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Phorbol Dibutyrate Induces Contractions in Bovine Cerebral Arteries by an Extracellular Calcium-independent Mechanism

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Abstract—The aim of the present study was to analyse the ability of phorbol 12,13-dibutyrate (PDB) to activate protein kinase C (PKC), measured by its capacity to translocate the enzyme from the cytosol to the membrane fraction, as well as to induce vasconstrictive responses in segments from branches of bovine cerebral arteries. PDB (0·1 μM) produced a marked translocation of PKC activity from the cytosolic to the membranous fraction. This drug induced concentration-dependent contractions which were slow in onset. The contraction elicited by PDB was reduced by the PKC inhibitor, staurosporine (1 and 10 nM), but unaltered by both Ca^{2+}-free medium containing 3 mM EGTA and the Ca^{2+}-channel antagonist, nifedipine (1 μM). Preincubation of segments with PDB (10 and 30 nM) reduced the vasoconstriction elicited by 5-hydroxytryptamine (5-HT) in a concentration- and preincubation time-dependent manner. These data indicate that bovine cerebral arteries possess cytosolic and membranous PKC activities, that the vasoconstrictive responses induced by PDB were independent of extracellular Ca^{2+}, that cytosolic C-kinase is translocated to the membrane and probably down-regulated by PDB, and that this enzyme is not involved in 5-HT responses, but is down-regulated by PDB.

Ca^{2+}-activated, phospholipid-dependent protein kinase (protein kinase C, PKC) plays an important role in transmembrane signalling (Nishizuka 1984; Abdel-Latif 1986; Haller et al 1990). PKC is an ubiquitous protein demonstrated to be present at high concentrations in smooth muscle (Nishizuka 1984; Kariya & Takai 1987). The unique manner in which PKC is activated is by its spatial translocation from the cytosol, mainly located in basal conditions, to the membrane by agonist stimulation. The physiologic activator of this enzyme is diacylglycerol (Nishizuka 1984; Abdel-Latif 1986; Haller et al 1990). This activator and inositol 1,4,5,-trisphosphate (IP_3) (intracellular messengers) are formed on stimulation of receptor by agonists; IP_3 causes Ca^{2+} release from intracellular compartments, and diacylglycerol and IP_3 complement each other to produce cellular responses in several tissues (Nishizuka 1984; Abdel-Latif 1986; Bell 1986). Furthermore, it has been suggested that PKC activation is responsible for the tension-maintenance phase of smooth muscle contraction (Rasmussen et al 1987).

Tumour-promoting phorbol esters are more potent activators of PKC than diacylglycerol (Castagna et al 1982; Nishizuka 1984; Abdel-Latif 1986). These agents usually produce both smooth muscle contraction (Rasmussen et al 1984; Baraban et al 1985; Itoh & Lederis 1987; Singer & Baker 1987) and an increase or reduction in the responsiveness to agonists (Baraban et al 1985) or K^+ (Rembold & Murphy 1988). On the other hand, phorbol ester-induced contractions are independent (Sybertz et al 1986; Jiang & Morgan 1987), partially dependent (Singer & Baker 1987; Jiang & Morgan 1987; Itoh & Lederis 1987) or dependent (Rasmussen et al 1984) on extracellular Ca^{2+}.

Recently, it has been reported that PKC is involved in the vasospasm following subarachnoid haemorrhage (Matsui et al 1991) and also in the changes in contractile responses elicited by agonists in this pathological condition (Kamata et al 1991). In addition, 5-hydroxytryptamine (5-HT), in contrast to noradrenaline, produces potent vasoconstrictor responses in the cerebrovascular bed (Marín et al 1979; Edvinsson et al 1985; Van Nueten et al 1985), and it is implicated in the vasospasm following subarachnoid haemorrhage (Allen et al 1976). To our knowledge little is known about the involvement of PKC in the regulation of contractile apparatus and the mechanism of action of phorbol esters in bovine cerebral arteries. Consequently, we studied the ability of the potent PKC activator phorbol 12,13-dibutyrate (PDB) (Castagna et al 1982; Nishizuka 1984) to cause translocation of PKC activity from the cytosol to the membrane fraction, and the mechanisms implicated on the responses elicited by this phorbol, and the capacity of PDB to modify the contractions caused by 5-HT, activating C-kinase (Abdel-Latif 1986).

Materials and Methods

Drugs and chemicals

The drugs and chemicals used were: phorbol 12,13-dibutyrate, 5-HT creatinine sulphate, HEPES, phenylmethylsulphonylfluoride, leupeptin, Nonidet P-40, phosphatidylserine (brain extract, type III: Folch Fraction III), 1,2-dioleoyl-*rac*-glycerol and histone HI (Sigma Chemical Co., St Louis, MO, USA), phentolamine hydrochloride (Ciba-Geigy, Basel, Switzerland), staurosporine (Boehringer Mannheim, Mannheim, Germany), nifedipine (gift from Bayer, Leverkusen, Germany), and [^{32}P]ATP (Amersham, Buckinghamshire, UK).

PKC translocation

Bovine cerebral arteries of two to three animals were isolated and placed in a Petri dish containing Krebs-Henseleit

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solution (KHS) at 4°C. Adherent tissues and endothelium were carefully removed. Arteries were cut in 5–10 mm segments and pooled. Pooled arteries, 50–100 mg, were added to 5 mL of KHS containing 5 mM glucose in 20 mL incubation vials, in which the air phase was substituted by a 95% O_2–5% CO_2 mixture. Arteries were incubated in a shaking water-bath at 37°C for 20 min, and then PDB (0·1 μM) or vehicle was added and maintained for 5 min. The time was reported as that which produces the maximum translocation from the cytosol to the membrane (Weiss et al 1989; Haller et al 1990). At the end of the incubation period, the arteries were frozen with liquid nitrogen and kept at −70°C until analysis. Before determination of PKC activity, some segments of arteries isolated from each brain were used to assess the ability of 0·1 μM PDB to induce vasocontractile responses. When this agent induced usual contractions, the rest of the cerebral arteries of the corresponding brain were used in experiments of C-kinase activation. This was the case in the great majority of vascular preparations used.

For enzyme extraction, the arteries were homogenized in a Polytron blender in 2 mL of a medium containing 0·3 M sucrose, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES) pH 7·5, 2 mM EDTA, 2 mM EGTA, 10 mM β-mercaptoethanol, 0·1% trasylol, 1 mM phenylmethylsulphonylfluoride and 10 μM leupeptin. The homogenate was centrifuged at 100 000 *g* for 1 h and the supernatant taken as the cytosolic fraction. The sediment was resuspended in the homogenization medium containing 1% Nonidet P-40, sonicated, and incubated on an ice bath for 30 min. It was then centrifuged at 100 000 *g* for 1 h and the supernatant was taken as the membranous fraction.

PKC was partially purified from both supernatants by DEAE chromatography according to Takai et al (1979). Extracts were applied to DE-52 (Whatman) columns equilibrated with homogenization medium and eluted with 3 mL of 200 mM NaCl. More than 90% PKC from the extract was recovered in this fraction. The assay was made essentially according to Kishimoto et al (1980). Briefly, incorporation of ^{32}P from [^{32}P]ATP into histone HI was allowed for 20 min at 30°C in a total volume of 80 μL containing 20 mM HEPES pH 7·5, 50 μg histone HI, 5 μM [^{32}P]ATP (0·5 μCi), 5 μM magnesium acetate, 1 mM $CaCl_2$, 5 mM β-mercaptoethanol, 0·1 μg 1,2-dioleoyl-*rac*-glycerol and 4 μg phosphatidylserine. A blank was performed for each sample; for this, an aliquot was incubated in the same conditions, but in the absence of phosphatidylserine and 1,2-dioleoyl-*rac*-glycerol. The counts obtained in each blank were substracted from the counts of the sample; samples and blanks were processed in duplicate. At the end of the incubation, an aliquot was added to ice-cold 10 mM trichloroacetic acid adsorbed in phosphocellulose paper and was stirred for 30 min. The trichloroacetic acid solution was changed three times, and the phosphocellulose pieces of paper were washed with ethanol, air dried, and measured in a scintillation counter to determine the incorporation of ^{32}P into protein.

Vascular reactivity

Branches of middle cerebral arteries were placed in a Petri dish containing KHS at 4°C and divided into segments of 5 mm in length. Each arterial cylinder was prepared for isometric tension recording in an organ bath according to the method of Nielsen & Owman (1971). The organ bath contained 5 mL of KHS at 37°C continuously bubbled with a 95% O_2–5% CO_2 mixture, which gave a pH of 7·4. Two stainless steel pins were passed through the lumen of the arterial segment. One pin was fixed to the organ bath wall, while the other was connected to a strain gauge for isometric tension recording. The latter pin was placed parallel with the former and was movable, permitting the application of resting tension in a perpendicular plane to the long axis of the vascular cylinder. The isometric contraction was recorded through a force-displacement transducer (Grass FTO3C) connected to a Grass model 7D polygraph. A resting tension of 1 g (optimal resting tone, i.e. the tone which produces maximal response to 75 mM K^+) was applied to cylindrical segments. This tension was readjusted every 15 min during 60–90 min equilibration period (for base line stabilization), before addition of the drugs.

The segments were initially exposed to 75 mM K^+ to test their functional integrity. Afterwards, the bath medium was changed several times until the resting tone was achieved. Then, either single PDB concentrations were added to the bath, or a concentration–response curve to this agent was performed. Endothelium influence on the phorbol response was analysed by careful removal of the endothelium from arterial segments, by introducing an appropriate stainless-steel rod through the lumen. The effective removal was tested by the inability of acetylcholine (1 μM) to produce vasodilation in segments precontracted with 5-HT (0·1 μM). The effects of staurosporine (1 and 10 nM), nifedipine (1 μM) and phentolamine (1 μM) on the actions of PDB were investigated by addition of these agents to the bath 15 min in advance. The influence of extracellular Ca^{2+} on the responses induced by PDB was also assessed. For this purpose, the arteries were incubated for 20 min in Ca^{2+}-free KHS containing 1 or 3 mM EGTA.

The influence of PDB on 5-HT (0·01 and 0·1 μM)-induced contractions was studied. For this purpose, three consecutive cumulative additions, separated by an interval of 30 min, of these 5-HT concentrations were carried out in a group of segments; these experiments were performed to demonstrate that the responses were maintained. Afterwards, different PDB concentrations (10 or 30 nM) were applied to the segments, and when the responses reached a plateau, 5-HT was added cumulatively. The effect of exposure time (0·5 and 1 h) to PDB (10 and 30 nM) on 5-HT responses was also studied. In this case, segments were exposed to the phorbol ester for 0·5 and 1 h, and the above 5-HT concentrations were then added. The arterial segments were subsequently washed out with KHS until base line recovery. Subsequently, the 5-HT process was repeated at 0·5 h intervals to determine the degree of contraction recovery.

Solutions and statistics

The composition of KHS was as follows (mM): NaCl 115, $CaCl_2$ 2·5, KCl 4·6, KH_2PO_4 1·2, $MgSO_4 \cdot 7H_2O$ 1·2, $NaHCO_3$ 25, glucose 11·1, Na_2 EDTA 0·03 (to prevent the oxidation of unstable substances). The composition of Ca^{2+}-free medium was similar except that $CaCl_2$ and Na_2EDTA were omitted and 1 or 3 mM EGTA was added. Stock solutions (all at 10 mM) of nifedipine were prepared in 99·5% ethanol and kept at −20°C, and those of PDB and

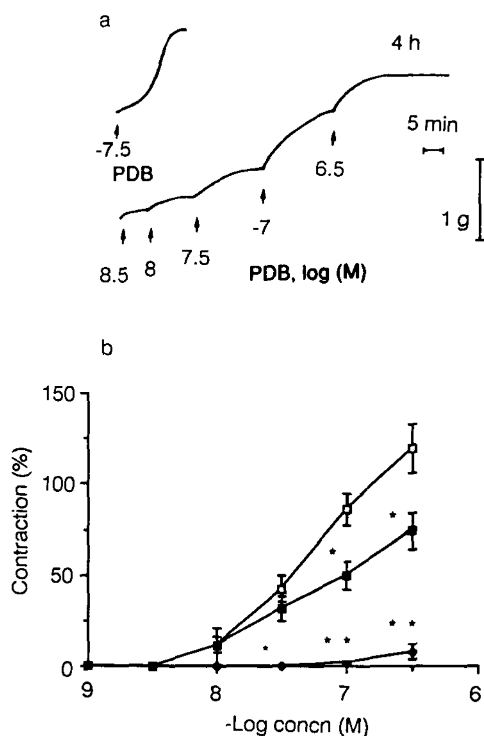

FIG. 1. a. Representative tracings showing the action of a single concentration of PDB and a concentration-response curve to PDB in segments from branches of bovine middle cerebral arteries. b. The effect of staurosporine on the concentration–response curve to PDB. Responses (means ± s.e.m.) are expressed as percentage of concentration induced by 75 mM K^+ (1765 ± 120 mg). $*P<0.05$; $**P<0.001$. □Control, n = 11; ■ 1 and ◆ 10 nM staurosporine, both n = 5.

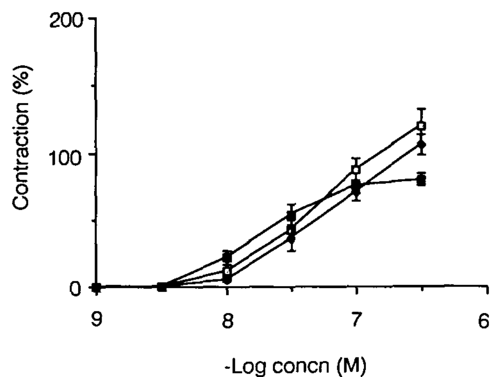

FIG. 2. Effect of nifedipine and EGTA concentration in Ca^{2+}-free medium on the contractions elicited by PDB in segments from branches of bovine middle cerebral arteries. Responses (means ± s.e.m.) are expressed as percentage of contraction induced by 75 mM K^+ (1780 ± 195 mg). □ Control, n = 11; ■ Ca^{2+}-free 3 mM EGTA, n = 6; ◆ 1 μM nifedipine, n = 6.

staurosporine were dissolved in dimethylsulphoxide and kept at −78°C. Dimethylsulphoxide did not produce any effects on these arteries. The solutions of nifedipine and staurosporine were light-protected, and the experiments with these drugs were performed under sodium light.

Contractions were expressed as a percentage of responses induced by 75 mM K^+. Results are given as means ± s.e.m. Statistical analysis was by Student's *t*-test for paired or unpaired experiments; a *P* value of less than 0·05 was considered significant.

Results

Effects of PDB on PKC translocation

In spite of great care taken in artery manipulation, it was observed that in 50% of the experiments, PKC was translocated to the membrane or even practically absent in both cytosolic and membrane fractions; we do not know the mechanism by which this phenomenon occurs. In the remaining experiments, at least 50% of PKC activity in the control was found in the cytosolic fraction. The basal value of PKC activity (taken as 100%) in the cytosol was 414 ± 89·6 and in the membrane, 187 ± 7·7 pmol min^{-1} (mg protein)$^{-1}$. The incubation of the arteries for 5 min with PDB (0·1 μM) increased the PKC activity in the membrane (284 ± 42, $P<0.05$) and concomitantly reduced that present in the cytosol (165 ± 23 pmol min^{-1} (mg protein)$^{-1}$, $P<0.05$).

PDB-induced contractile responses

The contractions induced by single (30 nM) or cumulative PDB concentrations (1–300 nM) were slow-developing sustained (more than 4 h) contractions and were not modified by a prolonged washout period (Fig. 1a). PDB responses were not affected by endothelium removal or phentolamine pretreatment (1 μM). Indeed, EC50 values (PDB concentrations producing 50% of 75 mM K^+-induced contractions) were: control, 51·2 ± 14·1; endothelium removal, 52·1 ± 12·2; phentolamine, 50·5 ± 12·3 nM, and maximal contractile responses were: control, 1502 ± 160; endothelium removal, 1615 ± 158; phentolamine, 1683 ± 135 mg, n = 6 − 11. Staurosporine (1 and 10 nM) reduced the contractions elicited by different concentrations (Fig. 1b). At 1 nM, 75 mM K^+-produced responses were not altered, but were reduced by 50% at 10 nM staurosporine (control, 1680 ± 190 mg; 10 nM, 740 ± 180 mg, n = 5, $P<0.05$).

Nifedipine (1 μM) did not modify the contraction induced by any of the PDB concentrations used (Fig. 2). This Ca^{2+} antagonist markedly reduced the contractions induced by 75 mM K^+ (control, 1980 ± 170 mg; nifedipine, 410 ± 90 mg, n = 4, $P<0.001$). The effect of the exposure of the segments for 20 min to Ca^{2+}-free KHS containing 1 or 3 mM EGTA on the PDB-, 5-HT (0·01 and 0·1 μM)-induced contractions was explored; at 1 mM EGTA, the contractions elicited by 5-HT were practically abolished (control 0·01 and 0·1 μM 5-HT, 175 ± 90 and 958 ± 391 mg, and in Ca^{2+}-free with 1 mM EGTA, 0 ± 0 and 8 ± 5 mg, respectively; n = 4, $P<0.001$), whereas those evoked by PDB were not affected, even with 3 mM EGTA (Fig. 2). The EGTA concentration did not damage the vascular smooth muscle, since 75 mM K^+-elicited contractions were not changed when the medium was substituted by normal KHS (results not shown).

Influence of PDB on 5-HT-induced contractions

The exposure to 30 nM PDB for 1 h (according to the protocol described in Material and Methods) abolished the

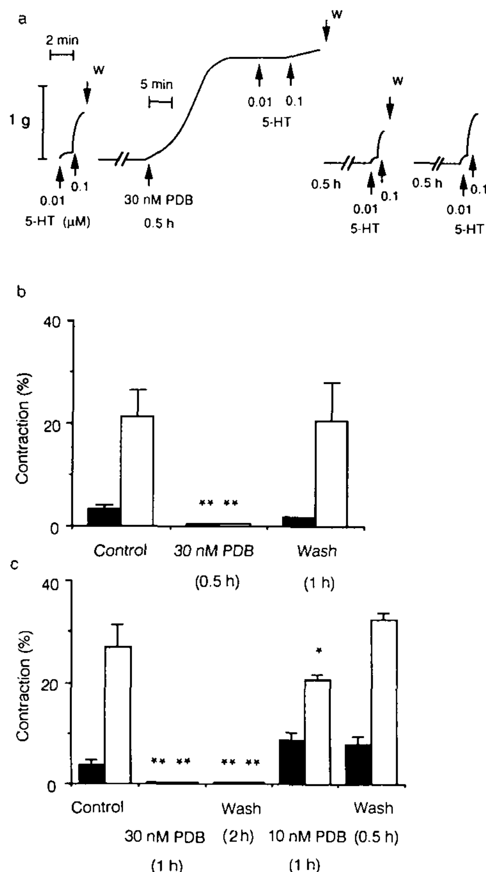

FIG. 3. a. Representative tracing of at least 5 experiments showing the effect of preincubation for 0·5 h with PDB (30 nM) on the responses to 5-HT (0·01 and 0·1 μM) in segments from branches of bovine middle cerebral arteries as well as the time period needed for recovering the initial 5-HT responses. b and c. Effect of incubation time (0·5 or 1 h) with PDB (10 and 30 nM) on the contractions to 5-HT (■ 0·01, □ 0·1 μM), and the washout period necessary to recover the initial 5-HT responses. Responses (mean ± s.e.m.) are expressed as percentage of contraction induced by 75 mM K^+ (1905 ± 190 mg). (n = 5–8), W = washed. *$P < 0·05$; **$P < 0·001$.

contractions elicited by 5-HT (0·01 and 0·1 μM), which were not recovered after a 2 h washout. However, when the incubation time was 0·5 h, 5-HT responses were abolished, and the washout period necessary to recover completely the initial 5-HT contraction was 1 h (Fig. 3). In another set of experiments, PDB concentration was reduced to 10 nM and the incubation time was fixed to 1 h. In these conditions, the contractions elicited by 0·1 μM 5-HT were reduced; the washout period required to recover the initial contractions of 5-HT was 0·5 h (Fig. 3).

Discussion

The present results show that, in those bovine cerebral arteries in which PKC was not down-regulated, about 50% of the total PKC activity was present in the cytosol. A high cytosolic fraction of PKC activity (85%) was found in bovine cerebral microvessels (Catalán et al 1989), whereas in brain microvessels from adult rats, PKC activity was found in the membrane fraction (Markovac & Goldstein 1988). Taking into account that in most tissues the effect of phorbol esters involves PKC translocation from the cytosol to the plasma membrane (Nishizuka 1984; Abdel-Latif 1986; Rana & Hokin 1990; Haller et al 1990), we studied whether this process takes place in bovine cerebral arteries. Incubation of the vessels for 5 min with PDB (0·1 μM) resulted in a redistribution of PKC activity from the cytosol into the membrane, suggesting that PKC is operative. Similar effects have been obtained with certain phorbol esters in other vessels (Catalán et al 1989; Haller et al 1990).

PDB induced potent slowly-developing contractions in segments of bovine cerebral arteries. The responses elicited by PDB were sustained, which is the usual response caused by phorbol esters (Rasmussen et al 1984; Rembold & Murphy 1988; Salaíces et al 1990), although relaxation or no effect has also been reported (Baraban et al 1985; Menkes et al 1986). The contractile responses caused by PDB were unaltered by the α-adrenoceptor antagonist phentolamine, and by endothelium removal, since the concentration–response curve was similar to that obtained in the control situation. Hence, PDB does not appear to act indirectly by release of noradrenaline or endothelium-derived contracting factor, but by direct action on the smooth muscle.

Staurosporine, a drug with relative selectivity to inhibit PKC (Tamaoki et al 1986; Rüegg & Burgess 1989), reduced PDB-elicited contractions. The smallest staurosporine concentration (1 nM) used failed to alter the contraction elicited by 75 mM K^+. These findings, together with the fact that PDB is a potent and specific activator of PKC (Castagna et al 1982; Nishizuka 1984), and that it is able to activate this enzyme by translocation from the cytosol to the membrane, suggest that this vasoconstrictive agent appears to produce its effects mainly by PKC activation.

Ca^{2+}-free medium containing 3 mM EGTA did not alter the contractile responses induced by PDB. The Ca^{2+}-entry blocker nifedipine, which has selectivity for voltage-operated Ca^{2+} channels (VOCs) (Cauvin et al 1983; Marín 1988), did not modify the contraction elicited by PDB, although K^+ (75 mM)-evoked responses were strongly inhibited. These results indicate that the vasoconstriction induced by PDB may be due to intracellular Ca^{2+} mobilization by the phorbol (Sybertz et al 1986; Jiang & Morgan 1987), direct phosphorylation of the myosin light chain by activated PKC (Abdel-Latif 1986), and smooth muscle activation by mechanisms independent of myosin light chain phosphorylation (Chatterjee & Tejada 1986).

The present study also attempted to determine whether PKC participates in the responses elicited by 5-HT, a potent vasoconstrictor agonist of cerebral arteries that causes a rapid hydrolysis of phosphatidylinositol 4,5-bisphosphate to produce diacylglycerol and IP_3. For this purpose, we can use PKC inhibitors, such as staurosporine (Hidaka et al 1984; Tamaoki et al 1986; Rüegg & Burgess 1989) or long-term exposure of the tissues to phorbol esters, resulting in a down-regulation or depletion of the C-kinase, which may feed-back to terminate phospholipase C-mediated IP_3 formation (Rana & Hokin 1990; Karibe et al 1991). Long-term exposure to phorbol esters usually reduces or abolishes the responses induced by several agonists which activate PKC (Baraban et al 1985; Lefkowitz & Caron 1986; Rana & Hokin 1990; Salaíces et al 1990). This depressor effect was observed in the

response caused by 5-HT in bovine cerebral arteries. Such an effect was directly dependent on both PDB concentration and incubation time, while the reversibility of the phenomenon was inversely related to both parameters. These findings suggest that the increased changes on the receptors or the processes involved in the smooth muscle contraction and down-regulation of PKC are directly related to the level of PKC activation. Such a desensitization may be specific for agonists, but not for K^+, since the contractions elicited by K^+ were not affected by PDB (results not shown). This indicates that VOCs, stimulated by K^+, are not modified by PKC, as reported (Baraban et al 1985; Salaíces et al 1990). It is interesting to note the possible pathological implications of PKC in cerebral arteries; it has been reported that this enzyme participates in the prolonged vasospasm that appears in the subarachnoid haemorrhage (Matsui et al 1991).

5-HT responses were reduced in Ca^{2+}-free medium, whereas C-kinase activation by PDB produced responses in this medium (even containing 3 mM EGTA). This suggests that the main mechanisms whereby PDB and the agonist 5-HT produce their effects are different. Indeed, phorbol esters directly activate PKC, whereas the agonists produce an activation of phospholipase C with subsequent formation of diacylglycerol and IP_3 and opening of Ca^{2+} channels (Nishizuka 1984; Rasmussen et al 1984; Abdel-Latif 1986). In addition, phorbol esters have the ability to activate PKC at resting intracellular Ca^{2+} levels (0·1 μM) and may remain active in the absence of Ca^{2+} (Nishizuka 1984; Rasmussen et al 1984; Abdel-Latif 1986). However, 5-HT responses are more dependent on extracellular Ca^{2+}, and intracellular Ca^{2+} stores used by the agonist are more rapidly depleted under these conditions. All these results suggest that PKC may not play an important role in 5-HT-induced contraction, but it may participate in the 5-HT-receptor desensitization, as reported for other agonists (Jiang & Morgan 1987; Salaíces et al 1990).

In summary, PDB activates PKC of bovine cerebral arteries resulting in a translocation of PKC activity from the cytosol to the membrane. This mechanism appears to be involved in the generation of slow-developing sustained contractile responses. These responses are dependent on intracellular Ca^{2+} and independent of endothelium. In addition, PKC minimally affects 5-HT contraction, and is down-regulated by PDB; the latter effect is involved in the desensitization of 5-HT response caused by PDB.

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