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The structural requirements for phorbol esters to enhance noradrenaline and dopamine release from rat brain cortex

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1 The effects of various protein kinase C (PKC) activators on the stimulation-induced (S-I) release of noradrenaline and dopamine was studied in rat cortical slices pre-incubated with [^{3}H]-noradrenaline or [^{3}H]-dopamine. The aim was to investigate a possible structure-activity relationship for these agents on transmitter release.

2 4β-Phorbol 12,13-dibutyrate (4βPDB, 0.1-3.0 μM), enhanced S-I noradrenaline and dopamine release in a concentration-dependent manner whereas the structurally related inactive isomer 4α-phorbol 12, 13-dibutyrate (4αPDB, 0.1-3.0 μM) and phorbol 13-acetate (PA, 0.1-3.0 μM) were without effect on noradrenaline release. Another group of phorbol 12, 13-diesters containing a common 13-ester substituent (phorbol 12, 13-diacetate, PDA, 0.1-3.0 μM; phorbol 12-myristate 13-acetate, PMA, 0.1-3.0 μM; phorbol 12-methylaminobenzoate 13-acetate, PMBA, 0.03-3.0 μM) also enhanced S-I noradrenaline and dopamine release in a concentration-dependent manner with PMA being the least potent.

3 The 12-deoxyphorbol 13-substituted monoesters, 12-deoxyphorbol 13-acetate (dPA, 0.1-3.0 μM), 12-deoxyphorbol 13-angelate (dPAng, 0.1-3.0 μM), 12-deoxyphorbol 13-isobutyrate (dPiB, 0.03-3.0 μM) and 12-deoxyphorbol 13-phenylacetate (dPPhen, 0.1-3.0 μM) enhanced S-I noradrenaline and dopamine release in a concentration-dependent manner. In contrast, 12-deoxyphorbol 13-tetradecanoate (dPT, 0.1-3.0 μM) was without effect.

4 The involvement of PKC in mediating the effects of the various phorbol esters was further investigated. PKC was down-regulated by 20 h exposure of the cortical slices to 4β-phorbol 12,13-dibutyrate (1 μM). In this case the facilitatory effect of 4βPDB and dPA was abolished whilst that of dPAng was significantly attenuated. This indicates that these agents were acting selectively at PKC. In support of this the PKC inhibitors, polymyxin B (21 μM) and bisindolylmaleimide I (3 μM), attenuated the facilitatory effect of 4βPDB and dPAng although that of dPA was not significantly altered.

5 The effects of these agents on transmitter release were not correlated with their *in vitro* affinity and isozyme selectivity for PKC. Short chain substituted mono- and diesters of phorbol were more potent enhancers of action-potential evoked noradrenaline and dopamine release than the long chain esters. Interestingly, these former agents are the least potent or non effective (e.g. dPA, PDA) tumour promoters. We suggest that the reason for the poor effects of lipophilic long chain phorbol esters (PMA, dPT) on transmitter release is that they are sequestered in the plasmalemma and do not access the cell cytoplasm where the PKC may be located.

Keywords: Phorbol esters; protein kinase C; noradrenaline release; dopamine release; brain cortex

Introduction

Tumour promoting phorbol esters enhance action-potential evoked transmitter release from a variety of neurones (e.g. Nichols *et al.*, 1987; Allgaier *et al.*, 1988; Musgrave *et al.*, 1991). Since these drugs activate protein kinase C (PKC) analogous to the endogenous activator, diacylglycerol (Bell & Burns, 1991), it is likely that this family of enzymes is involved in the effect. Indeed both PKC inhibitors (Versteeg & Ulenkate, 1987; Daschmann *et al.*, 1988; Musgrave & Majewski, 1989) and PKC down-regulation (Foucart *et al.*, 1991; Schroeder *et al.*, 1995) can prevent or reduce the effects of phorbol esters on transmitter release. Phorbol esters have diverse PKC-mediated biological effects including tumour promotion (Hecker, 1971; Hergenhahn *et al.*, 1974; Brooks *et al.*, 1989) and skin inflammation (Schmidt & Evans, 1980; Zayed *et al.*, 1984; Brooks *et al.*, 1989) making therapeutic application of their property to enhance transmitter release difficult. The structure-activity requirements for tumour promotion are partially known (Baird & Boutwell, 1971; Hecker, 1971; Fürstenberger & Hecker, 1972; Hergenhahn *et al.*, 1974; Zayed *et al.*, 1984; Brooks *et al.*, 1989) and these seem to differ from those underlying skin inflammation (Schmidt & Evans, 1980; Zayed *et al.*, 1984; Brooks *et al.*, 1989). However, little is known about the structural requirements of phorbol esters for transmitter release effects.

Several developments suggest that targeted biological actions of PKC activators may be possible. Firstly, molecular cloning has demonstrated the existence of multiple isozymes of PKC: cPKC (α, β_1, β_2, γ which are Ca^{2+}-dependent, phorbol ester sensitive), nPKC (δ, ε, η, θ, μ which are Ca^{2+}-independent, phorbol ester sensitive) and aPKC (ζ, λ, ι which are Ca^{2+}-independent, phorbol ester insensitive) (Nishizuka, 1992; Dekker & Parker, 1994). These isozymes appear to be differentially distributed (Mochly-Rosen *et al.*, 1991; Wetsel *et al.*, 1992; Leach *et al.*, 1992) and perhaps fulfil different functions (Buchner, 1995). Secondly, there are multiple phorbol ester receptors (e.g. Dunn & Blumberg, 1983) and it is still not known if these are linked to different isozymes.

There are suggestions in the literature that the effects of phorbol esters in neurones may be different from that in other tissues. For example, phorbol 12-myristate 13-acetate (PMA) is more potent than phorbol 12, 13-dibutyrate (4βPDB) in activating PKC *in vitro*. The K_d for PMA is 0.06 nM and that for 4βPDB is 0.4 nM for rat brain PKC (Kazanietz *et al.*, 1992). Nevertheless, it has frequently been observed that 4βPDB is more potent and more effective in enhancing noradrenaline release than PMA (rabbit hippocampus, Allgaier *et*

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al., 1987; mouse atria, Musgrave *et al.*, 1991; canine saphenous vein, Takata *et al.*, 1991; mouse brain cortex, Schroeder *et al.*, 1995). It may be that noradrenergic neurones possess isozymes of PKC that are selective for 4βPDB or have other characteristics which confer selectivity for 4βPDB.

The aim of the present study was to explore further the structural requirements for PKC activators in enhancing transmitter release from noradrenergic and dopaminergic neurones. The first group of drugs used were those thought to show some isozyme selectivity (thymeleatoxin α, β, γ selective and 12-deoxyphorbol 13-phenylacetate 20-acetate β_1 selective, see Evans *et al.*, 1991; Ryves *et al.*, 1991; Kazanietz *et al.*, 1993). The second group consisted of phorbol esters which possessed different ester substituents to enable structure-activity questions to be answered including a series of phorbol 12, 13-diesters and a series of 12-deoxyphorbol 13-monoesters. Finally, we examined a group of structurally diverse agents which have been shown to bind and/or activate PKC (mezerein, Brooks *et al.*, 1989; Kazanietz *et al.*, 1993), 5-chloro-N-heptylnaphthalene-1-sulphonamide (SC-10, Ito *et al.*, 1986), 3-(N-acetylamino)-5-(N-decyl-N-methylamino) benzyl alcohol (ADMB, Wender *et al.*, 1986) and resiniferonol 9, 13, 14-orthophenylacetate (ROPA, Szallasi *et al.*, 1989). The studies were performed in rat cortex brain slices which were pre-incubated with either [^{3}H]-noradrenaline or [^{3}H]-dopamine and the electrically-evoked release of radioactivity was used as an index of noradrenaline and dopamine release, respectively.

Methods

Noradrenaline and dopamine release from rat cerebral cortex

Outbred Sprague-Dawley rats (150–250 g) were decapitated and the brains rapidly excised and placed in ice-cold physiological salt solution (PSS) previously gassed with a mixture of 5% CO_2 and 95% O_2. Slices from cerebral cortex (400 μm thick) were obtained with a Campden vibroslice. Slices were incubated in PSS maintained at 37°C and gassed with a mixture of 5% CO_2 and 95% O_2 containing either [^{3}H]-noradrenaline (10 μCi ml^{-1}, 0.2 μM for 20 min) or [^{3}H]-dopamine (5 μCi ml^{-1}, 0.1 μM for 30 min). For dopamine release studies the noradrenergic neuronal uptake blocking agent, desipramine (0.3 μM), was present during the incubation with [^{3}H]-dopamine to prevent incorporation of [^{3}H]-dopamine into noradrenergic neurones (see Hoffmann *et al.*, 1988). Following incubation, the slices were rinsed, transferred to flow cells (4 cells per bank with electrodes connected in series) and continuously superfused at 0.5 ml min^{-1} with PSS (in the absence of ^{3}H transmitter) maintained at 37°C. The slices were superfused for 60 min before sample collection began (washing period). After 30 min of washing, an electrical priming stimulation was delivered through a pair of parallel platinum electrodes on either side of the brain slice (field strength 34 V cm^{-1}, 22 mA, square wave pulses of 2 ms duration at a frequency of 1 Hz for 60 s for noradrenaline release and 30 s for dopamine release). After the washing period was completed, the collection period began in which the superfusate fractions were collected over consecutive 5 min periods for a total of 120 min. At 10, 55, 80 and 105 min after the commencement of the collection period, the cortical slices were stimulated (each at 1 Hz for 60 s, S_1–S_4). The effect of PKC activators on the electrical stimulation-induced outflow of radioactivity was determined by adding them in increasing concentrations to the superfusate solution 15 min before the second, third and fourth stimulation. The PKC inhibitors, polymyxin B and bisindolylmaleimide I, were added to the superfusate solution 35 min before the second stimulation where they remained until the completion of the experiment. At the completion of the experiments the cortical slices were removed from the flow cells and placed in 0.5 ml Soluene (Packard Instruments, Melbourne, Australia) for 24 h to solubilise the tissue. The radioactivity present in the superfusate solution and brain slices were determined after the solutions were mixed with 3.0 ml Picofluor-40 (Packard Instruments, Melbourne, Australia) followed by liquid scintillation counting. Corrections for counting efficiency were made by external standardisation and results are expressed as disintegrations per min (d min^{-1}).

Long-term treatment with 4β-phorbol 12,13-dibutyrate

Rat cortical brain slices were prepared as described previously. The slices were placed in 50 ml modified PSS containing 0.1 mM Ca^{2+}, dextran (50 g l^{-1}, average MW = 70,000) and either 4β-phorbol 12, 13-dibutyrate (1 μM) or vehicle (dimethyl sulphoxide, DMSO 0.06% v:v) in an open dish and maintained at 32°C in a tissue culture incubator for 20 h. The atmosphere of the incubator was a mixture of 5% CO_2 and 95% O_2. At the end of the 20 h incubation, the brain slices were removed from the culture medium and washed in 100 ml PSS. The slices were then incubated with [^{3}H]-noradrenaline (10 μCi ml^{-1}, 0.2 μM for 20 min) in normal PSS and then followed the identical protocol as described for acute experiments.

Calculation of results

The resting (spontaneous) outflow of radioactivity for each stimulation period was taken as the radioactive content of the bathing solution during the 5 min period immediately before the start of the respective stimulation. The stimulation-induced (S-I) component of the outflow of radioactivity for S_1–S_4 was calculated by subtracting the resting radioactive outflow from the radioactive content of each of the two 5 min samples collected immediately after the commencement of each stimulation. These values were then expressed as a ratio of the radioactivity present in the tissue at the onset of the stimulation (the fractional S-I outflow, FR). Drug effects on the fractional S-I outflow of radioactivity were evaluated by comparing the ratio of FR_2/FR_1, FR_3/FR_1 and FR_4/FR_1.

Statistics

The values are given as mean and s.e.mean, n indicates the number of slices used; within each experimental group, the slices came from different animals and one experiment was performed on each tissue slice. The results were analysed with Scheffé's test after a two-way analysis of variance (ANOVA) or unpaired 2-tailed Student's t test with Bonferroni correction. Where appropriate two-way analysis of variance was also carried out to determine whether there was an interaction between the effects of the phorbol esters on transmitter release (A) and a PKC inhibitor or PKC down-regulation (B). In this case the significance was determined from an F-test on the interaction term A*B in the analysis of variance table. In all cases, a probability of falsely concluding that two identical means are different (type 1 error) of less than 5% ($P<0.05$) was taken to indicate statistical significance. The statistical package GB-Stat (Dynamic Microsystems, Silver Spring, U.S.A.) was used for analysis.

Materials

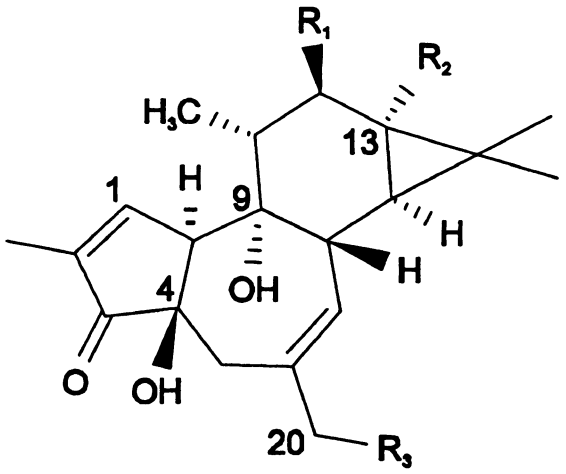
The physiological salt solution (PSS) consisted of (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.03, $NaHCO_3$ 25.0, D-(+)-glucose 11.1, $MgSO_4$ 1.2, $CaCl_2$ 1.3, ascorbic acid 0.14 and disodium EDTA 0.067.

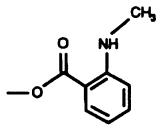
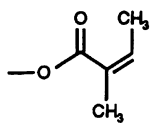
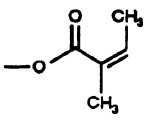
Radiochemicals and drugs

Drugs used were (−)-[ring-2,5,6-^{3}H]-noradrenaline (specific activity 43.7 Ci mmol^{-1}) and [ring-2,5,6-^{3}H]-dopamine (specific activity 53.2 Ci mmol^{-1}) (DuPont NEN Products; Boston, U.S.A.); 5-chloro-N-heptylnaphthalene-1-sulphonamide (SC-10) was obtained from Research Biochemicals Interna-

tional (Natick, U.S.A.); 4β-phorbol 12, 13-dibutyrate (4βPDB), 4α-phorbol 12, 13-dibutyrate (4αPDB), phorbol 12-myristate 13-acetate (PMA) and bisindolylmaleimide I hydrochloride (Bis I) were obtained from LC Laboratories (Woburn, U.S.A.); phorbol 13-acetate (PA), phorbol 12, 13-diacetate (PDA), phorbol 12-methylaminobenzoate 13-acetate (PMBA, sapintoxin-D), 12-deoxyphorbol 13-acetate (dPA), 12-deoxyphorbol 13-angelate (dPAng), 12-deoxyphorbol 13-isobutyrate (dPiB), 12-deoxyphorbol 13-phenylacetate (dPPhen), 12-deoxyphorbol 13-tetradecanoate (dPT, synonymous with 12-deoxyphorbol 13-myristate), 12-deoxyphorbol 13-phenylacetate 20-acetate (dPPhenA), 12-deoxyphorbol 13-angelate 20-acetate (dPAngA), thymeleatoxin (THYM), mezerein (MEZ), 3-(N-acetylamino)-5-(N-decyl-N-methylamino)

Table 1 Structures of the phorbol esters used in this study



Phorbol ester	*R_1 (C12)*	*R_2 (C13)*	*R_3 (C20)*
4β-Phorbol 12, 13-dibutyrate (4βPDB)	$-OCO(CH_2)_2CH_3$	$-OCO(CH_2)_2CH_3$	-OH
Phorbol 13-acetate (PA)	-OH	$-OCOCH_3$	-OH
Phorbol 12, 13-diacetate (PDA)	$-OCOCH_3$	$-OCOCH_3$	-OH
Phorbol 12-methylaminobenzoate 13-acetate (PMBA)		$-OCOCH_3$	-OH
Phorbol 12-myristate 13-acetate (PMA)	$-OCO(CH_2)_{12}CH_3$	$-OCOCH_3$	-OH
12-Deoxyphorbol 13-acetate (dPA)	-H	$-OCOCH_3$	-OH
12-Deoxyphorbol 13-angelate (dPAng)	-H		-OH
12-Deoxyphorbol 13-isobutyrate (dPiB)	-H	$-OCOCH(CH_3)_2$	-OH
12-Deoxyphorbol 13-phenylacetate (dPPhen)	-H	$-OCOCH_2C_6H_5$	-OH
12-Deoxyphorbol 13-tetradecanoate (dPT)	-H	$-OCO(CH_2)_{12}CH_3$	-OH
12-Deoxyphorbol 13-phenylacetate 20-acetate (dPPhenA)	-H	$-OCOCH_2C_6H_5$	$-OCOCH_3$
12-Deoxyphorbol 13-angelate 20-acetate (dPAngA)	-H		$-OCOCH_3$

benzyl alcohol (ADMB) and resiniferonol 9, 13, 14-orthophenylacetate (ROPA) were obtained from Sapphire Bioscience (Alexandria, Australia). Dextran, desipramine hydrochloride and polymyxin B sulphate were obtained from Sigma (St Louis, U.S.A.) and were dissolved and diluted with PSS. Stock solutions of the protein kinase C activators were initially made up in dimethyl sulphoxide (DMSO) and stored at −20°C. Similarly, bisindolylmaleimide I hydrochloride was made as a stock solution in H_2O and stored at −20°C. On the day of the experiment these drugs were further diluted in PSS. Throughout the text all 12-deoxyphorbol and phorbol derivatives are of the 4β configuration except in the case of 4α-phorbol 12,13-dibutyrate. Control experiments were conducted with the corresponding concentration of DMSO (up to 0.18% v:v).

Results

The structures and abbreviations used for all phorbol derived PKC activators are given in Table 1.

[^{3}H]-noradrenaline release from rat cerebral cortex

[^{3}H]-noradrenaline was incorporated into the noradrenergic transmitter stores of rat cerebral cortical slices and the electrical field stimulation evoked a stimulation-induced (S-I) outflow of radioactivity which was taken as an index of noradrenaline release. There were four periods of electrical stimulation (S_1, S_2, S_3, S_4 all at 1 Hz for 60 s) and the fractional S-I outflow in the first period (S_1) was 0.015 ± 0.001 ($n=11$), the fractional resting outflow immediately before S_1 was 0.016 ± 0.001 per 5 min ($n=11$) and the tissue radioactivity at the beginning of the sample collection was 427618 ± 44826 d min^{-1} ($n=11$). Both tetrodotoxin (0.3 μM) added for the second (S_2) stimulation and the removal of calcium from the bathing fluid (after addition of the Ca^{2+} chelator EGTA, 0.1 mM) almost abolished the fractional outflow of radioactivity in the second stimulation (control $FR_2/FR_1 = 0.94 \pm 0.03$, $n=11$; tetrodotoxin $FR_2/FR_1 = 0.15 \pm 0.02$*, $n=3$; zero Ca^{2+} $FR_2/FR_1 = 0.03 \pm 0.02$*, $n=3$; * significantly different from control, $P<0.05$, Student's t test with Bonferroni correction). This indicates that the S-I outflow involved the conduction of action-potentials and Ca^{2+} entry at the nerve terminal. None of these treatments affected the resting outflow of radioactivity (not shown).

Effect of phorbol esters on noradrenaline release

Concentration-response curves were constructed by superfusing tissue with increasing concentrations of phorbol esters during S_2-S_4. The phorbol 12, 13-diester, 4βPDB enhanced the fractional S-I outflow of radioactivity during S_2-S_4 in a concentration-dependent manner (Figure 1) whilst the structurally related but inactive isomer, 4αPDB, was without effect (Figure 1).

Another group of phorbol esters containing a common 13-acetate ester substituent: PDA, PMBA and PMA also enhanced the fractional S-I outflow of radioactivity during S_2-S_4 in a concentration-dependent manner (Figure 1). However, phorbol 13-acetate (PA) was without effect (Figure 1). For the phorbol 12, 13-diesters with a 13-acetate (PDA, PMBA, PMA), the degree of enhancement and the potency (Figure 1, Table 2) were significantly less for PMA which had the lar-

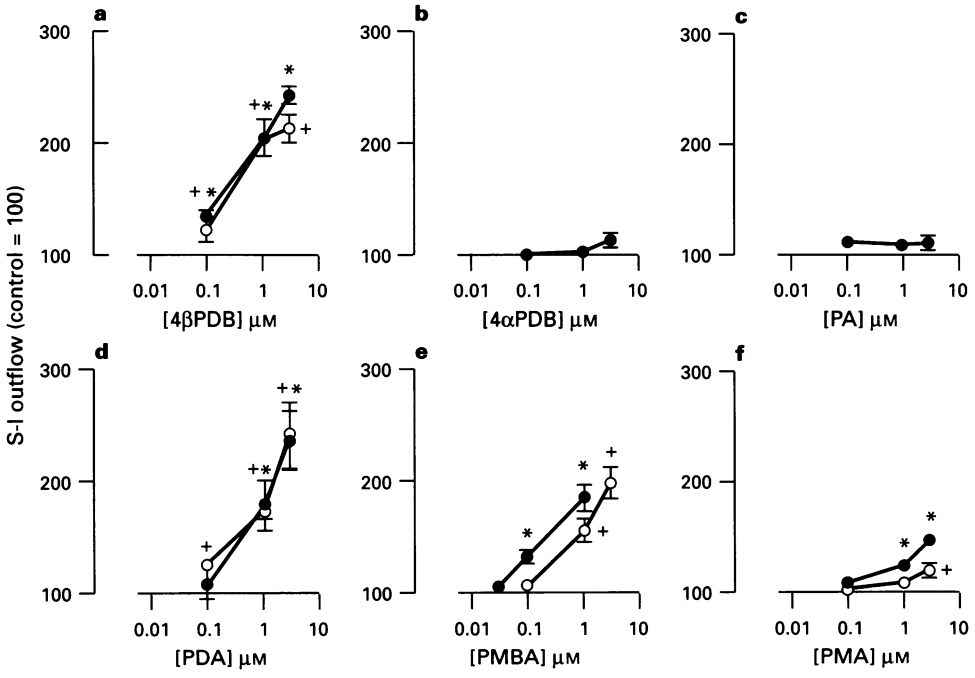


Figure 1 The effect of phorbol esters on the fractional stimulation-induced (S-I) outflow of radioactivity from rat cortex slices preincubated with either [^{3}H]-dopamine (○) or [^{3}H]-noradrenaline (●). There were four periods of electrical stimulation (each 1 Hz for 60 s) and drugs (a, 4βPDB; b, 4αPDB; c, PA; d, PDA; e, PMBA; f, PMA) were present during the second, third and fourth stimulation in increasing concentrations. The fractional S-I outflow in the second, third and fourth stimulation was expressed as a percentage of that in the first. All results are normalised such that control = 100 for each stimulation period (x), thus the ratio of FR_x/FR_1 in the presence of drug was expressed as a percentage of the ratio of FR_x/FR_1 in the absence of drug (control series). For control in noradrenaline release slices, $FR_2/FR_1 = 0.95 \pm 0.03$, $FR_3/FR_1 = 0.89 \pm 0.03$, $FR_4/FR_1 = 0.83 \pm 0.04$ ($n=8$). For control in dopamine release slices, $FR_2/FR_1 = 0.89 \pm 0.03$, $FR_3/FR_1 = 0.77 \pm 0.02$, $FR_4/FR_1 = 0.74 \pm 0.02$ ($n=10$). Each symbol represents the mean and the vertical lines the s.e.mean. The number of experiments was between 4–10 for each drug. *Represents a significant difference from control, noradrenaline release ($P<0.05$, Scheffé's test after two-way ANOVA). $^+$Represents a significant difference from control, dopamine release ($P<0.05$, Scheffé's test after two-way ANOVA). 4βPDB = 4β-phorbol 12, 13-dibutyrate; 4αPDB = 4α-phorbol 12, 13-dibutyrate; PA = phorbol 13-acetate; PDA = phorbol 12, 13-diacetate; PMBA = phorbol 12-methylaminobenzoate 13-acetate; PMA = phorbol 12-myristate 13-acetate. No experiments were performed with 4αPDB and PA for dopamine release.

gest 12-ester substituent (Table 1). None of these agents altered the resting outflow of radioactivity during S_2-S_4 (not shown).

Effect of 12-deoxyphorbol esters on noradrenaline release

The 12-deoxyphorbol 13-monoesters: dPA, dPAng, dPiB, dPPhen, dPPhenA and dPAngA enhanced the fractional S-I outflow of radioactivity during S_2-S_4 in a concentration-dependent manner (Figure 2, Table 2). In contrast, dPT which had the largest 13-ester substituent (Table 1) was without effect (Figure 2, Table 2). None of these agents altered the resting outflow of radioactivity during S_2-S_4 (not shown). Addition of a 20-acetate group did not appreciably affect activity in the case of dPPhen (compare with dPPhenA; Figure 2, Table 2) but in the case of dPAng there was a reduction (compare with dPAngA; Figure 2, Table 2).

Effect of long-term treatment with 4βPDB on noradrenaline release

In order to down-regulate PKC, cortical slices were treated in modified PSS medium containing 0.1 mM Ca^{2+}, dextran and either 4βPDB (1 μM) or vehicle (DMSO) for 20 h before being incubated with [^{3}H]-noradrenaline. The vehicle did not affect the fractional S-I or resting outflow of radioactivity when compared to freshly excised cortical slices (compare vehicle-treated and untreated, Table 3). Compared to vehicle-treated tissues, 4βPDB treatment significantly increased the fractional S-I outflow and resting outflow of radioactivity (Table 3).

In rat cortical slices which were treated for 20 h with vehicle before incubation with [^{3}H]-noradrenaline, 4βPDB, PMA, dPA and dPAng all enhanced the fractional S-I outflow of radioactivity in a concentration-dependent manner (Figure 3) and in a manner similar to that of freshly excised slices (compare with Figures 1 and 2). However, when the slices were treated for 20 h in PSS with 4βPDB (1 μM), subsequent application of 4βPDB, PMA and dPA failed to enhance the fractional S-I outflow of radioactivity (Figure 3). Although not abolished, the facilitatory effect of dPAng was significantly attenuated (Figure 3).

Influence of PKC antagonists on the effects of PKC activators on noradrenaline release

Two PKC inhibitors were used: polymyxin B (21 μM) and bisindolylmaleimide I (3 μM). Polymyxin B slightly but significantly inhibited S-I noradrenaline release and bisindolylmaleimide I slightly but significantly enhanced S-I noradrenaline release (Figure 4). The facilitatory effect of 4βPDB and dPAng was significantly attenuated by both polymyxin B and bisindolylmaleimide I (Figure 4). Neither polymyxin B (21 μM) nor bisindolylmaleimide I (3 μM) significantly altered the facilitatory effect of dPA on the S-I outflow of radioactivity (Figure 4). Polymyxin B (21 μM) and bisindolylmaleimide I (3 μM) did not alter the resting outflow of radioactivity (not shown).

Effect of non-phorbol ester PKC activators on noradrenaline release

The non-phorbol ester PKC activators, MEZ and THYM (0.03–1.0 μM), slightly enhanced S-I outflow of radioactivity at the highest concentration tested (1 μM) (Figure 5) without altering the resting outflow (not shown). In contrast, SC-10 (0.03–1.0 μM), ROPA (0.1–3.0 μM) and ADMB (0.1–3.0 μM) were without effect on either S-I (Figure 5) or the resting outflow of radioactivity (not shown).

[^{3}H]-dopamine release from rat cerebral cortex

[^{3}H]-dopamine was incorporated into the dopaminergic transmitter stores of rat cerebral cortical slices in the presence

Table 2 The percentage increase in S-I outflow of radioactivity from rat cortical slices pre-incubated with [^{3}H]-noradrenaline and [^{3}H]-dopamine, respectively, in the presence of the activator drug (at 1 μM) and the calculated EC_{40} (concentration of drug that increases transmitter release by 40%)

Activator	*Increase S-I outflow at 1 μM, [^{3}H]-noradrenaline incubation* (% increase over control)	*EC_{40} (–log M), [^{3}H]-noradrenaline incubation*	*Increase S-I outflow at 1 μM [^{3}H]-dopamine incubation* (% increase over control)	*EC_{40} (–log M) [^{3}H]-dopamine incubation*
4βPDB	104±5♣	6.85±0.05♣	104±17♣	6.65±0.10
4αPDB	3±6	<5.50	NT	NT
PA	10±6	<5.50	NT	NT
PDA	79±22♣	6.33±0.15♣	74±9♣	6.62±0.11
PMBA	85±12♣	6.97±0.12*♣	56±10♣	6.24±0.14*
PMA	24±2*	5.61±0.10	10±4*	<5.50
dPA	78±7♥	6.53±0.06	63±12♥	6.38±0.17
dPAng	100±12♥	6.72±0.11	88±9♥	6.69±0.09
dPiB	107±6♥	6.91±0.05*	84±19♥	6.56±0.14*
dPPhen	72±7♥	6.74±0.11	73±9♥	6.59±0.09
dPT	4±7	<5.50	0±4	<5.50
dPPhenA	100±15*	6.79±0.11*	46±3*△	6.16±0.07*△
dPAngA	49±1◆	6.25±0.15◆	52±19	6.15±0.15◆
MEZ	36±7*	5.68±0.34	13±4*	<5.50
THYM	41±6	5.93±0.18	NT	NT
SC-10	11±5	<5.50	9±6	<5.50
ROPA	2±7	<5.50	7±4	<5.50
ADMB	0±5	<5.50	0±7	<5.50

*Represents a significant difference of activator between transmitter systems. $P<0.05$, Student's t-test. ◆Represents a significant difference of dPAngA from dPAng, $P<0.05$, Student's t test. △Represents a significant difference of dPPhenA from dPPhen, $P<0.05$, Student's t test. ♣Represents a significant difference of phorbol 12, 13-diesters from PMA, $P<0.05$, Student's t test. ♥Represents a significant difference of 12-deoxyphorbol 13-monoesters from dPT, $P<0.05$, Student's t test. NT = not tested.

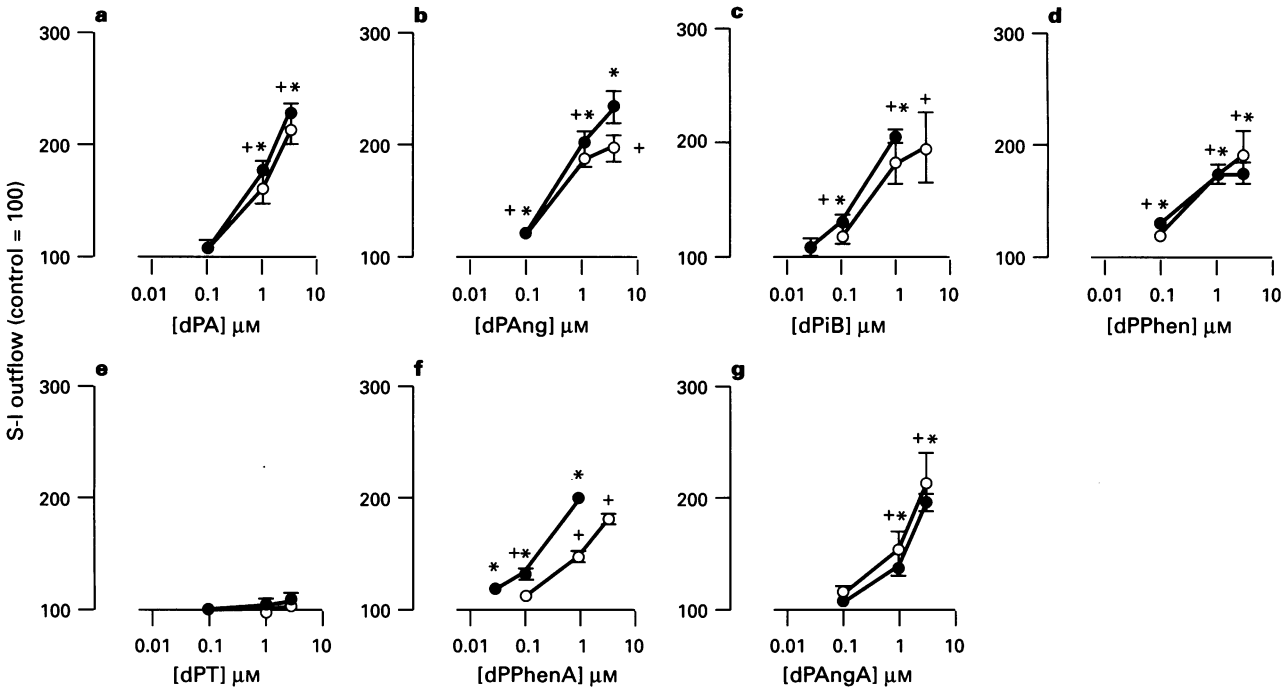


Figure 2 The effect of 12-deoxyphorbol 13-substituted monoesters on the fractional stimulation-induced (S-I) outflow of radioactivity from rat cortex slices pre-incubated with either [^{3}H]-dopamine (○) or [^{3}H]-noradrenaline (●). There were four periods of electrical stimulation (each 1 Hz for 60 s) and drugs (a, dPA; b, dPAng; c, dPiB; d, dPPhen; e, dPT; f, dPPhenA; g, dPAngA) were present during the second, third and fourth stimulation in increasing concentrations. The fractional S-I outflow in the second, third and fourth stimulation was expressed as a percentage of that in the first. All results are normalised such that control = 100 for each stimulation period (x), thus the ratio of FR_x/FR_1 in the presence of drug was expressed as a percentage of the ratio of FR_x/FR_1 in the absence of drug (control series). For control in noradrenaline release slices, $FR_2/FR_1 = 0.97 \pm 0.01$, $FR_3/FR_1 = 0.80 \pm 0.03$, $FR_4/FR_1 = 0.78 \pm 0.03$ ($n = 5$). For control in dopamine release slices, $FR_2/FR_1 = 0.89 \pm 0.03$, $FR_3/FR_1 = 0.77 \pm 0.02$, $FR_4/FR_1 = 0.74 \pm 0.02$ ($n = 10$). Each symbol represents the mean and the vertical lines s.e.mean. The number of experiments was between 4–10 for each drug. *Represents a significant difference from control, noradrenaline release ($P < 0.05$, Scheffé's test after two-way ANOVA). $^+$Represents a significant difference from control, dopamine release ($P < 0.05$, Scheffé's test after two-way ANOVA). dPA = 12-deoxyphorbol 13-acetate; dPAng = 12-deoxyphorbol 13-angelate; dPiB = 12-deoxyphorbol 13-isobutyrate; dPPhen = 12-deoxyphorbol 13-phenylacetate; dPT = 12-deoxyphorbol 13-tetradecanoate; dPPhenA = 12-deoxyphorbol 13-phenylacetate 20-acetate; dPAngA = 12-deoxyphorbol 13-angelate 20-acetate.

Table 3 The fractional resting (R_1) and stimulation-induced (FR_1) outflow of radioactivity associated with the first stimulation period (S_1) from rat cortical slices pre-incubated with either [^{3}H]-noradrenaline or [^{3}H]-dopamine

	R_1	FR_1
[^{3}H]-noradrenaline pre-incubation		
Untreated, $n = 172$	0.0150 ± 0.0002	0.0129 ± 0.0003
Vehicle-treated (20 h), $n = 27$	0.0152 ± 0.0003	0.0115 ± 0.0005
4βPDB-treated (20 h), $n = 26$	0.0168 ± 0.0004*	$0.0141 + 0.0005$*
[^{3}H]-dopamine pre-incubation		
Untreated, $n = 99$	0.0256 ± 0.0003	0.0203 ± 0.0005

There were several types of experiments. Firstly, untreated freshly excised cortical slices, secondly cortical slices placed in an incubator for 20 h with either 4βPDB or vehicle (DMSO) in the bathing solution before being taken for labelling with radioactive transmitter. R_1 represents the outflow of radioactivity over a 5 min sampling period immediately before the first stimulation period (S_1) expressed as a fraction of tissue radioactivity. FR_1 represents the total stimulation-induced portion of the radioactive outflow at S_1 (1 Hz for 60 s) expressed as a fraction of the tissue radioactivity. *Represents a significant difference from vehicle-treated slices, $P < 0.05$, Student's t test. 4βPDB = 4β-phorbol 12, 13-dibutyrate.

of desipramine and the S-I outflow of radioactivity was taken as an index of dopamine release. There were four periods of electrical stimulation (S_1, S_2, S_3, S_4 all at 1 Hz for 60 s) and the fractional S-I outflow in the first period (S_1) was 0.023 ± 0.002 ($n = 10$), the fractional resting outflow immediately before S_1 was 0.023 ± 0.001 per 5 min ($n = 10$) and the tissue radioactivity at the beginning of sample collection was 376542 ± 25749 d min^{-1} ($n = 10$). Tetrodotoxin (0.3 μM) added for the second (S_2) stimulation and the removal of calcium from the bathing fluid (after addition of the Ca^{2+} chelator EGTA, 0.1 mM) both almost abolished the fractional outflow of radioactivity in the second stimulation (control $FR_2/FR_1 = 0.91 \pm 0.02$, $n = 6$; tetrodotoxin $FR_2/FR_1 = 0.13 \pm 0.07$*, $n = 3$; zero Ca^{2+} $FR_2/FR_1 = 0.02 \pm 0.01$*, $n = 3$; * significantly different from control, $P < 0.05$, Student's t test with Bonferroni correction). This indicates that the S-I outflow involved the conduction of action-potentials and Ca^{2+} entry at the nerve terminal. None of these treatments affected the resting outflow of radioactivity (not shown).

Effect of phorbol esters on dopamine release

Concentration-response curves were constructed by superfusing tissue with increasing concentrations of phorbol esters during $S_2 - S_4$ as above. 4βPDB enhanced the fractional S-I outflow of radioactivity during $S_2 - S_4$ in a concentration-dependent manner (Figure 1). The phorbol esters containing a common 13-acetate ester substituent: PDA, PMBA, PMA also enhanced the fractional S-I outflow of radioactivity during $S_2 - S_4$ in a concentration-dependent manner (Figure 1). For these compounds the degree of enhancement and the potency (Figure 1, Table 2) were significantly less for PMA which had the largest 12-ester substituent (Table 1). None of these agents altered the resting outflow of radioactivity during $S_2 - S_4$ (not shown). No experiments were performed with 4αPDB and PA on dopamine release.

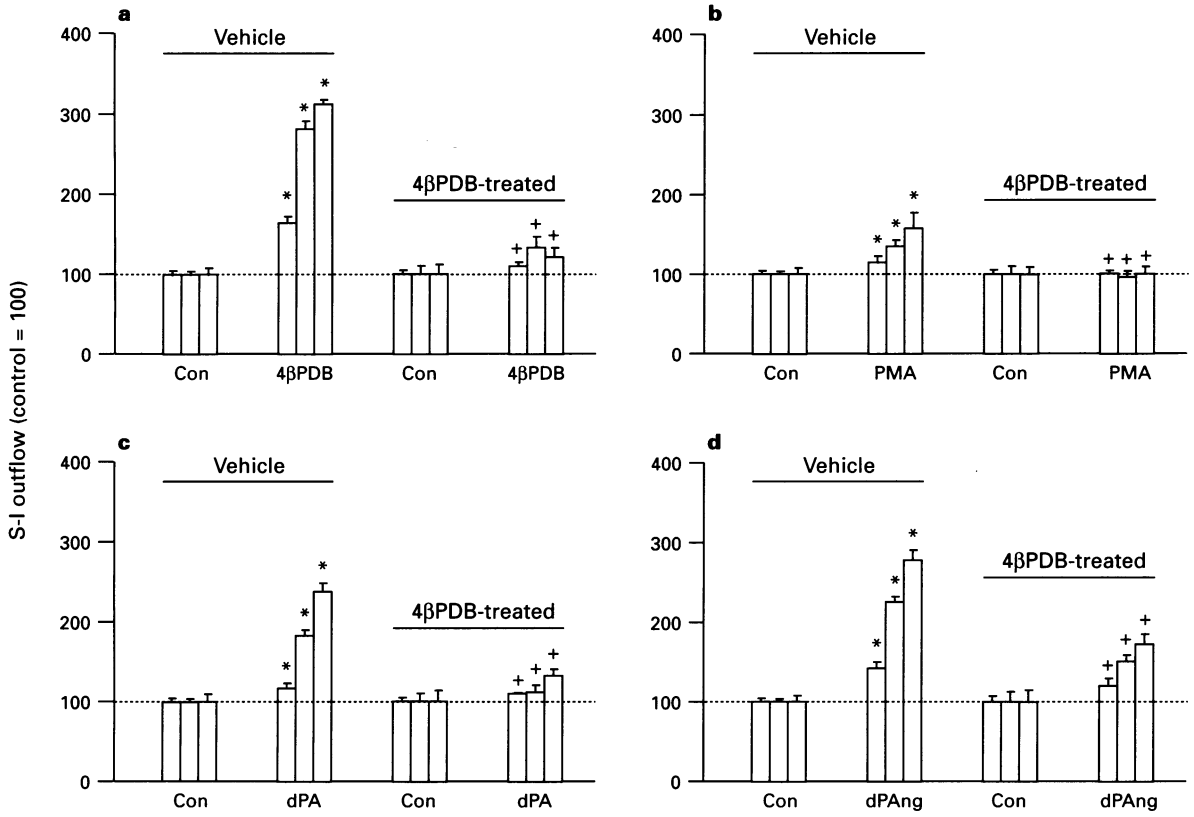


Figure 3 The influence of pretreatment with 4β-phorbol 12, 13-dibutyrate (4βPDB, 1 μM for 20 h) on the effects of protein kinase C (PKC) activators on the fractional stimulation-induced (S-I) outflow of radioactivity from rat cortex slices subsequently pre-incubated with [^{3}H]-noradrenaline. There were four periods of electrical stimulation (each 1 Hz for 60 s) and drugs ((a) 4βPDB; (b) PMA; (c) dPA; (d) dPAng) were present during the second, third and fourth stimulation in increasing concentrations (0.1, 1.0 and 3.0 μM). The fractional S-I outflow in the second, third and fourth stimulation was expressed as a percentage of that in the first. All results are normalised and the ratio of FR_x/FR_1 in the presence of drug was expressed as a percentage of the ratio of FR_x/FR_1 in the absence of drug (control series). For control in vehicle-treated slices, $FR_2/FR_1 = 0.93 \pm 0.04$, $FR_3/FR_1 = 0.91 \pm 0.05$, $FR_4/FR_1 = 0.86 \pm 0.07$ ($n = 9$). For control in 4βPDB-treated slices, $FR_2/FR_1 = 0.91 \pm 0.06$, $FR_3/FR_1 = 0.92 \pm 0.09$, $FR_4/FR_1 = 0.90 \pm 0.11$ ($n = 9$). Each column represents the mean and the vertical lines the s.e.mean. The three adjoining columns represent FR_2/FR_1, FR_3/FR_1 and FR_4/FR_1, respectively, which also represents increasing concentrations of phorbol ester (0.1, 1.0 and 3.0 μM). The number of experiments was between 4–9 for each drug. *Represents a significant difference from control ($P < 0.05$, two-way ANOVA). $^+$Represents a significant attenuation of the facilitatory effect of 4βPDB, PMA, dPA and dPAng after 4βPDB treatment ($P < 0.05$, two-way ANOVA). 4βPDB = 4β-phorbol 12, 13-dibutyrate; PMA = phorbol 12-myristate 13-acetate; dPA = 12-deoxyphorbol 13-acetate; dPAng = 12-deoxyphorbol 13-angelate.

Effect of 12-deoxyphorbol esters on dopamine release

The 12-deoxyphorbol 13-monoesters: dPA, dPAng, dPiB, dPPhen, dPPhenA and dPAngA enhanced the fractional S-I outflow of radioactivity during S_2-S_4 in a concentration-dependent manner (Figure 2). In contrast, dPT was without effect (Figure 2). None of these agents altered the resting outflow of radioactivity (not shown).

Effect of non-phorbol ester PKC activators on dopamine release

The non-phorbol ester PKC activators, MEZ (0.1–3.0 μM) and ROPA (0.1–3.0 μM), slightly enhanced the S-I outflow of radioactivity at the highest concentration (3 μM) (Figure 5) without altering the resting outflow (not shown). In contrast, SC-10 (0.1–3.0 μM) and ADMB (0.1–3.0 μM) were without effect on either S-I (Figure 5) or resting outflow of radioactivity (not shown). No experiments were performed with THYM on dopamine release.

Comparison of effects on dopamine and noradrenaline release

For most of the compounds tested there were no significant differences between their effects on dopamine and noradrenaline release in terms of magnitude of enhancement at a concentration of 1 μM and potency as measured by EC_{40} (concentration of compound to enhance transmitter release by 40%). There were four exceptions: PMBA, PMA, dPPhenA and MEZ all of which showed statistically significant selectivity towards elevating noradrenaline release versus dopamine release (Table 2).

Discussion

We assessed the effects of a series of protein kinase C (PKC) activators on noradrenaline and dopamine release in rat cortical slices by use of radiolabelled noradrenaline and dopamine. For both transmitters, the stimulation-induced (S-I) release of radioactivity was Ca^{2+}-dependent and tetrodotoxin-sensitive in line with neural release and previous studies (e.g. Nichols *et al.*, 1987; Schroeder *et al.*, 1995). In the present study true maximal effects of the PKC activators were not obtained in most cases at the highest concentrations employed (3 μM). Higher concentrations were not used because several of the agents have limited solubility. The PKC activators had markedly different effects on both noradrenaline and dopamine release ranging from large enhancements to no effect and this is discussed below in terms of structure and activity.

In order to confirm that the facilitatory effects of phorbol esters on noradrenaline release were mediated by PKC, some representative phorbol esters were examined in detail. Firstly,

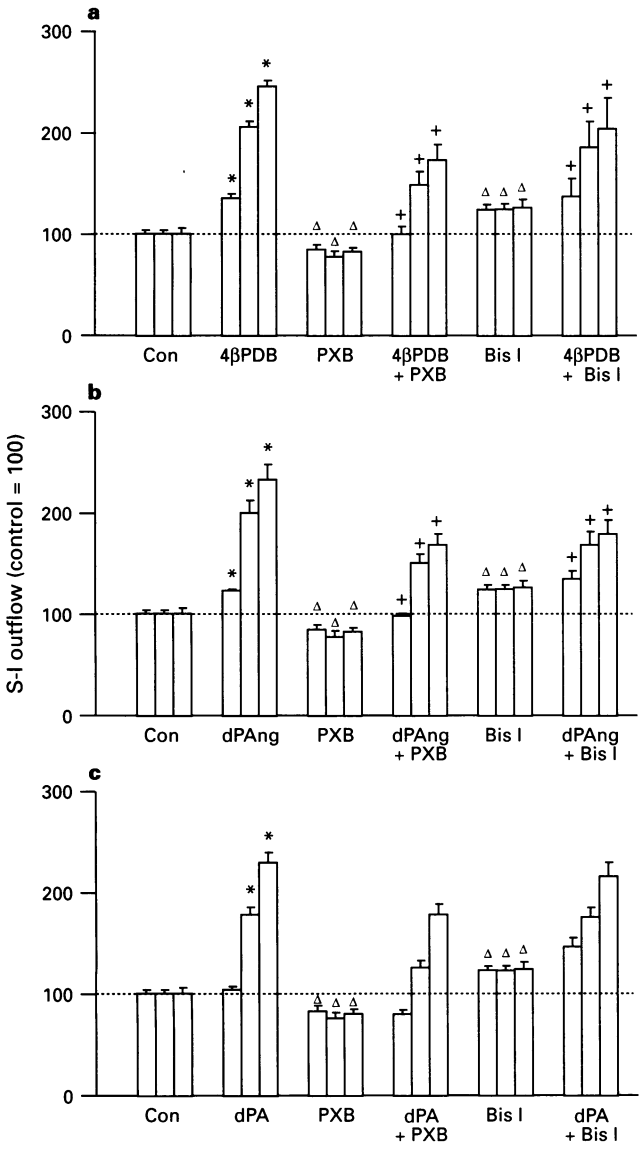


Figure 4 The influence of the protein kinase C (PKC) inhibitors, polymyxin B and bisindolylmaleimide I, on the effects of PKC activators on the fractional stimulation-induced (S-I) outflow of radioactivity from rat cortex slices pre-incubated with [^{3}H]-noradrenaline. There were four periods of electrical stimulation (each 1 Hz for 60 s) and drugs ((a) 4βPDB; (b) dPAng; (c) dPA) were present during the second, third and fourth stimulation in increasing concentrations (0.1, 1.0 and 3.0 μM). The drugs, polymyxin B (PXB, 21 μM) and bisindolylmaleimide I (Bis I, 3 μM) were also present during the second, third and fourth stimulation but were added 35 min (instead of 15 min for the phorbol esters) prior to the second stimulation. The fractional S-I outflow in the second, third and fourth stimulation was expressed as a percentage of that in the first. All results are normalised and the ratio of FR_x/FR_1 in the presence of drug was expressed as a percentage of the ratio of FR_x/FR_1 in the absence of drug (control series). For control slices, $FR_2/FR_1 = 0.95 \pm 0.03$, $FR_3/FR_1 = 0.89 \pm 0.03$, $FR_4/FR_1 = 0.83 \pm 0.04$ ($n = 8$). Each column represents the mean and the vertical lines the s.e.mean. The three adjoining columns represent FR_2/FR_1, FR_3/FR_1 and FR_4/FR_1, respectively, which also represents increasing concentrations of phorbol ester (0.1, 1.0 and 3.0 μM). The number of experiments was between 4–9 for each drug. *Represents a significant difference from control ($P < 0.05$, two-way ANOVA). $^{\Delta}$Represents a significant effect of the respective antagonist alone ($P < 0.05$, two-way ANOVA). $^{+}$Represents a significant reduction in the effect of the phorbol ester by the antagonist ($P < 0.05$, interaction term, two-way ANOVA). 4βPDB = 4β-phorbol 12, 13-dibutyrate; dPAng = 12-deoxyphorbol 13-angelate; dPA = 12-deoxyphorbol 13-acetate.

PKC is known to be down-regulated after prolonged exposure to phorbol esters and this has been used to deplete PKC from biological systems (Matthies *et al.*, 1987; Adams & Gullick, 1989; Bader *et al.*, 1989; Burgess *et al.*, 1989; Foucart *et al.*, 1991; Schroeder *et al.*, 1995). In the present study in cortex slices in which the PKC was down-regulated by prior exposure to 4βPDB (see Foucart *et al.*, 1991; Schroeder *et al.*, 1995), the facilitatory effects on noradrenaline release of 4βPDB, PMA and dPA were abolished and in the case of dPAng it was markedly reduced. Similarly, the PKC inhibitors, polymyxin B and bisindolylmaleimide I, also partially reduced the effects of 4βPDB and dPAng although dPA was not significantly affected. This may be because the inhibitors did not achieve sufficient blockade of PKC. Higher concentrations of the inhibitors were not used since they are known to interact with other pathways (Mazzei *et al.*, 1982; Schächtele *et al.*, 1989; Toullec *et al.*, 1991; Foucart *et al.*, 1991). By itself polymyxin B inhibited noradrenaline release and this involves blockade of PKC since this can be prevented by PKC down-regulation (Schroeder *et al.*, 1995) and is consistent with PKC activators enhancing transmitter release. Surprisingly, bisindolylmaleimide I enhanced noradrenaline release suggesting that it possessed a non-PKC action.

The phorbol esters 4αPDB and PA, which do not bind to or activate PKC (Kreibich & Hecker, 1970; Blumberg, 1980; Kikkawa *et al.*, 1983), were without effect on noradrenaline release in accord with previous studies (e.g. Wakade *et al.*, 1985; Musgrave *et al.*, 1991; Schroeder *et al.*, 1995). Together, these results suggest that PKC is involved in the facilitatory effects of the phorbol esters on noradrenaline release.

For phorbol derivatives with a common C13-acetate substituent several structure-activity requirements to facilitate S-I noradrenaline and dopamine release were observed. The structures are detailed in Table 1. The 12-deoxyphorbol dPA, which differs from the inactive PA (see above) only in having an H instead of OH at C12, was a potent enhancer of noradrenaline and dopamine release. If the C12 substituent is an extremely lipophilic group (e.g. myristate) as in PMA, then activity is greatly diminished compared to H (dPA) or acetate (PDA). For 12-deoxyphorbol derivatives which have a common H at the C12 position when the C13 ester substituent was made more lipophilic the ability to enhance noradrenaline and dopamine release was reduced [i.e. acetate (dPA) as well as angelate (dPAng), isobutyrate (dPiB), phenylacetate (dPPhen) >> tetradecanoate (dPT)]. Taken together it would appear that the lipophilicity of either the C12 or C13 ester group selects against activity on transmitter release. Those 12-deoxyphorbols with an acetate at C20 (dPAngA, dPPhenA) had either increased or no change in activity compared to the respective 12-deoxyphorbols without a 20-acetate (dPAng, dPPhen) making it unclear as to the activity effects of 20 acetate. In 4 cases (PMBA, PMA, dPPhenA, MEZ) the compounds showed some slight selectivity towards enhancing noradrenaline release over dopamine release (up to 0.7 log units difference). There appeared to be no structural similarities between the compounds and it is unclear whether this is an indication of neural differences.

The affinity differences between the compounds for PKC isozymes do not synchronise with the order of potency of the compounds on noradrenaline or dopamine release observed in the present study. For example, dPPhenA has a lower affinity than PMA for PKC α, β_1, β_2, γ, δ, ε (Dimitrijevic *et al.*, 1995) yet is more effective than PMA in enhancing noradrenaline and dopamine release. Similarly, dPA has a lower affinity than either thymeleatoxin or mezerein for PKC α, β, γ, δ, ε, η (Kazanietz *et al.*, 1993) yet is more effective in enhancing noradrenaline and dopamine release. Furthermore, for the two phorbol ester binding sites detailed in mouse skin the order of affinity is PMA > 4βPDB >> dPiB >> PDA (Dunn & Blumberg, 1983) which is markedly different to the order of potency on noradrenaline or dopamine release (4βPDB = PDA = dPiB >> PMA).

The activity (as opposed to affinity) of these compounds for

isolated PKC isozymes is also not a predictor of effects on transmitter release. Thus, PMA produces a greater activation of the isozyme activity (α, β, γ, δ, ε) than either dPPhen or dPPhenA (Ryves *et al.*, 1991), yet both of these agents had greater effects than PMA on noradrenaline or dopamine release. Furthermore, the rank order of effectiveness for insertion of PKC into phospholipid vesicles (PMA > dPPhen > 4βPDB >> dPA >> PDA, Kazanietz *et al.*, 1992), is clearly different to the rank order of effectiveness to enhance noradrenaline or dopamine release (4βPDB = PDA = dPA = dPPhen >> PMA).

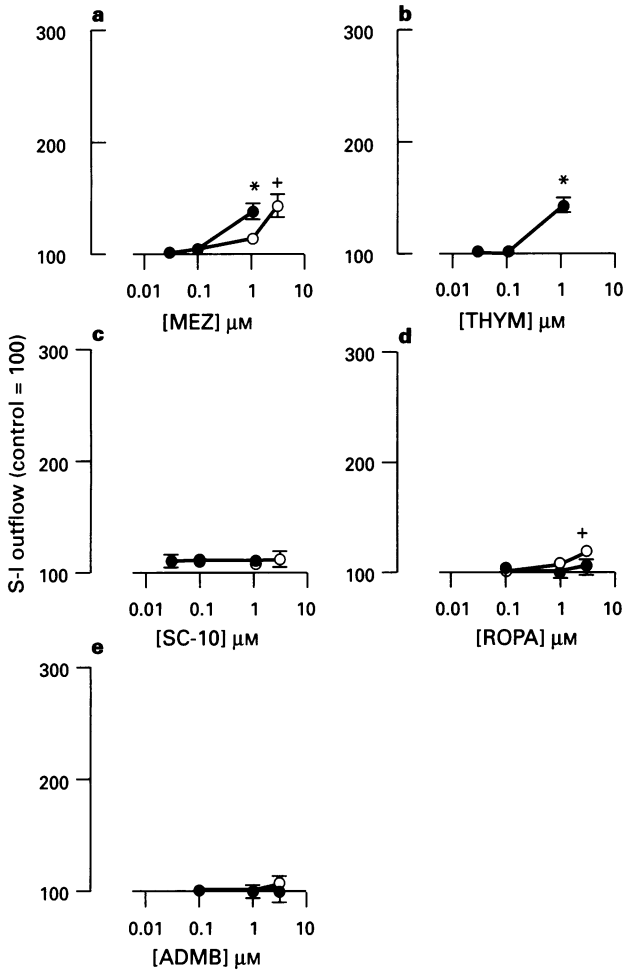


Figure 5 The effect of various non-phorbol ester protein kinase C (PKC) activators on the fractional stimulation-induced (S-I) outflow of radioactivity from rat cortex slices pre-incubated with either [^{3}H]-dopamine (○) or [^{3}H]-noradrenaline (●). There were four periods of electrical stimulation (each 1 Hz for 60 s) and drugs ((a) MEZ; (b) THYM; (c) SC-10; (d) ROPA; (e) ADMB) were present during the second, third and fourth stimulation in increasing concentrations. The fractional S-I outflow in the second, third and fourth stimulation was expressed as a percentage of that in the first. All results are normalised such that control = 100 for each stimulation period (x), thus the ratio of FR_x/FR_1 in the presence of drug was expressed as a percentage of the ratio of FR_x/FR_1 in the absence of drug (control series). For control in noradrenaline release slices, $FR_2/FR_1 = 0.94 \pm 0.03$, $FR_3/FR_1 = 0.86 \pm 0.06$, $FR_4/FR_1 = 0.78 \pm 0.05$ ($n = 11$). For control in dopamine release slices, $FR_2/FR_1 = 0.89 \pm 0.03$, $FR_3/FR_1 = 0.77 \pm 0.02$, $FR_4/FR_1 = 0.74 \pm 0.02$ ($n = 10$). Each symbol represents the mean and the vertical lines the s.e.mean. The number of experiments was between 4–11 for each drug. *Represents a significant effect of drug on noradrenaline release from control ($P < 0.05$, Scheffé's test after two-way ANOVA). $^+$Represents a significant effect of drug on dopamine release from control ($P < 0.05$, Scheffé's test after two-way ANOVA). MEZ = mezerein; THYM = thymeleatoxin; SC-10 = 5-chloro-N-heptyl-naphthalene-1-sulphonamide; ROPA = resiniferonol 9,13,14-ortho-phenylacetate; ADMB = 3-(N-acetylamino)-5-(N-decyl-N-methylamino) benzyl alcohol. No experiments were performed with THYM on dopamine release.

Our data suggest that increased lipophilicity of PKC activators reduces the effects on transmitter release, although it is generally regarded that increased lipophilicity produces a more potent PKC activator (Baird & Boutwell, 1971; Hecker, 1971; Fürstenberger & Hecker, 1972; Zayed *et al.*, 1984). This may implicate novel phorbol ester binding sites on PKC in neurones which have not been previously described or may reveal pharmacokinetic/distribution problems with lipophilic drugs. PMA is more lipophilic than 4βPDB (Jacobson *et al.*, 1975) although 4βPDB produces a consistently greater maximum effect than PMA on transmitter release (e.g. mouse atria, Musgrave *et al.* 1991; see also Introduction). It has been proposed by Allgaier *et al.* (1988) that highly lipophilic phorbol esters may take longer to achieve a response thus accounting for the differences. However, this seems unlikely given that in the present study the tissue contact time with PMA (at least 1 μM) was up to 45 min in contrast to that of Allgaier *et al.*, (1988) (10 min). Furthermore, in mouse atria 4βPDB was also more effective than PMA in enhancing noradrenaline release and the effect of PMA was not altered by changing tissue contact time from 15 min to 90 min (Musgrave *et al.*, 1991).

The current orthodoxy is that phorbol esters in the plasma membrane cause translocation of PKC (see Sharkey & Blumberg, 1985). However, there are suggestions that PKC isozymes may be compartmentalized within cells and possibly colocalised with target substrates (Mochly-Rosen *et al.*, 1991; Wetsel *et al.*, 1992; Leach *et al.*, 1992; Buchner, 1995). Based on the present findings we propose that the less lipophilic phorbol esters enter the cell cytoplasm to access the functional pool of PKC and that access to these sites is limited for lipophilic drugs such as PMA which would be sequestered in the plasma membrane. It is well known that PMA washes out of cell membranes less effectively than 4βPDB (Szalassi *et al.*, 1994) and presumably this indicates that PMA is also slower to enter the cell cytoplasm. Although with time an equilibrium should be established, this may not occur if the phorbol esters undergo intracellular metabolic inactivation. This would tend to reduce the activity of the more lipophilic compounds more markedly since these compounds would be present intracellularly in lower concentrations and if metabolized would not be replenished as quickly as the less lipophilic compounds. Metabolism of phorbol esters occurs by esterases with the 12-ester group being preferentially removed (Muller *et al.*, 1990). To our knowledge the metabolism of phorbol esters by neurones has not been studied and may be of particular interest given the present suggestion of different sites of actions for compounds of differing lipophilicities.

Tumour formation by phorbol esters is thought to involve PKC translocation at the cell membrane with nuclear communication via signal transduction cascades and is favoured by the lipophilic compounds (see Blumberg *et al.*, 1984). It is interesting in this regard that some of the best phorbol enhancers of noradrenaline release are in fact not (PDA, dPA, dPPhen, dPAngA), or very poor (dPAng, dPPhenA, dPiB), tumour promoters (Baird & Boutwell, 1971; Hecker, 1971; Fürstenberger & Hecker, 1972; Hergenhahn *et al.*, 1974; Zayed *et al.*, 1984; Brooks *et al.*, 1989). Similarly, skin irritation is also less evident with the 12-deoxyphorbols (Schmidt & Evans, 1980; Zayed *et al.*, 1984) which nevertheless enhance transmitter release.

In addition to phorbol esters, several non-phorbol compounds were tested. Firstly thymeleatoxin and mezerein, which have anomalously low tumour promoting activity yet are strong skin irritants (Fürstenberger & Hecker, 1972; Hergenhahn *et al.*, 1974; Brooks *et al.*, 1989). These highly lipophilic PKC activators were found to be poor enhancers of noradrenaline and dopamine release again supporting the hypothesis that lipophilicity is an important consideration underlying the effects of these compounds on transmitter release. Another

compound examined was ADMB, a rationally designed PKC activator with lower potency than phorbol esters (Wender *et al.*, 1986); in the same concentration range as the phorbol esters it had no effect on noradrenaline release. Finally, SC-10 an isoquinolone sulphonamide, which has been shown to be a PKC activator (Ito *et al.*, 1986), and ROPA, which is a tumour promoter and PKC activator related to resiniferatoxin (Szallasi *et al.*, 1989), also had no effect.

In conclusion the facilitatory effects of the phorbol esters on action-potential evoked noradrenaline and dopamine release do not correlate with *in vitro* affinity and activity at purified PKC isozymes and we suggest that differential distribution within cells may explain the results. The basic characteristics required to enhance noradrenaline/dopamine release are: for phorbol 13-monoesters, at C12 conversion of an OH to H confers activity on transmitter release and this is optimal if the C13 ester substituent is small and non-lipophilic. For phorbol 12, 13-diesters, both ester groups need to be small and non-lipophilic for maximal activity. These structural requirements are different from those required for tumour formation and we suggest that a high degree of selectivity for transmitter release over tumour formation exists for some of the test compounds (PDA, dPA, dPPhen, dPAngA).

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