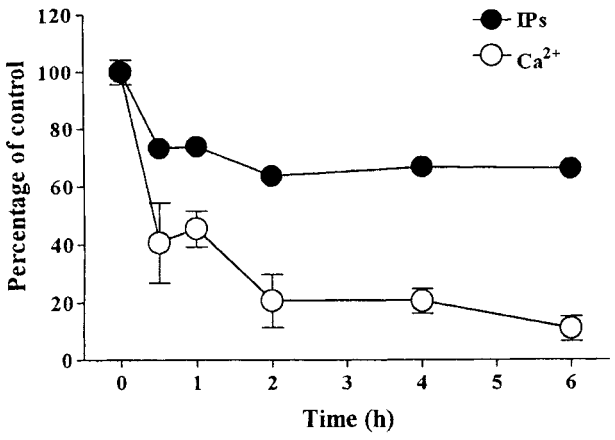

Figure 8 Time course of PMA inhibition of BK-stimulated IPs accumulation and Ca^{2+} mobilization in TECs. [^{3}H]-inositol labelled TECs were preincubated with 1 μM PMA for various times and then exposed to 10 μM BK for 60 min. Reactions were terminated by the addition of 5% PCA. IPs and $[Ca^{2+}]_i$ were determined as described under Methods. Values are expressed as the mean $\pm$ s.e.mean from at least three separate experiments.

Discussion

It has been established that receptor activation caused a rapid PI breakdown in several tissues in response to stimuli, such as neurotransmitters, growth factors, hormones, and light (Rana & Hokin, 1990). BK-induced hydrolysis of PI, with the subsequent generation of IPs and DAG and the rise in $[Ca^{2+}]_i$, can be attenuated by short-term activation of PKC in several cell types (Osugi *et al*., 1987; Garristen & Cooper, 1992; Yang *et al*., 1995; Sena *et al*., 1996; Yang *et al*., 1997). Furthermore, such a modulation of signal transduction in Ca^{2+}-mobilizing cells by PKC has been proposed to be involved in homologous desensitization in DDT1 MF-2 cells (Leeb-Lundberg *et al*., 1985), vascular smooth muscle cells (Pfeilschifter *et al*., 1989) and tracheal smooth muscle cells (Yang *et al*., 1995, 1997). In this study, we have shown that PMA treatment blocks BK receptor-mediated PI hydrolysis and Ca^{2+} mobilization in TECs. The concomitant loss of hormone-stimulated IPs accumulation and Ca^{2+} mobilization induced by short-term PMA treatment supports the existence of a causal relationship between these responses as suggested by previous studies

Table 1 Distribution and translocation of protein kinase C isozymes by PMA treatment in TECs

PKC	α	β*I*	β*II*	δ	ε	γ	θ
(Cytosol)							
5 min	72	70	89	72	63	58	79
30 min	65	64	75	52	52	36	76
2 h	67	69	92	51	29	27	82
4 h	53	65	82	43	29	24	85
6 h	38	59	55	44	36	23	92
(Membrane)							
5 min	194	221	232	349	121	113	172
30 min	197	227	250	327	116	115	153
2 h	195	198	268	415	157	86	123
4 h	159	157	222	289	112	59	94
6 h	133	148	186	156	99	70	93

For the translocation and down-regulation of PKC isozymes, the results from the scanning of autoradiographs from repetitive experiments in Figure 7B were pooled. Membrane-bound and cytosolic fractions were normalized as the percentage over the level seen in respective controls. Results shown are the means of two separate experiments.

Table 2 Effect of PMA treatment on [^{3}H]-BK binding in cultured canine TECs

Treatment	B_{max} (fmol/mg protein)	K_D (nM)
Control	50.5±4.9	1.7±0.3
PMA (30 min)	48.7±5.7	1.5±0.4
PMA (6 h)	52.3±6.1	1.8±0.3

Cultured TECs were incubated in the absence (control) or presence of PMA (1 μM) for 30 min and 6 h. Binding assays were performed in triplicate with concentrations of [^{3}H]-BK ranging from 0.2–10 nM and incubated at 4°C for 4 h. Data are expressed as the mean±s.e.mean of three individual experiments.

(Osugi *et al*., 1987; Pfeilschifter *et al*., 1989; Garristen & Cooper, 1992; Sena *et al*., 1996; Yang *et al*., 1995; 1997).

Because PKC activation is associated with several cellular responses, phorbol ester-mediated inhibition of IPs formation might occur at one or several different sites. In a number of cell types, elevation of intracellular Ca^{2+} by Ca^{2+}-mobilizing agonists known to act by receptor-mediated stimulation of PI turnover has been shown to be inhibited by phorbol esters (Yang *et al*., 1997; Sena *et al*., 1996; Daykin *et al*., 1993; Murray *et al*., 1989; Kotlikoff *et al*., 1987). It has been suggested that protein phosphorylation mediated by interaction of phorbol esters with PKC may be the mechanism by which PMA modulates hormone-sensitive PI metabolism. According to some reports (Connolly *et al*., 1986; Lapetina *et al*., 1986), phorbol esters might attenuate a rise in IP_3 through increasing its degradation by activation of a phosphomonoesterase specific for IP_3. The activity of this cytosolic enzyme increases after phosphorylation by PKC which provides a mechanism for inhibiting the agonist-induced rise in IP_3 accumulation in platelets. Our finding that PMA rapidly inhibits the BK-stimulated IPs accumulation and Ca^{2+} mobilization is consistent with the view that PMA acts through activation of PKC, since staurosporine, a potent PKC inhibitor, blocks the inhibitory effect of PMA.

To determine which PKC isozymes were involved in the regulation of receptor-mediated signal transduction, Western blot analysis was performed using PKC isozyme-specific antibodies. Short-term PMA treatment induced translocation of PKC-α, βI, βII, γ, δ, ε and θ from cytosol to membrane and attenuated BK-induced responses in TECs. In contrast, long-term exposure to PMA, these PKC isozymes underwent down-regulation close to the level of control cells. We further looked insight into a down-regulation protocol to investigate the roles of the PKC isozymes in the inhibition of responses to BK by PMA. Extended (6 h) treatment resulted in down-regulation of these PKC isozymes except PKC-ζ and the BK-induced responses were close to those of control cells. These results are consistent with the dual action of PMA on PKC isozymes in several cell types (Ozawa *et al*., 1993; Chen *et al*., 1995; Sena *et al*., 1996; Yang *et al*., 1997). Phorbol esters are shown to activate PKC and to increase the rate of degradation (Young *et al*., 1987) with possible differences in sensitivities among isoforms (Nishizuka, 1992).

PMA inhibited BK-induced IPs accumulation in cultured canine TECs in a time-dependent manner. PMA did not affect the basal level of IPs, thus ruling out the possibility that PMA caused its effect by depleting an agonist-sensitive pool of membrane PI. In addition, the inhibitory effect of PMA on BK-induced Ca^{2+} mobilization appeared to be directly related to the inhibition of IPs formation.

One site at which hormone-stimulated PI hydrolysis could be inhibited by PMA is located at the receptor level. It has been reported that phorbol esters induced phosphorylation of α_1-adrenergic receptors associated with antagonism of PI breakdown in DDT1 MF-2 cells and suggested that altered receptor binding may be a mechanism of PKC-induced inhibitory effect (Leeb-Lundberg *et al*., 1985). Moreover, pretreatment with PMA for either 30 min or 6 h did not change the B_{max} and K_D of BK receptors in canine TECs. It seems that BK receptor is not a site for the inhibitory effect of PMA on BK-induced responses. The ability of PMA to block histamine-stimulated IPs accumulation also suggests that the target of PMA is a more general component of the PI cycle than a specific receptor (Daykin *et al*., 1993; Murray *et al*., 1989), and that PMA affects unknown transducers that couple receptor occupation to response. Several lines of evidence suggest that a post-receptor site is the best unifying hypothesis for the location of the phorbol ester inhibitory effect. Phorbol esters blocked vasopressin-induced IP_3 accumulation in A10 cells without changing receptor binding and abolished the guanine nucleotide shift, indicating that coupling of the receptor to Gp was altered (Aiyar *et al*., 1987). Since PMA has no effect on the basal level of PI turnover, PKC can uncouple the G protein from PLC. It has been shown that activation of PKC affects the G protein coupling process in astrocytes (Chen *et al*., 1995), neutrophils (Matsumoto *et al*., 1986) and inhibits the function of Gi protein in platelets (Katada *et al*., 1985). In addition, activation of PKC by phorbol esters has been shown to phosphorylate PI-PLC in rat basophilic leukaemia cells, providing an additional mechanism for receptor-PLC uncoupling (Bennet & Crooke, 1987). Regardless of the precise mechanism, an implication of a post-receptor site of phorbol ester inhibition is that other agonists that act through PI-PLC stimulation might be uncoupled from cytosolic Ca^{2+} mobilization.

The inhibition of BK-stimulated increase in $[Ca^{2+}]_i$ by PMA is in agreement with the inhibitory effect of PMA on agonist-induced IPs accumulation and Ca^{2+} mobilization in TECs (McCarthy *et al*., 1989; Buchan & Martin, 1991; Sena *et al*., 1996; Yang *et al*., 1997). The fact that the same experimental conditions block IP_3 accumulation and Ca^{2+} mobilization suggest that the mechanism this PKC-mediated inhibition is not confined to the IP_3-sensitive Ca^{2+} release site. This is consistent with the observation that the purified IP_3 receptor

from brain is phosphorylated and not functionally modified by PKC (Supattapone *et al*., 1988).

In conclusion, we have demonstrated that short-term PMA treatment causes an inhibition on BK-induced IPs accumulation and Ca^{2+} mobilization in canine TECs. The inhibition by PMA on BK-induced responses was associated with translocation of PKC-α, βI, βII, δ, ε, γ and θ from cytosol to membrane. These results suggest that physiological activation of PKC might serve as a modulator of cellular responses induced by IPs. The site of PMA inhibition appears to be at a post-receptor location and may be involved in PI-PLC itself.

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