British Journal of Pharmacology (1997) 121, 853–860

Inhibition of 5-hydroxytryptamine-induced phosphoinositide hydrolysis and Ca^{2+} mobilization in canine cultured tracheal smooth muscle cells by phorbol ester

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1 Regulation of the increase in inositol-1,4,5-trisphosphate (IP_3) production and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$ by protein kinase C (PKC) was investigated in canine cultured tracheal smooth muscle cells (TSMCs). Stimulation of TSMCs by 5-hydroxytryptamine (5-HT) caused an initial transient $[Ca^{2+}]_i$ peak followed by a sustained elevation of $[Ca^{2+}]_i$ in a concentration-dependent manner.

2 Pretreatment of TSMCs with phorbol 12-myristate 13-acetate (PMA, 1 μM) for 30 min blocked the 5-HT-induced IP_3 formation and Ca^{2+} mobilization. This inhibition was reduced after the cells had been incubated with PMA for 8 h, and within 48 h the 5-HT-induced Ca^{2+} mobilization reached the same extent as control cells.

3 The concentration of PMA that gave half-maximal inhibition of 5-HT-induced increase in $[Ca^{2+}]_i$ was 4 nM. Pretreatment of TSMCs with staurosporine (1 μM) of GF109203X (0.1 μM), PKC inhibitors, inhibited the ability of PMA to attenuate 5-HT-induced responses, suggesting that the inhibitory effect of PMA was mediated through the activation of PKC.

4 In parallel with the effect of PMA on 5-HT-induced IP_3 formation and Ca^{2+} mobilization, the translocation and down-regulation of PKC isozymes were determined by Western blot analysis in TSMCs. Analysis of cell extracts by Western blotting with antibodies against different PKC isozymes revealed that TSMCs expressed PKC-α, βI, βII, δ, ε, θ and ζ. With PMA treatment of the cells for various times, translocation of PKC-α, βI, βII, δ, ε and θ from the cytosol to the membrane was seen after 5 min, 30 min, 2 h, and 4 h treatment. However, 24 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 In conclusion, these results suggest that activation of PKC may inhibit the receptor-mediated phosphoinositide hydrolysis and consequently attenuate the $[Ca^{2+}]_i$ increase or inhibit both responses independently. The translocation of PKC-α, βI, βII, δ, ε, and θ induced by PMA caused an attenuation of 5-HT-stimulated IP_3 accumulation and Ca^{2+} mobilization in TSMCs.

Keywords: Phorbol ester; 5-hydroxytryptamine; protein kinase C; inositol phosphates; Ca^{2+}; tracheal smooth muscle cells

Introduction

It is established that many agonists cause contraction of tracheal smooth muscle through the activation of phosphoinositide (PI) hydrolysis to generate inositol 1,4,5-trisphosphate (IP_3) and subsequently an increase in intracellular free calcium concentration ($[Ca^{2+}]_i$) (Coburn & Baron, 1990). 5-Hydroxytryptamine (5-HT) has been shown to stimulate PI hydrolysis and/or calcium mobilization in several cell types including C_6 glioma cells (Ananth *et al*., 1987), smooth muscle cells (Roth *et al*., 1986), and C6BU-1 glioma cells (Kagaya *et al*., 1993). 5-HT is known to stimulate IP_3 accumulation in guinea-pig (Cohen & Wittenauer, 1987) and canine (Yang *et al*., 1994c tracheal smooth muscle and subsequently increase $[Ca^{2+}]_i$ (Yang *et al*., 1994a). Thus, in terms of second messenger generation, contraction of tracheal smooth muscle may be mediated by IP_3-induced Ca^{2+} mobilization from intracellular stores.

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 PI hydrolysis (Pearce *et al*., 1988; Pfeilschifter *et al*., 1989; Daykin *et al*., 1993), intracellular Ca^{2+} mobilization (McCarthy *et al*., 1989), or both responses (Chuang *et al*., 1991). These data suggest that there are many candidates for target sites of PKC in the regulation of 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 biological systems (Nishizuka, 1992). To date, twelve isozymes with distinct enzymological characteristics and differential tissue expression and intracellular localization have been identified (Nishizuka, 1992; Dekker & Parker, 1994). 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 about their individual roles. Therefore, the translocation and down-regulation of PKC isozymes involved in the modulation of 5-HT-induced PI hydrolysis and

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Ca^{2+} mobilization needs to be investigated in tracheal smooth muscle cells (TSMCs).

The precise molecular and biochemical mechanisms of phorbol esters related to inhibition of 5-HT-mediated physiological responses in tracheal smooth muscle are not clear. Hence, the purpose of the present study was to investigate the role of the activating PKC pathway in the modulation of PI breakdown, change in $[Ca^{2+}]_i$ and the involvement of PKC isozymes in the inhibition of 5-HT-mediated responses in TSMCs by phorbol esters.

Methods

Animals

Mongrel dogs, 10–20 kg, purchased from a local supplier, were used throughout this study. Dogs were housed in animal facilities under automatically controlled temperature and light cycle and fed standard laboratory chow and tap water *ad libitum*. Dogs of either sex were anaesthesized with ketamine (20 mg kg^{-1} i.m.) and pentobarbitone (30 mg kg^{-1} i.v.) and the lungs were ventilated mechanically via an orotracheal tube. The tracheae were surgically removed.

Isolation of tracheal smooth muscle cells

The TSMCs were isolated according to the methods previously described (Yang *et al*., 1994a, c). The trachea was cut longitudinally through the cartilage rings and the smooth muscle was dissected. The muscle was minced and transferred to the dissociation medium containing 0.2% collagenase IV, 0.01% deoxyribonuclease I, 0.01% elastase IV, and antibiotics (100 u ml^{-1} penicillin G, 100 μg ml^{-1} streptomycin and 250 ng ml^{-1} fungizone) in physiological solution. The physiological solution contained (mM): NaCl 137, KCl 5, $CaCl_2$ 1.1, $NaHCO_3$ 20, NaH_2PO_4 1, glucose 1 and HEPES 2.5 (pH 7.4). The tissue pieces were gently agitated at 37°C in a rotary shaker for 1 h. The released cells were collected and the residue was again digested with fresh enzyme solution for an additional h at 37°C. The released cells were washed twice with DMEM/F-12 medium (1 : 1 v/v). The cells, suspended in DMEM/F-12 containing 10% FBS, were plated onto a 60 mm culture dish and incubated at 37°C for 1 h to remove fibroblasts which attached to the dish more rapidly than TSMCs. The cell number was counted and the suspension diluted with DMEM/F-12 to 2×10^5 cells ml^{-1}. The cell suspension was plated onto (1 ml/well) 12-well, or (2 ml/well) 6-well culture plates containing glass coverslips coated with collagen for IP accumulation of Ca^{2+} measurement, respectively. The medium was changed after 24 h and then every 3 days. After a 5-day culture, cells were changed to DMEM/F-12 containing 1% FBS for 24 h at 37°C. Then, the cells were incubated in DMEM/F-12 containing 1% FBS supplemented with IGF-I (10 ng ml^{-1}) and insulin (1 μg ml^{-1}) for 12–14 days.

In order to characterize the isolated and cultured TSMCs and to exclude contamination by epithelial cells and fibroblasts, the cells were identified by an indirect immunofluorescent staining method by use of a monoclonal antibody recognition of smooth muscle light chain myosin (Gown *et al*., 1985). Over 95% of the cell preparation was composed of smooth muscle cells.

Accumulation of inositol phosphates

Effect of 5-HT on the hydrolysis of PI was assayed by monitoring the accumulation of ^{3}H-labelled IPs as described by Berridge *et al*. (1983). Cultured TSMCs were incubated with 5 μCi ml^{-1} *myo*-[^{3}H]-inositol at 37°C for 2 days, washed twice with PBS and incubated in Krebs-Henseleit buffer (KHS, pH 7.4) containing (in mM): Nacl 117, KCl 4.7, $MgSO_4$ 1.1, KH_2PO_4 1.2, $NaHCO_3$ 20, $CaCl_2$ 2.4, glucose 1, HEPES 20 and LiCl 10, at 37°C for 30 min. In all experiments (other than 5 and 30 min incubation with phorbol esters) the cells were treated with 1 μM PMA in the KHS buffer for the appropriate time during the *myo*-[^{3}H]-inositol labelling stage. Control cells were preincubated in parallel with 0.1% DMSO for 24 h. The cells were washed three times with KHS and stimulated with 5-HT in the presence of 10 mM LiCl for another 20 min. The incorporation of *myo*-[^{3}H]-inositol was not affected by 24 h PMA treatment. Reactions were terminated by addition of 5% perchloric acid (PCA) followed by sonication and centrifugation at $3000 \times g$ for 15 min.

The PCA 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 *myo*-[^{3}H]-inositol and glycerophosphoinositol, respectively. Sequential washes with 5 ml of 0.2 M ammonium formate/0.1 M formic acid, 0.4 M ammonium formate/0.1 M formic acid, and 1 M ammonium formate/0.1 M formic acid were used to elute inositol monophosphate (IP_1), inositol bisphosphate (IP_2), and inositol trisphosphate (IP_3), repsectively. The amount of [^{3}H]-IP was determined in a radiospectrometer (Beckman TA5000).

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 were 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): NaCl 125, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 2, NaH_2PO_4 0.5, $NaHCO_3$ 5, HEPES 10 and glucose 1; pH 7.4. The cells were incubated in PBS for a further 30 min to complete dye deesterification. 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 an SLM 8000C spectrofluorometer (SLM Aminco, Urbana, IL, U.S.A.). Continuous stirring was achieved with a magnetic stirrer. Fluorescence of Ca^{2+}-bound and unbound fura-2 was measured by rapidly alternating the dual excitation wavelengths between 340 and 380 nm and electronically separating the resultant fluorescence signals at an emission wavelength of 510 nm. The autofluorescence of each monolayer was subtracted from the fluorescence data. The ratios (R) of the fluorescence at the two wavelengths were 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).

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 as indicated in Figure 5. Dimethylsulphoxide (0.1%) was added to control cells for 24 h. When staurosporine, GF109203X (PKC inhibitors) or ketanserin (a 5-HT_{2A} receptor antagonist) was used, they were added to the cells 30 min before treatment with PMA or 5-HT. The cells were then rapidly washed with ice-cold PBS, scraped and collected by centrifugation at $1000 \times g$ for 10 min. The preparation of cell extracts and immunoblot analysis were performed as previously described (Chen *et al*., 1995). Briefly, the collected cells were lysed in ice-cold homogenization buffer containing (mM): Tris-HCl 20, pH 7.4; dithiothreitol 1, EGTA 5, EDTA 2, glycerol 10%, PMSF 0.5 and 5 μg ml^{-1} leupeptin. The homogenates were centrifuged at $45{,}000 \times g$ 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 by use of a 10% running gel. Proteins were transferred to nitrocellulose membrane and the membrane was incubated successively at room temperature with 0.1% dried milk in TTBS for 1 h, with rabbit antibodies specific for PKC isoforms (α, βI, βII, γ, δ, ε, η, θ, ζ, ι, λ, and μ) for 1 h, and with anti-rabbit horseradish peroxidase antibody 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 brain cortex, used as a positive control (data not shown).

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). Insulin and insulin-like growth factor I (IGF-I) were from Boehringer Mannheim (GmbH, Germany). *Myo*-[^{3}H]-inositol (18 Ci mmol^{-1}) was from Amersham (Buckinghamshire, U.K.). Fura-2/AM was from Molecular Probes Inc (Eugene, OR). 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). Enzymes and other chemicals were from Sigma Co (St. Louis, MO). GF109203X (L) (3-[1-[3-(dimethylamino) propyl]-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione) was obtained from Biomol Research Lab (Plymouth Meeting, PA).

Analysis of data

Concentration-effect curves were fitted by Graph Pad Programme (GraphPad, San Diego, CA, U.S.A.). EC_{50} values were estimated by the same programme. Data are expressed as the mean$\pm$s.e.mean and analysed with a two-tailed Student's t test at a $P<0.01$ level of significance.

Results

Effects of PMA on the $[Ca^{2+}]_i$ response to 5-HT

In order to examine a possible role of PKC in the regulation of $[Ca^{2+}]_i$ in TSMCs, we tested the effect of PMA on 5-HT-induced changes in $[Ca^{2+}]_i$. Figure 1 shows the time course of PMA treatment on 5-HT-induced changes in $[Ca^{2+}]_i$. PMA treatment for various periods did not change the resting level of $[Ca^{2+}]_i$. However, it did significantly inhibit the transient elevation of $[Ca^{2+}]_i$ induced by 5-HT in TSMCs treated with PMA between 30 min and 2 h ($P<0.001$, as compared with control cells). Treatment with PMA for more than 8 h reduced its inhibitory effect, while the $[Ca^{2+}]_i$ response to 5-HT after treatment of TSMCs with PMA for 48 h showed the same response as that of the control cells (Figure 1). Figure 2A depicts tracings of 5-HT-induced $[Ca^{2+}]_i$ changes in TSMCs following treatment with various concentrations of PMA for 30 min. In control cells, the resting level of $[Ca^{2+}]_i$ was 110$\pm$14 nM ($n=30$). Addition of 5-HT (100 μM) resulted in a biphasic elevation of $[Ca^{2+}]_i$ consisting of a rapid, transient component at 627$\pm$25 nM within 30 s, which was followed by a lower sustained component (386$\pm$31 nM, $n=16$). PMA (0.1–1000 nM) did not significantly alter the resting level of $[Ca^{2+}]_i$ (107$\pm$12 nM). As shown in Figure 2A, pretreatment of TSMCs with PMA followed by subsequent exposure to 100 μM 5-HT markedly inhibited the Ca^{2+} mobilization. Figure 2B shows the effects of various concentrations of PMA on 5-HT-induced increase in $[Ca^{2+}]_i$. PMA induced half-maximal and maximal inhibition of 5-HT-stimulated $[Ca^{2+}]_i$ changes at 3.9$\pm$0.4 nM and 1 μM ($n=8$), respectively. The inhibitory effect of PMA resulted in a decrease in the maximal response and a shift to the right in the concentration-effect curve for 5-HT-induced $[Ca^{2+}]_i$ changes (Figure 3). The half-maximal value (EC_{50}) for the stimulating effect of 5-HT on Ca^{2+} mobilization in the presence of 10 nM PMA (2.1$\pm$0.7 μM) was higher ($P<0.01$, as compared with control) than that of control cells (0.30$\pm$0.12 μM).

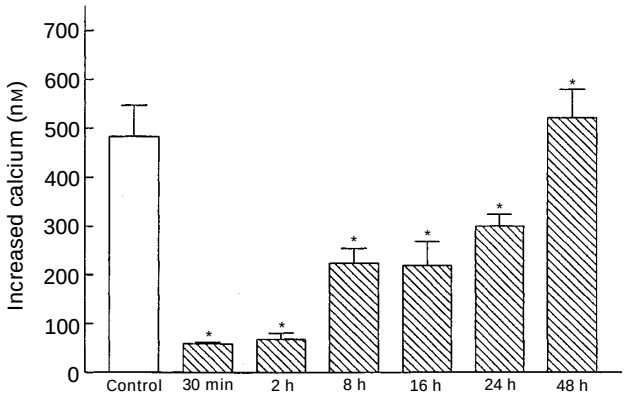

Figure 1 Time course of PMA-induced inhibition of 5-HT-stimulated $[Ca^{2+}]_i$ change in cultured TSMCs. Cells were incubated in the absence (control) and presence of 1 μM PMA for various times. TMSCs grown on glass coverslips were loaded with 5 μM fura-2/AM and fluorescent measurement of $[Ca^{2+}]_i$ was carried out in a dual excitation wavelength spectrofluorometer, with excitation at 340 and 380 nm, when 100 μM 5-HT was added. Values are expressed as the mean$\pm$s.e.mean from eight separate experiments. *$P<0.001$ as compared with control.

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

We examined the effects of staurosporine and GF109203X, PKC inhibitors, on PMA-induced inhibition of $[Ca^{2+}]_i$ responses to 5-HT. As shown in Figure 4, TSMCs were treated with phorbol esters (1 μM) and then stimulated with 100 μM 5-HT. Treatment of TSMCs with 1 μM PMA for 30 min inhibited the 5-HT-stimulated $[Ca^{2+}]_i$ response by 83% ($P<0.001$, $n=8$, as compared with the control). When TSMCs were preincubated with either staurosporine (1 μM) or GF109203X (0.1 μM), the inhibitory effect of PMA on 5-HT-stimulated Ca^{2+} mobilization was reversed, although pretreatment with these PKC inhibitors alone did not affect the $[Ca^{2+}]_i$ response to 5-HT. It is clear that this inhibition could be prevented by the PKC inhibitors. The inactive phorbol ester, 4α-phorbol 12,13-didecanoate (4α-PDD, 1 μM), did not block 5-HT-induced Ca^{2+} mobilization (data not shown).

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

Several studies have shown that activation with PMA results in a translocation of PKC from cytosol to membranes, and that this translocation differs between the various isozymes. To determine which PKC isozyme is associated with the regulation of 5-HT-induced responses, the expression of PKC isozymes in cultured TSMCs was characterized by a Western blot analysis. Immunoblot analysis of whole cell extracts by use of antibodies against PKC-α, βI, βII, γ, δ, ε, η, θ, ζ, ι, λ and μ, revealed the presence of PKC-α, βI, βII, δ, ε, θ, and ζ in cultured TSMCs (data not shown), consistent with recent findings with the exception that PKC-α was not detected (Donnelly *et al*., 1995). This difference may be due to different experimental conditions. Furthermore, to evaluate the effect of PMA treatment on the level of these detectable PKC isozymes in TSMCs, cell extracts were subjected to immunoblotting with specific antibodies to various PKC isozymes. As shown in Figure 5, a short (5 min, 30 min, and 2 h) ex-

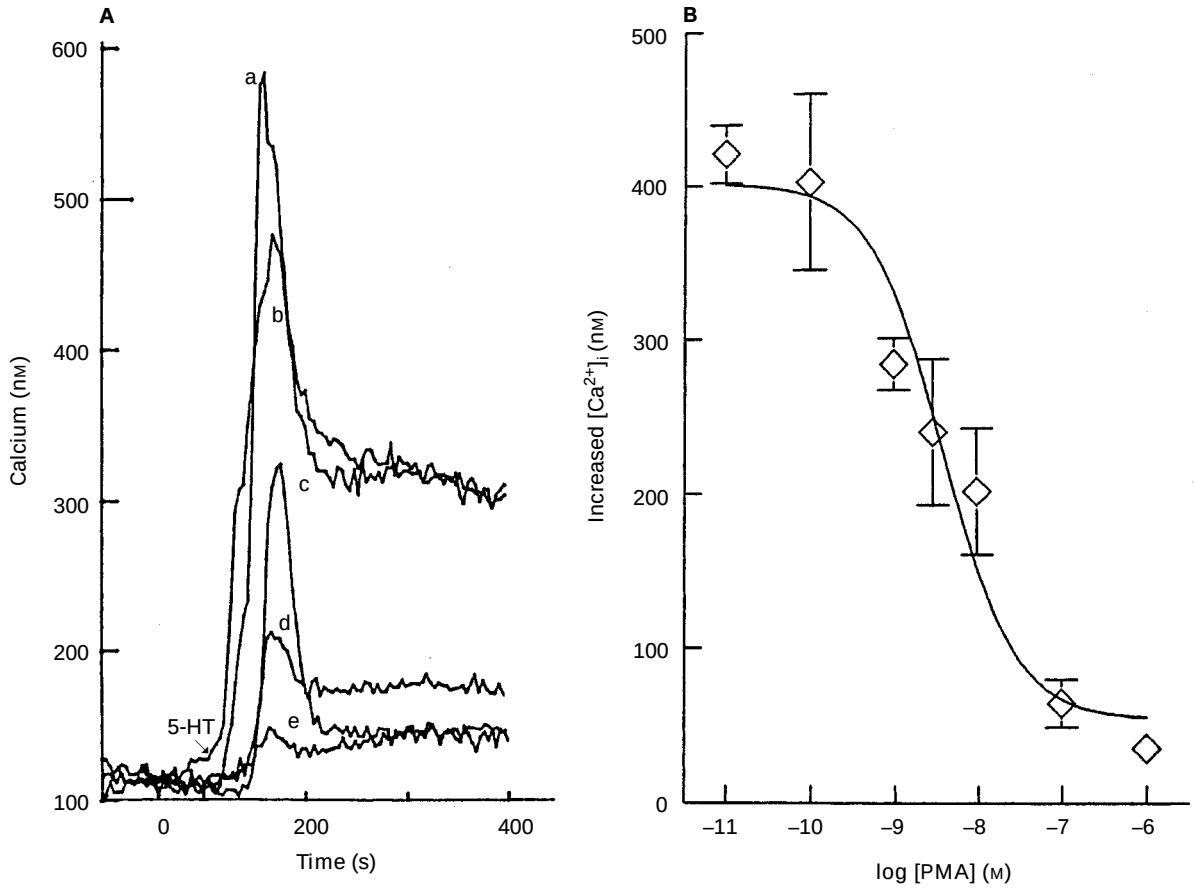

Figure 2 Concentration-dependence of PMA inhibition of 5-HT-stimulated $[Ca^{2+}]_i$ change in cultured TSMCs. (A) Cells were incubated with various concentrations of PMA (a, 0.1 nM; b, 1 nM; c, 10 nM; d, 100 nM; e, 1 μM) for 30 min. $[Ca^{2+}]_i$ was measured when 5-HT (100 μM) was added, indicated by an arrow. Data expressed as the mean $\pm$ s.e.mean (vertical lines) from eight separate experiments are shown in (B).

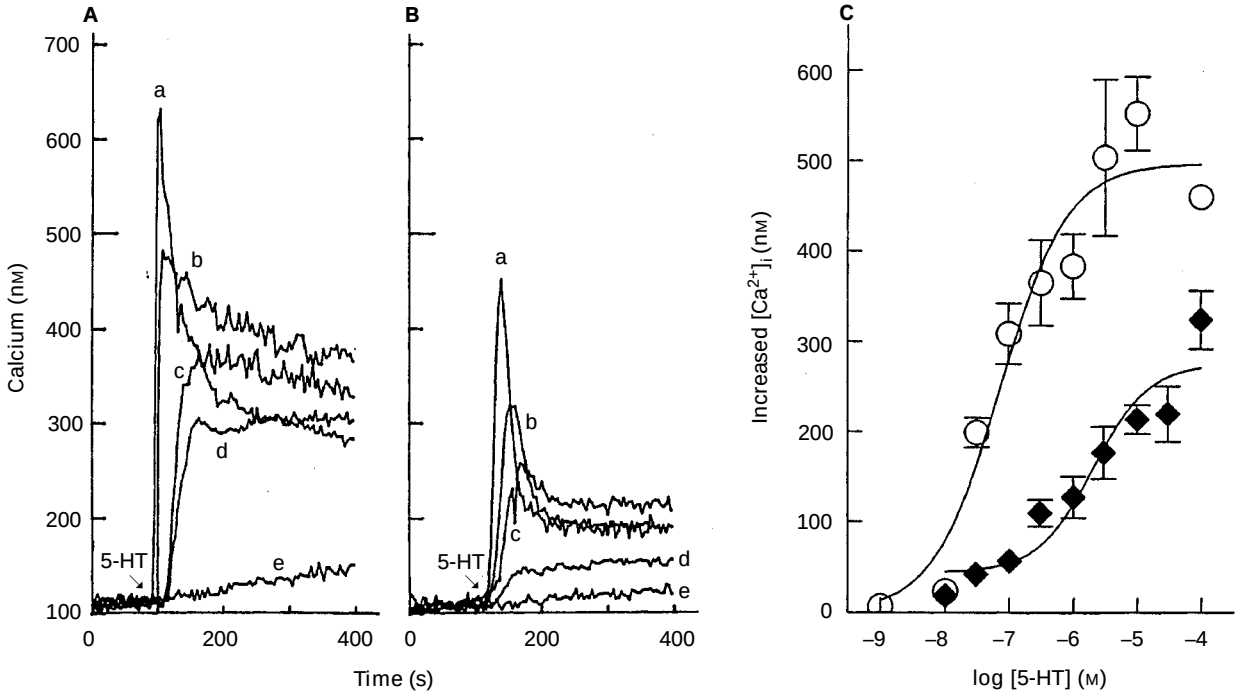

Figure 3 Effect of PMA on concentration-response curves for 5-HT-induced change in $[Ca^{2+}]_i$ in cultured TSMCs. Cells were incubated in the absence (A) and presence of 10 nM PMA (B) at 37°C for 30 min. $[Ca^{2+}]_i$ was measured after various concentrations of 5-HT (a, 100 μM; b, 10 μM; c, 1 μM; d, 100 nM; e, 10 nM) had been added, indicated by an arrow. Data expressed as the mean $\pm$ s.e.mean (vertical lines) from eight separate experiments are shown in (C); (○), control; (◆) PMA treatment.

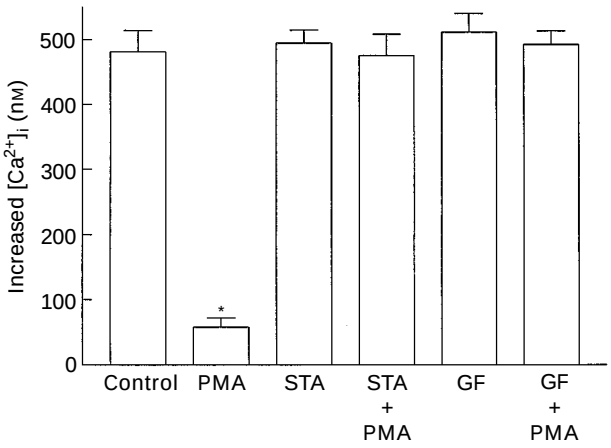

Figure 4 Effects of phorbol esters and PKC inhibitors on 5-HT-induced increase in $[Ca^{2+}]_i$ in cultured TSMCs. Cells were incubated with PMA (1 μM), staurosporine (STA, 1 μM), STA plus PMA, GF109203X (0.1 μM), or GR109203X plus PMA, at 37°C for 30 min. $[Ca^{2+}]_i$ was measured after the addition of 5-HT (100 μM). Values are expressed as the mean ± s.e.mean from eight separate experiments.

posure of the TSMCs to 1 μM PMA resulted in a rapid translocation of PKC-α, βI, βII, δ, ε and θ from cytosolic to membrane fractions, but not PKC-ζ, and this was sustained over the levels in controls for 4 h. When the cells were exposed to PMA for 24 h, the PKC-α, βI, βII, δ, ε and θ translocated from the cytosol to the membrane underwent down-regulation. However, there was no evidence of translocation of these detectable PKC isozymes from cytosol to membrane in response to 5-HT for 30 min (Figure 6). Moreover, PKC inhibitors staurosporine and GF109203X did not significantly block the translocation of these isozymes induced by PMA (Figure 6).

Effects of PMA on 5-HT-stimulated IP_3 accumulation

To investigate whether PKC activation by PMA causes a change in 5-HT-induced IP_3 accumulation, the effect of PMA treatment was examined in TSMCs. The inhibitory effect of PMA occurred in a concentration-dependent manner (Figure 7a), consistent with previous findings in several cell types (Leeb-Lundberg *et al*., 1985; Pearce *et al*., 1988; Pfeilschifter *et al*., 1989; Yang *et al*., 1994c). PMA induced half-maximal inhibition of 5-HT-stimulated IP_3 formation at 3.0 ± 2.0 nM, $n=4$. It had no effect on the basal levels of IP_3 at any of the concentrations tested. The inhibitory action of PMA resulted in a decrease in the maximal response and a shift to the right in

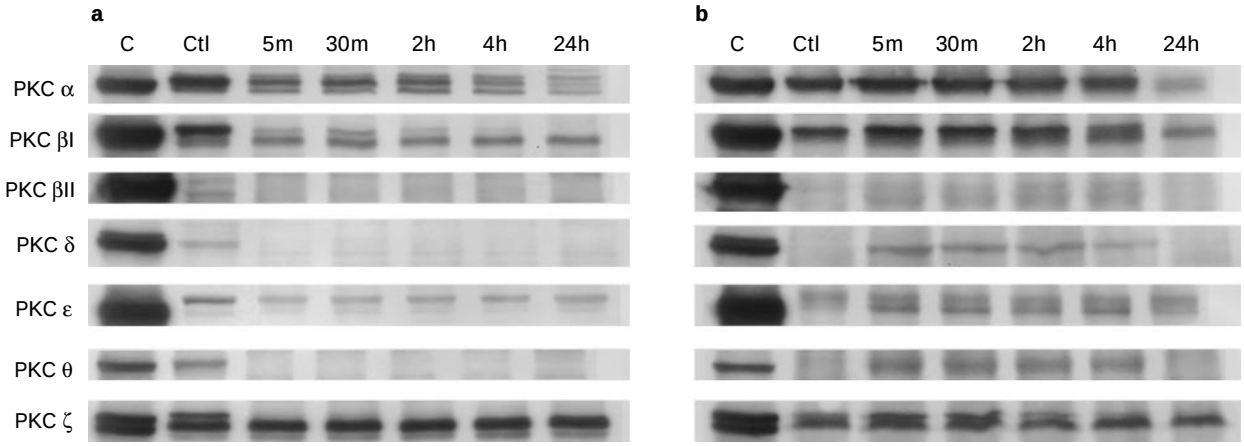

Figure 5 Time course of translocation and down-regulation of PKC isozymes in TSMCs, (a) in the cytosol and (b) in the membrane. Cells were exposed to 1 μM PMA for the indicated times. Membrane (b) and cytosolic (a) fractions were prepared. The proteins were separated by 10% SDS-PAGE, transferred to nitrocellulose 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 by ECL. C; canine brain cortex as a positive control; Ctl, non-PMA treatment; m, min; h, hour.

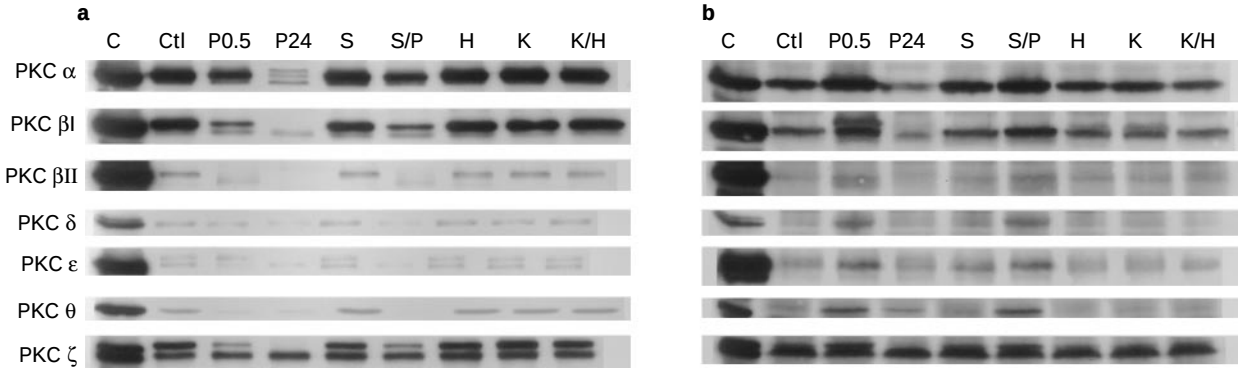

Figure 6 Effects of pretreatment with PMA, 5-HT, staurosporine and ketanserin (a selective 5-HT_{2A} receptor antagonist) on the intracellular distribution of PKC isozymes in TSMCs, (a) in the cytosol and (b) in the membrane. Cells were exposed to 1 μM PMA (P) for 0.5 and 24 h, 1 μM staurosporine (S), staurosporine plus PMA (S/P), 100 μM 5-HT (H), 10 μM ketanserin (K) and ketanserin plus 5-HT (K/H) for 30 min. 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 by ECL. C: canine brain cortex as a positive control; Ctl: untreated cells.

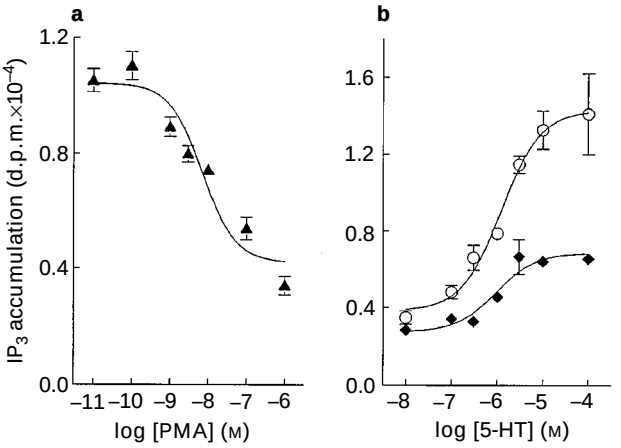

Figure 7 Effect of PMA on 5-HT-stimulated [^{3}H]-IP_3 accumulation in TMSCs. (a) Concentration-dependence of PMA inhibition of 5-HT-stimulated IP_3 accumulation. Cells prelabelled with [^{3}H]-inositol were washed with KHS, treated with various concentrations of PMA (0.1 nM – 1 μM) for 30 min and then exposed to 5-HT (100 μM) for 20 min. (b) Effect of PMA on concentration-effect curves for 5-HT-stimulated IP_3 accumulation. [^{3}H]-inositol labelled cells were incubated in the absence (○) and presence of 10 nM PMA (◆) for 30 min and then exposed to various concentrations of 5-HT for 20 min. The accumulation of IP_3 was determined, as described in Methods. Values are expressed as the mean from four separate experiments determined in triplicate; vertical lines show s.e.mean.

the concentration-effect curve for 5-HT-induced IP_3 accumulation (Figure 7b). The half-maximal value (EC_{50}) for the stimulating effect of 5-HT on IP_3 formation in the presence of 10 nM PMA (1.4 ± 0.2 μM, $n=4$) was higher than that of control (0.9 ± 0.3 μM, $n=7$).

The effect of PMA on the 5-HT-induced response and the translocation and down-regulation of PKC isozymes after various periods of treatment is shown in Figure 8. Pretreatment of TSMCs with PMA for 5 min resulted in a rapid inhibition of the 5-HT-induced IP_3 accumulation by 45% (Figure 8a). Following preincubation, maximal inhibition of 5-HT-stimulated IP_3 accumulation by 69 ± 5% was obtained within 30 min. After longer periods of PMA treatment, the inhibitory effect of PMA on 5-HT response was reduced and almost returned to those in controls after 24 h (Figure 8a). Under similar experimental conditions, the results from scanning of autoradiographs from repetitive experiments were pooled (Figure 5), the loss from the cytosolic fraction was expressed as a percentage of the level found 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 Figure 8b, translocation of PKC-δ was the most profound among these PKC isoforms detected in TSMCs, whereas PKC-ζ showed no significant translocation (data not shown). After 5 min of PMA treatment, the levels of PKC-δ, θ, α, βI and βII were significantly increased over the level seen in controls by 223%, 180%, 153%, 142%, 140% and 122%, respectively ($P<0.01$). However, after longer periods of PMA treatment the levels of these PKC isozymes, except PKC-βII, had declined by 30 min, 2 h and 4 h; for PKC-βII this loss was not apparent until 4 h. The membrane levels of these PKC isoforms were identical to those in controls after 4 h of treatment. Almost complete down-regulation of these PKC isoforms below those in controls was seen after 24 h of treatment with PMA. In the cytosolic fraction, after 5 min of PMA treatment, the loss of PKC-δ, θ, α, βI and βII from the cytosol was 74%, 80%, 45%, 32%, 53% and 56%, respectively ($P<0.01$). The maximal loss in the cytosol was reached by 2 h, with no further depletion of cytosolic levels at later times.

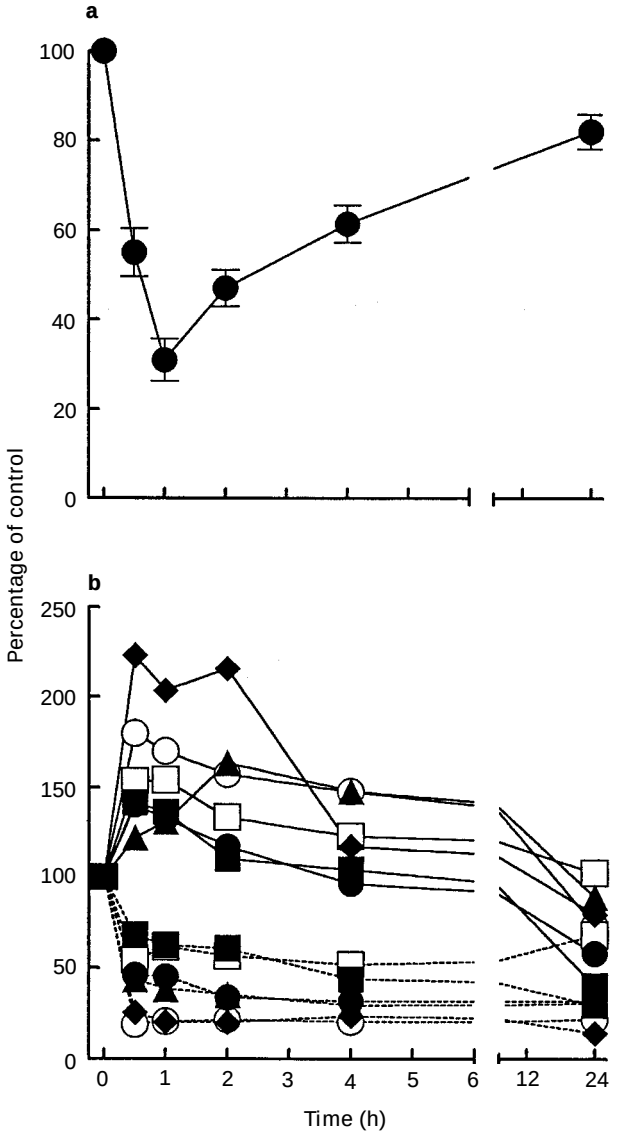

Figure 8 The translocation of PKC isozymes and inhibition of 5-HT-stimulated IP_3 accumulation by PMA treatment of TMSCs. (a) For IP_3 accumulation, [^{3}H]-inositol labelled TMSCs were preincubated with 1 μM PMA for various times and then exposed to 100 μM 5-HT for 20 min. Reactions were terminated by the addition of 5% PCA. Inositol trisphosphate (IP_3, ●) was separated as described in Methods. (b) For the translocation and down-regulation of PKC isozymes, the results from the scanning of autoradiographs from repetitive experiments in Figure 5 were pooled. Membrane-bound (solid line) and cytosolic (broken line) fractions were normalized as the percentage over the level seen in respective controls. Results shown are the mean of two separate experiments. PKC-α (□), βI (▲), βII (○), δ (■), ε (◆), and θ (△).

Discussion

A rapid PI breakdown after receptor activation has been observed in several tissues in response to stimuli, such as neurotransmitters, growth factors, hormones and light (Rana & Hokin, 1990). 5-HT-induced hydrolysis of PI, with the subsequent generation of IP_3 and DAG and the rise in $[Ca^{2+}]_i$, can be attenuated by short-term activation of PKC in smooth muscle (Roth *et al.*, 1986) and cardiomyocytes (Hamamori *et al.*, 1990). 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) and vascular smooth muscle cells (Pfeilschifter *et al.*, 1989). In this study, we have shown

that PMA treatment blocks 5-HT receptor-mediated PI hydrolysis and Ca^{2+} mobilization in TSMCs. The concomitant loss of hormone-stimulated IP_3 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 (Roth *et al*., 1986; Pfeilschifter *et al*., 1989; Hamamori *et al*., 1990; Daykin *et al*., 1993).

Because PKC activation is associated with several cellular responses, phorbol ester-mediated inhibition of IP_3 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 (Murray *et al*., 1989; Daykin *et al*., 1993). 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 studies (Connolly *et al*., 1986), phorbol esters might attenuate a rise in IP_3 by increasing its degradation via 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 inhibited 5-HT-stimulated IP_3 accumulation and Ca^{2+} mobilization is consistent with the view that PMA acts through activation of PKC, since staurosporine and GF109203X, potent PKC inhibitors, blocked the inhibitory effect of PMA.

To determine which PKC isozymes are involved in the regulation of receptor-mediated signal transduction, Western blot analysis was performed with PKC isozyme-specific antibodies. Short-term PMA treatment induced translocation of PKC-α, βI, βII, δ, ε and θ from cytosol to membrane and attenuated 5-HT-induced responses in TSMCs. In contrast, during long-term exposure to PMA, these PKC isozymes underwent down-regulation to a level below those of control cells. However, PKC-ζ may not be significantly changed during treatment of TSMCs with PMA, since this PKC isozyme was not activated by DAG or phorbol esters (Wilkinson & Hallam, 1994). We further looked into a down-regulation protocol to investigate the roles of the PKC isozymes in the inhibition of responses to 5-HT by PMA. Extended (24 h) treatment resulted in down-regulation of these PKC isozymes and the 5-HT-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). Phorbol esters are shown to activate PKC and to increase the rate of degradation (Young *et al*., 1987), with possible differences in sensitivities between isoforms (Nishizuka, 1992).

PMA inhibited 5-HT-induced IP_3 accumulation in canine cultured TSMCs in a time-dependent manner. PMA did not affect the basal level of IP_3, thus ruling out the possibility that PMA caused its effect by depleting an agonist-sensitive pool of membrane PI. In addition, PMA did not change the resting level of $[Ca^{2+}]_i$. The inhibitory effect of PMA on 5-HT-induced Ca^{2+} mobilization appeared to be directly related to the inhibition of IP_3 formation.

One site at which hormone-stimulated PI hydrolysis could be inhibited by PMA is located at the receptor level. It has been shown that phorbol esters induce phosphorylation of α_1-adrenoceptors 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 of TSMCs with PMA for either 2 or 24 h does not change the B_{max} or K_D values of muscarinic receptors in canine TSMCs (Yang *et al*., 1994b). It seems that the 5-HT receptor is not a site for the inhibitory effect of PMA on 5-HT-induced responses. The ability of PMA to block histamine-stimulated accumulation of IPs also suggests that the target of PMA is a more general component of the PI cycle than a specific receptor (Murray *et al*., 1989; Daykin *et al*., 1993), 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 block vasopressin-induced IP_3 accumulation in A10 cells without changing receptor binding and abolish the guanine nucleotide shift, indicating that coupling of the receptor to Gp is 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 (Bennett & Crooke, 1987). Regardless of the precise mechanism, the implication of a post-receptor site for phorbol ester inhibition is that other agonists that act through PI-PLC stimulation might be uncoupled from cytosolic Ca^{2+} mobilization.

The inhibition of 5-HT-stimulated increase in $[Ca^{2+}]_i$ by PMA is in agreement with the inhibitory effect of PMA on agonist-induced IP_3 accumulation and Ca^{2+} mobilization in TSMCs (Murray *et al*., 1989). The fact that the same experimental conditions block IP_3 accumulation and Ca^{2+} mobilization suggests that the mechanism of 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 inhibits 5-HT-induced IP_3 accumulation and Ca^{2+} mobilization in canine TSMCs, while long-term PMA treatment is associated with a down-regulation of PKC and the loss of its inhibitory function. The inhibition by PMA of 5-HT-induced responses was associated with translocation and down-regulation of PKC-α, βI, βII, δ, ε and θ from cytosol to membrane. These results suggest that physiological activation of PKC might serve as a modulator of cellular responses induced by IP_3. The site of PMA inhibition appears to be at a post-receptor location and may be involved in PI-PLC itself.

This work was supported by grants CMRP-680 from Chang Gung Medical Research Foundation and NSC85-2331-B182-114M10 from National Science Council, Taiwan. The authors are greatly indebted to Dr Anthony Herp at Chang Gung College of Medicine and Technology for his critical reading of the manuscript and suggestions.

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(Received February 20, 1997
Accepted March 17, 1997)