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Differential abilities of phorbol esters in inducing protein kinase C (PKC) down-regulation in noradrenergic neurones

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1 The ability of several phorbol ester protein kinase C (PKC) activators (phorbol 12, 13-dibutyrate, PDB; phorbol 12, 13-diacetate, PDA; and 12-deoxyphorbol 13-acetate, dPA) to down-regulate PKC was studied by assessing their effects on electrical stimulation-induced (S-I) noradrenaline release from rat brain cortical slices and phosphorylation of the PKC neural substrate B-50 in rat cortical synaptosomal membranes.

2 In cortical slices which were incubated for 20 h with vehicle, acute application of PDB, PDA and dPA (0.1–3.0 μM) enhanced the S-I noradrenaline release in a concentration-dependent manner to between 200–250% of control in each case. In slices incubated with PDB (1 μM for 20 h), subsequent acute application of PDB (0.1–3.0 μM) failed to enhance S-I release, indicating PKC down-regulation. However, in tissues incubated with PDA or dPA (3 μM) for 20 h, there was no reduction in the facilitatory effect of their respective phorbol esters or PDB (0.1–3.0 μM) when acutely applied, indicating that PKC was not down-regulated. This was confirmed using Western blot analysis which showed that PDB (1 μM for 20 h) but not PDA (3 μM for 20 h) caused a significant reduction in PKCα.

3 Incubation with PDB for 20 h, followed by acute application of PDB (3 μM) failed to increase phosphorylation of B-50 in synaptosomal membranes, indicating down-regulation. In contrast, tissues incubated with PDA or dPA for 20 h, acute application of their respective phorbol ester (10 μM) or PDB (3 μM) induced a significant increase in B-50 phosphorylation.

4 Acutely all three phorbol esters elevate noradrenaline release to about the same extent, yet PDA and dPA have lower affinities for PKC compared to PDB, suggesting unique neural effects for these agents. This inability to cause functional down-regulation of PKC extends their unusual neural properties. Their neural potency and lack of down-regulation may be related to their decreased lipophilicity compared to other phorbol esters.

5 We suggest that PKC down-regulation appears to be related to binding affinity, where agents with high affinity, irreversibly insert PKC into artificial membrane lipid and generate Ca^{2+}-independent kinase activity which degrades and deplete PKC. We suggest that this mechanism may also underlie the ability of PDB to down-regulate PKC in nerve terminals, in contrast to PDA and dPA.

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Abbreviations: BSA, bovine serum albumin; DMSO, dimethyl sulphoxide; dPA, 12-deoxyphorbol 13-acetate; PDA, phorbol 12, 13-diacetate; PDB, phorbol 12, 13-dibutyrate; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PSS, physiological salt solution; S-I, stimulation-induced; TBS, TRIS-buffered saline

Introduction

Protein kinase C (PKC; ATP: protein phosphotransferase, EC 2.7.1.37) is a family of multiple related isoenzymes that are expressed in a variety of cell types (Nishizuka, 1992; Dekker & Parker, 1994). The PKC isoenzymes appear to be differentially distributed and may subserve a variety of biological functions including cell differentiation, cell expression and modulation of exocytosis of transmitters from neurones (Mochly-Rosen *et al*., 1991; Wetsel *et al*., 1992; Leach *et al*., 1992; Buchner, 1995; Majewski & Iannazzo, 1998).

Chronic activation of PKC with the tumour promoting phorbol ester, phorbol 12-myristate 13-acetate (PMA), can effectively deplete PKC from intact cells (e.g. Blackshear *et al*., 1985; Adams & Gullick, 1989; Lindner *et al*., 1991). The corresponding loss of PKC protein is due to a net increase in the proteolysis of activated PKC by protease(s) at a susceptible linker region between the regulatory and catalytic domain (Kishimoto *et al*., 1983, 1989). It is thought that PMA causes the translocation of both PKC and Ca^{2+}-dependent proteases I and II (also known as calpains) from cytosol to lipid membrane where proteolysis of PKC is

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exerted by calpains (Hong *et al*., 1995). The translocation of PKC is associated with a conformational change which increases the affinity of the kinase for calpains (Savart *et al*., 1992). Subsequent proteolysis generates a Ca^{2+} and co-factor independent kinase activity which further degrades PKC from the cell (Shea *et al*., 1994; Cressman *et al*., 1995).

It has been shown that only PKC activators which cause irreversible insertion of PKC into phospholipid vesicles or liposomes generate Ca^{2+}-independent kinase activity (see Moruzzi *et al*., 1990; Kazanietz *et al*., 1992) and this may give clues as to the factors underlying their ability to down-regulate PKC. It has been suggested that the differential ability of PKC activators to cause insertion in either vesicles or liposomes is related to their binding affinity for PKC (Moruzzi *et al*., 1990; Kazanietz *et al*., 1992). Interestingly, both phorbol 12,13-dibutyrate (PDB) and PMA which bind and activate PKC with similar low nanomolar potency (Dunn & Blumberg, 1983; Kazanietz *et al*., 1993; Dimitrijevic *et al*., 1995), are particularly good at irreversibly inserting PKC into artificial membranes and generating Ca^{2+}-independent kinase activity (Moruzzi *et al*., 1990; Kazanietz *et al*., 1992). In marked contrast, the phorbol esters phorbol 12,13-diacetate (PDA) and 12-deoxyphorbol 13-acetate (dPA), which bind with considerably lower affinity, reversibly insert PKC into membrane lipid and therefore do not generate Ca^{2+}-independent kinase activity (Kazanietz *et al*., 1992).

Surprisingly, PDA and dPA are more potent and efficacious than PMA in elevating action-potential evoked transmitter release from a variety of systems from rat cortex including noradrenergic, dopaminergic and serotonergic neurones (Kotsonis & Majewski, 1996; Iannazzo *et al*., 1999), despite having lower affinity for PKC in rat cortex synaptosomal membranes (Murphy *et al*., 1999). In the present study we investigated whether prolonged exposure to PDA and dPA causes PKC down-regulation, like that described for PDB in noradrenergic neurones (Foucart *et al*., 1991; Schroeder *et al*., 1995; Kotsonis and Majewski, 1996) and serotonergic neurones (Iannazzo *et al*., 1999). PKC down-regulation was assessed both functionally by measuring the output of [^{3}H]-noradrenaline release from rat brain cortex and, biochemically as a change in the level of phosphorylation of the neural substrate B-50 (Dekker *et al*., 1989) in synaptosomal membranes.

Methods

Noradrenaline release following long-term treatment of cerebral cortical slices with phorbol esters

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 using a Vibroslice 752, Campden Instruments) were placed in an open dish in 50 ml modified PSS containing 0.1 mM Ca^{2+}, dextran (50 g l^{-1}, average MW = 70,000) and incubated with either PDB (1 μM), PDA (3 μM), dPA (3 μM) or vehicle (dimethyl sulphoxide (DMSO) 0.06 or 0.18% v v^{-1}). The slices were maintained at 32°C in a tissue culture incubator for 20 h where the atmosphere 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.

Slices were then maintained at 37°C and gassed with a mixture of 5% CO_2 and 95% O_2 containing [^{3}H]-noradrenaline (10 μCi ml^{-1}, 0.2 μM for 20 min). Following incubation, the slices were rinsed, transferred to flow cells (four cells per bank with electrodes connected in series) and continuously superfused at 0.5 ml min^{-1} with PSS 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 pules of 2 ms duration at a frequency of 1 Hz for 60 s). 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 acutely applied PKC activators on the electrical stimulation-induced (S-I) outflow of radioactivity was determined by adding them in increasing concentrations to the superfusate solution 15 min before the second, third and fourth stimulation. 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 solubilize 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 standardization and results are expressed as disintegrations per min (d min^{-1}).

Calculation of noradrenaline release 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 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), Drug effects on the fractional S-I outflow of radioactivity were evaluated by comparing the ratio of S_2/S_1, S_3/S_1 and S_4/S_1.

B-50-phosphorylation following long-term incubation of cerebral cortical slices with phorbol esters

The rat cortical slices were prepared and incubated for 20 h as for noradrenaline release. They were then gently homogenized in 10 ml ice-cold sucrose (0.32 M) containing HEPES (5 mM) and EGTA (1 mM), pH 7.4 using a teflon/glass homogenizer. The suspension was centrifuged at $1000 \times g$ for 10 min at 4°C. The supernatant was recentrifuged at $15{,}000 \times g$ for 30 min at 4°C. The P2 pellet was resuspended in lysis buffer (leupeptin 10 μg ml^{-1}, sodium

vanadate 0.5 mM, phenylmethylsulphonylfluoride 1 mM, TRIS 30 mM, pH 7.4), and membranes were adjusted to a final protein concentration of 1 mg ml^{-1}.

The membrane preparation (5 μl) was incubated with 8.4 μl solution containing (mM) TRIS 24, pH 7.4, EGTA 1, $MgSO_4$ 10, $CaCl_2$ 0.38, sodium vanadate 0.05 and either the different phorbol esters or vehicles at 37°C for 5 min. The phosphorylation reaction was started by the addition of 4 μl of [^{33}P]-ATP (specific activity 200–400 Ci $mmol^{-1}$, 0.14 mM). The reaction was stopped after 30 s by the addition of reducing buffer (TRIS/HCl 130 mM, pH 7.4, 6% sodium dodecyl sulphate (SDS) (w v^{-1}), 30% glycerol (v v^{-1}), 0.003% bromophenol blue (w v^{-1}, 15% mercaptoethanol (v v^{-1})). Samples were stored at −20°C overnight.

Proteins were denatured by boiling the samples at 100°C for 3 min before being separated by gel electrophoresis. Protein samples (27 μg per well) and molecular weight markers were electrophoresed for 65–75 min at 150 V in running buffer (TRIS 25 mM, glycine 0.19 M, SDS 1% w v^{-1}, pH 8.1–8.4) on a 10% SDS–PAGE-gel (Laemmli, 1970). The gels were removed and fixed in a solution of 10% acetic acid and 30% methanol for 10 min and then washed in deionized water for 20 min. The gels were then dried on a gel drier for 120 min at 80°C. Autoradiography with Kodak BioMax MS films was then carried out.

Western blot analysis of B-50

The above protocol for the B-50 phosphorylation studies was adopted in the absence of [^{33}P]-ATP. Briefly, protein was separated using 10% SDS–PAGE gel electrophoresis, then samples were transferred to nitrocellulose membrane in transfer buffer (TRIS 25 mM, glycine 0.19 M and methanol 20%, pH 8.1–8.4) at 4°C, 100 V for 75 min. Non-specific binding sites on the membranes were blocked for 1 h with 5% bovine serum albumin (BSA) solution in TRIS-buffered saline (TBS: TRIS 20 nM, NaCl 137 nM). Membranes were then incubated at room temperature for 20 h with mouse anti-human-B-50 (diluted 1 : 1000 in 5% BSA). Membranes were then washed twice with TBS for 10 min, once with 0.1% Tween-20 in TBS for 15 min, then twice with TBS for 10 min. The membranes were then incubated for 1 h with goat anti-mouse secondary antibody conjugated to horseradish peroxidase (diluted 1 : 5000) in TBS. The membranes were then washed again with the above procedure. They were then reacted with hydrogen peroxide and luminol (enhanced-chemiluminescence system ECL; Amersham, U.K.) and B-50 was visualized by exposure of the membrane to Kodak Chemiluminescence film. Molecular weight markers were run parallel to all samples.

Calculations of phosphorylation and B-50 results

The optical density of the corresponding radioactive protein bands, as visualized on X-ray film was measured using Scion Image version 3b software (Scion Corporation, Maryland, U.S.A.), and the average density multiplied by the area of each band on the film. In each gel all values were then expressed as a percentage of the respective vehicle lane band which was run in each gel.

Western blot analysis of PKCα downregulation following incubation with phorbol esters

The rat cortical slices were prepared and incubated for 20 h as for noradrenaline release. They were then gently homogenized in 10 ml ice-cold sucrose (0.32 M) containing HEPES (5 mM) and EGTA (1 mM), pH 7.4 using a teflon/glass homogenizer. The suspension was centrifuged at $1000 \times g$ for 10 min at 4°C. The supernatant was recentrifuged at $15{,}000 \times g$ for 30 min at 4°C. The P2 pellet was resuspended in lysis buffer (leupeptin 10 μg ml^{-1}, sodium vanadate 0.5 mM, phenylmethylsulphonylfluoride 1 mM, TRIS 30 mM, pH 7.4) and the synaptosomal membranes and the cytosolic fraction were separated by centrifugation at $100{,}000 \times g$ for 60 min at 4°C. The resulting supernatant contained the cytosolic fraction and the pellet the membrane fraction. The protein concentration of each fraction was determined, the proteins denatured by boiling in a reducing buffer for 3 min and 7 μg of solubilized proteins were then separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE; 7.5% acrylamide). Proteins were then transferred as described for B-50 Western blot, however, proteins were probed with anti-PKCα (1 : 1000). The remainder of the protocol was as described for B-50 Western blot.

Phorbol ester metabolism during 20 h incubation

The chemical stability of the phorbol esters PDB and PDA following 20 h incubation in PSS was examined using high

Table 1 The fractional outflow of radioactivity associated with the first stimulation period from rat cortical slices loaded with [^{3}H]-noradrenaline after 20 h incubation

Incubation	n	R_1	FR_1	*Tissue radioactivity* (d min^{-1})	*Figure no.*
Vehicle	13	0.0150 ± 0.0004	0.0108 ± 0.0007	695.470 ± 42.539	1
PDB	13	0.1073 ± 0.0005*	0.0137 ± 0.0009*	632.985 ± 49.926	1
Vehicle	20	0.0162 ± 0.0003	0.0104 ± 0.0005	611.529 ± 27.619	2 and 3
PDA	20	0.0174 ± 0.0004**	0.0099 ± 0.0004	641.629 ± 25.540	2
dPA	15	0.0162 ± 0.0005	0.0078 ± 0.0005**	679.432 ± 33.912	3

Cortical slices placed in an incubator for 20 h with either PDB, PDA, dPA or vehicle (DMSO, 0.06 or 0.18% v v^{-1}) in the bathing solution before being incubated with [^{3}H]-noradrenaline. R_1 represents the outflow of radioactivity over a 5 min sampling period immediately before the first stimulation period expressed as a fraction of tissue radioactivity. FR_1 represents the total stimulation-induced portion of the radioactive outflow at the first stimulation (1 Hz for 60 s) expressed as a fraction of the tissue radioactivity. Tissue radioactivity indicates the amount of radioactivity present in the slices at the beginning of the sample collection. *Represents a significant difference from vehicle-incubated slices (DMSO 0.06% v v^{-1}, Figure 1), $P<0.05$, Student's *t*-test. **Represents a significant difference from vehicle-incubated slices (DMSO 0.18% v v^{-1}, Figures 2 and 3), $P<0.05$, Student's *t*-test.

pressure liquid chromatography [HPLC, octadecylsilane based reverse phase column with C18 packings (EXCIL C18 5M-Alt 230) methanol : water (70 : 30) mobile phase]. The radiolabelled phorbol esters, [^{3}H]-PDB and [^{3}H]-PDA, were incubated in PSS for 20 h in an incubation chamber analogous to transmitter release experiments (see Methods) in the presence or absence of rat brain cortical slices. The bathing solution was examined using HPLC.

Statistics

For the B-50-phosphorylation and release studies, the values indicate the mean and standard error of the mean (s.e.m.). '*n*' indicates the number of brains used for the membrane preparation or of slices used or the number of synaptosomal preparations used for phosphorylation experiments; within each experimental group, the slices came from different animals and one experiment was performed on each tissue slice; each synaptosomal preparation was derived from different rat brains.

Data obtained from the B-50 phosphorylation experiments was analysed using a Student's-*t*-test (comparison: variable versus constant). In release studies the results were analysed with Scheffe'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 acutely applied phorbol esters on transmitter release (A) and incubation with phorbol esters (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)

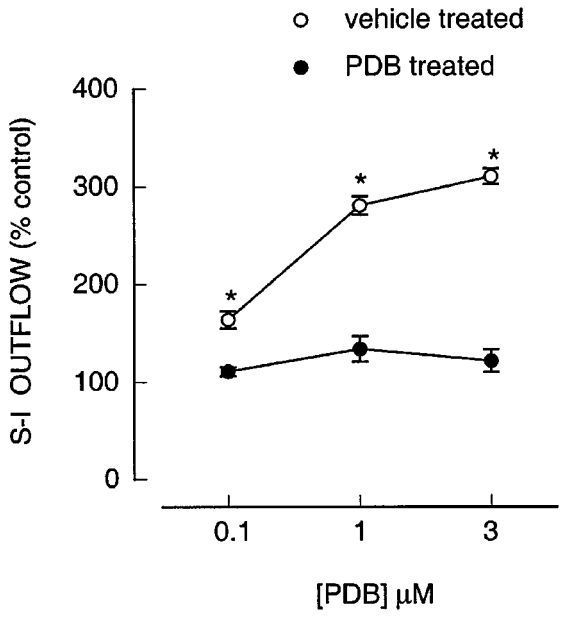

Figure 1 The influence of prior incubation with PDB (1 μM for 20 h) on the effects of acutely applied PDB on the fractional stimulation-induced (S-I) outflow of radioactivity from rat cortex slices subsequently loaded with [^{3}H]-noradrenaline. There were four periods of electrical stimulation (each 1 Hz for 60 s) and PDB was 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 normalized (control = 100%, not shown) and the ratio of S_x/S_1 in the presence of drug was expressed as a percentage of the ratio of S_x/S_1 in the absence of drug (control series). Each symbol represents the mean and the vertical lines the standard error of the mean (s.e.m.). Open circles indicate cortical slices incubated with vehicle (DMSO 0.06% v v^{-1} for 20 h) and filled circles indicate cortical slices incubated with PDB (1 μM for 20 h). The number of experiments was between 4–9 for each drug. *Represents a significant difference from control ($P<0.05$, two-way ANOVA). There was a significant attenuation of the facilitatory effect of PDB after prior PDB incubation ($P<0.05$, two-way ANOVA).

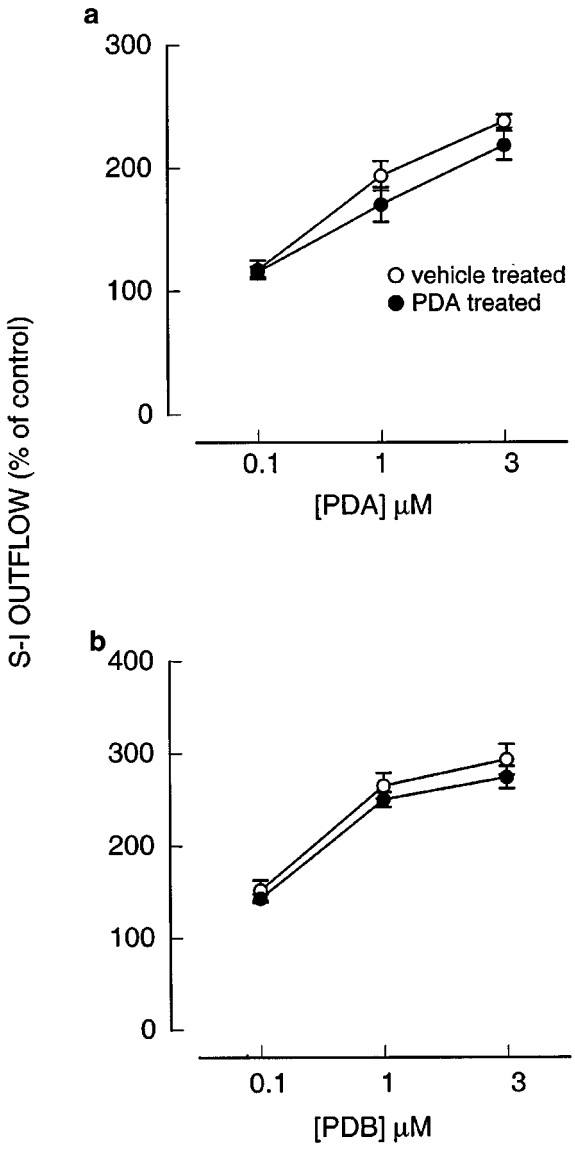

Figure 2 The influence of acutely applied PDA (upper panel, a) and PDB (lower panel, b) on the fractional stimulation-induced (S-I) outflow of radioactivity from rat cortex slices incubated with PDA (3 μM for 20 h) and loaded with [^{3}H]-noradrenaline. There were four periods of electrical stimulation (each 1 Hz for 60 s) and drugs (PDA and PDB) were present during the second, third and fourth stimulation in increasing concentrations. All results are expressed as described in Figure 1 legend. Each symbol represents the mean and the vertical lines the standard error of the mean (s.e.m.). Open circles indicate cortical slices incubated with vehicle (DMSO 0.18% v v^{-1} for 20 h) and filled circles indicate cortical slices incubated with PDA (3 μM for 20 h). The number of experiments was between 5–7 for each drug.

of less that 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 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.

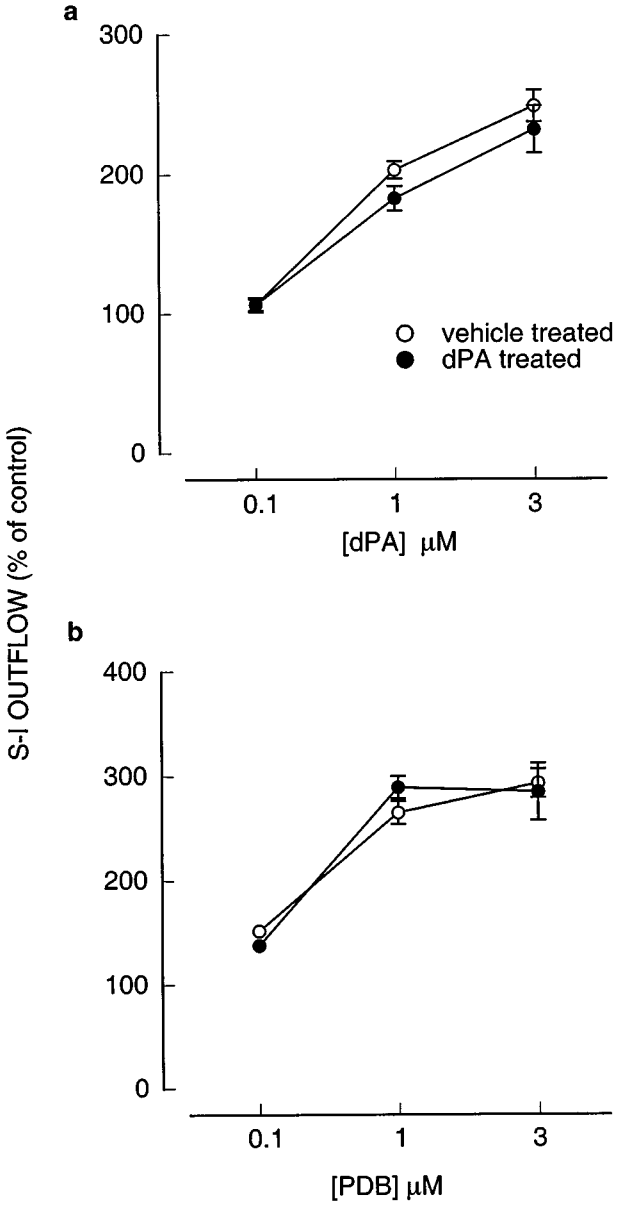

Figure 3 The influence of acutely applied dPA (upper panel, a) and PDB (lower panel, b) on the fractional stimulation-induced (S-I) outflow of radioactivity from rat cortex slices incubated with dPA (3 μM for 20 h) and loaded with [^{3}H]-noradrenaline. There were four periods of electrical stimulation (each 1 Hz for 60 s) and drugs (dPA and PDB) were present during the second, third and fourth stimulation in increasing concentrations. All results are expressed as described in Figure 1 legend. Each circle represents the mean and the vertical lines the standard error of the mean (s.e.m.). Open circles indicate cortical slices incubated with vehicle (DMSO 0.18% v v^{-1} for 20 h) and filled circles indicate cortical slices incubated with dPA (3 μM for 20 h). The number of experiments was five for each drug.

Radiochemicals and drugs

Drugs used were: (−)-[ring-2,5,6-^{3}H]-noradrenaline (specific activity 43.7 Ci mmol^{-1}), [20-^{3}H(N)]-PDB (specific activity 19.6 Ci mmol^{-1}), [20-^{3}H(N)]-4β-phorbol 12,13-diacetate (specific activity 18 Ci mmol^{-1}), ^{33}P-ATP Easytides (specific activity 2000 Ci mmol^{-1}) (DuPont NEN Products; Boston, U.S.A.), phorbol, 12,13-dibutyrate (PDB), phorbol 12,13-diacetate (PDA) and 12-deoxyphorbol 13-acetate (dPA) (LC Laboratories, Woburn, U.S.A.). ATP, bovine serum albumin, dextran, glycine, leupeptin, phenylmethylsulphonylfluoride, SDS, sodium vanadate and Tween-20 (Sigma, St Louis, U.S.A.), mouse anti-human-Neuromodulin/GAP-43 (B-50) primary antibody, mouse anti-PKCα primary antibody (Transduction Laboratories, Lexington, Kentucky, U.S.A.), goat anti-mouse secondary-HRP antibody (DAKO, Carpinteria, California, U.S.A.), nitrocellulose membrane, enhanced chemiluminescence reagents (Amersham, Buckinghamshire, U.K.), Western blot reagents (BioRad, Hercules, California, U.S.A.). Stock solutions of the PKC activators were initially made up in DMSO and stored at −20°C. On the day of the experiment these drugs were further diluted in PSS. Control experiments were conducted with the corresponding concentration of DMSO (up to 0.18% v v^{-1}).

Results

Effect of long-term treatment with phorbol esters on [^{3}H]-noradrenaline release

In order to down-regulate PKC, cortical slices were incubated for 20 h in modified PSS medium containing 0.1 mM Ca^{2+}, dextran and either PDB (1 μM), PDA (3 μM), dPA (3 μM) or vehicle (DMSO 0.06 or 0.18% v v^{-1} DMSO) before being loaded with [^{3}H]-noradrenaline. The electrical field stimulation evoked a stimulation-induced (S-I) outflow of radioactivity which was taken as an index of noradrenaline release. Table 1 details the fractional S-I outflow in the first period (S_1), the fractional resting outflow immediately before S_1 and the tissue radioactivity at the beginning of the sample collection for each series of experiments. There were some differences in absolute parameters, compared to vehicle-incubated tissues (DMSO 0.06% v v^{-1} for 20 h), PDB incubation (1 μM for 20 h) significantly increased the fractional S-I outflow and resting outflow of radioactivity (Table 1). PDA incubation (3 μM for 20 h) significantly

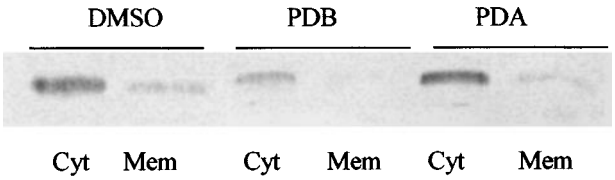

Figure 4 Western blot. A rat brain sample separated into cytosolic and membrane fractions was run on an SDS−PAGE-gel following 20 h exposure to either DMSO, PDB (1 μM) or PDA (3 μM). Lanes 1 and 2 = DMSO treated, lanes 3 and 4 = PDB treated, lanes 5 and 6 = PDA treated. Cytosol (Cyt) fraction and membrane (Mem) fractions are shown. Primary: mouse anti-PKCα antibody. Secondary: goat anti-mouse antibody conjugated with horse-radish peroxidase.

increased the fractional resting outflow radioactivity and dPA incubation (3 μM for 20 h) significantly decreased the fractional S-I outflow of radioactivity (Table 1).

In rat cortical slices which were incubated for 20 h with vehicle (0.06% v v^{-1} DMSO) before incubation with [^{3}H]-noradrenaline, acutely applied PDB (0.1 – 3.0 μM) enhanced the fractional S-I outflow of radioactivity in a concentration-dependent manner (Figure 1) without altering the resting outflow of radioactivity (not shown). In slices incubated with PDB (1 μM) for 20 h, acutely applied PDB failed to enhance the fractional S-I outflow of radioactivity (Figure 1).

In rat cortical slices which were incubated for 20 h with vehicle (0.18% v v^{-1} DMSO) acutely applied PDA (0.1 – 3.0 μM), dPA (0.1 – 3.0 μM) and PDB (0.1 – 3.0 μM) enhanced

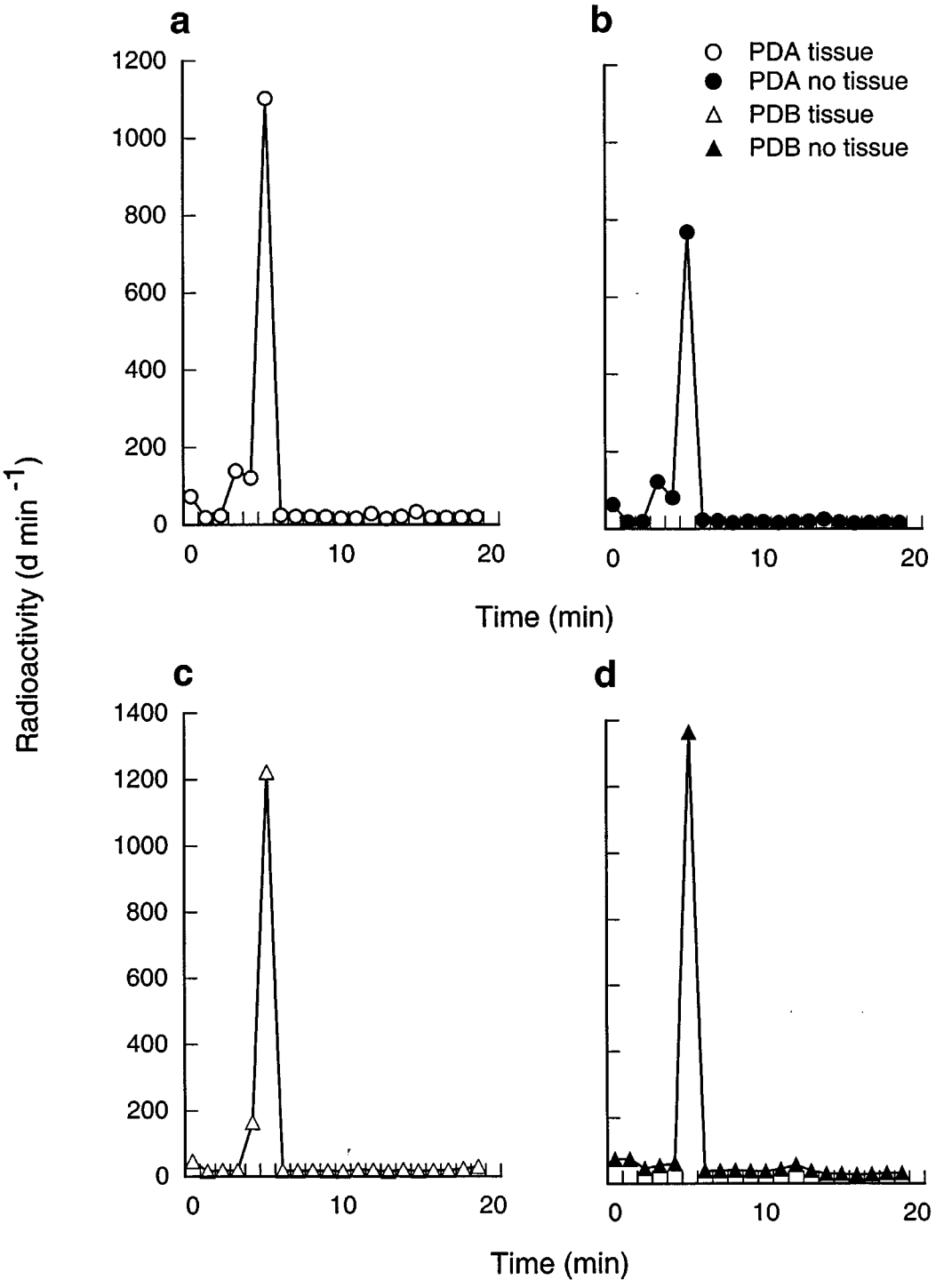

Figure 5 Chromatograms of HPLC of [^{3}H]-PDB and [^{3}H]-PDA following 20 h incubation. Rat cortex slices were incubated for 20 h with [^{3}H]-PDA (3 μM) and [^{3}H]-PDB (10 μM) respectively in a total volume of 50 ml. The supernatant was analysed by HPLC for degradation of phorbol ester. Single peaks indicate authentic PDB or PDA. (a,c) are in the absence of tissue, and (b,d) are in the presence of tissue.

the fractional S-I outflow of radioactivity in a concentration-dependent manner (Figures 2 and 3) without altering the resting outflow of radioactivity (not shown). In slices incubated with PDA (3 μM) for 20 h, subsequent acute application of PDA (0.1 – 3.0 μM) and PDB (0.1 – 3.0 μM) also enhanced the fractional S-I outflow of radioactivity (Figure 2) in a manner analogous to vehicle-incubated tissues. Similarly, in slices incubated with dPA (3 μM) for 20 h, subsequent acute application of dPA (0.1 – 3.0 μM) and PDB (0.1 – 3.0 μM) also enhanced the fractional S-I outflow of radioactivity (Figure 3) in a manner analogous to vehicle-incubated tissues.

Effect of long-term treatment with phorbol esters on PKCα

In rat cortical slices which were incubated for 20 h with vehicle (0.18% v v^{-1} DMSO), PKCα was detected in both the cytosolic and membrane fractions. However, in rat cortical slices which were incubated for 20 h with PDB (1 μM), PKCα was markedly downregulated in both cytosolic and membrane fractions whereas with PDA (3 μM), downregulation had not occurred (Figure 4).

Phorbol ester metabolism during 20 h incubation

The chemical stability of the phorbol esters PDB, PDA following 20 h incubation in modified physiological salt solution (PSS) was examined using the radiolabelled phorbol esters, [^{3}H]-PDB and [^{3}H]-PDA. Peaks of radioactivity (>80%) coincided with authentic [^{3}H]-phorbol ester indicating no chemical or metabolic breakdown (Figure 5a – d).

Effect of long-term treatment with phorbol esters and B-50-phosphorylation

In order to test for down-regulation of PKC, cortical slices were incubated with either PDB (1 μM), PDA (3 μM), dPA (3 μM) or vehicle (0.06 or 0.18% v v^{-1} DMSO) for 20 h before being washed and prepared into membranes for the phosphorylation reactions using [^{33}P]-ATP. After the phosphorylation reaction, the samples were run on SDS – PAGE gel and autoradiography was performed (Figure 6a). B-50, a neural PKC substrate was identified according to molecular weight and Western blot using the mouse anti-human-B-50 primary antibody (Figure 6b).

In membranes from rat cortical slices which were incubated for 20 h with vehicle, subsequent acute application of PDB (3 μM), PDA (10 μM) and dPA (10 μM) significantly enhanced the phosphorylation of B-50 (Figure 7a).

Acute treatment with PDB (3 μM) did not significantly increase B-50 phosphorylation in tissues incubated with PDB (1 μM) for 20 h. However in tissues incubated with PDA (3 μM) or dPA (3 μM) for 20 h, acute application with PDB (Figure 7b), PDA and dPA (Figure 7c) caused a significant increase in B-50-phosphorylation.

Compared to vehicle-incubated tissues (0.18% v v^{-1} DMSO), PDB (1 μM) incubation increased the basal phosphorylation of B-50 (Figure 8). PDA (3 μM) and dPA (3 μM) incubation had no effect on the basal phosphorylation of B-50. The acute application of the PKC inhibitor polymyxin B (21 μM), at a concentration which inhibits PDB-induced facilitation of noradrenaline release from rat brain cortex (Kotsonis & Majewski, 1996), did not significantly decrease B-50-phosphorylation indicating that baseline phosphorylation was not due to PKC activity (Figure 8).

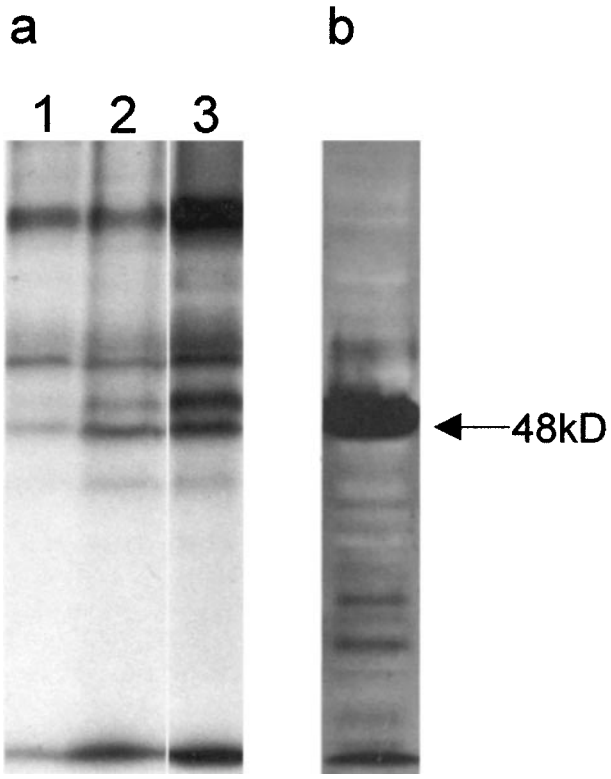

Figure 6 A typical experiment. (a) Autoradiography of phosphorylated proteins run on an SDS – PAGE gel with B-50 at 48 kD. Lane 1 has been incubated with DMSO and lane 2 with PDB (1 μM) for 20 h and both have had vehicle acutely applied. The difference between lanes 1 and 2 shows the influence of incubation with PDB on basal phosphorylation. Lane 3 shows the increase in B-50 phosphorylation when synaptosomes incubated for 20 h with vehicle are then acutely treated with 3 μM PDB. (b) Western blot. A rat brain sample was run on a SDS – PAGE gel. Primary mouse anti-human-B-50 antibody. Secondary goat-anti-mouse antibody conjugated with horse-radish peroxidase.

Discussion

Previous work by our group has shown that PDB, PDA and dPA elevate action-potential evoked noradrenaline release from rat brain cortex but that PDA and dPA had an unusual high potency when compared with their affinity for PKC (see Kotsonis & Majewski, 1996). In the present study we examined the ability of these phorbol esters to cause PKC down-regulation in rat brain cortical slices at concentrations that were equipotent in enhancing noradrenaline release (see Kotsonis & Majewski, 1996: PDB (1 μM), PDA (3 μM) and dPA (3 μM)).

The down-regulation protocol involved incubating rat cortex slices with phorbol ester for 20 h, followed by a wash period, and then examining the effect of acutely applied phorbol ester on either noradrenaline release or on phosphorylation of the neural PKC substrate B-50 (Rodnight &

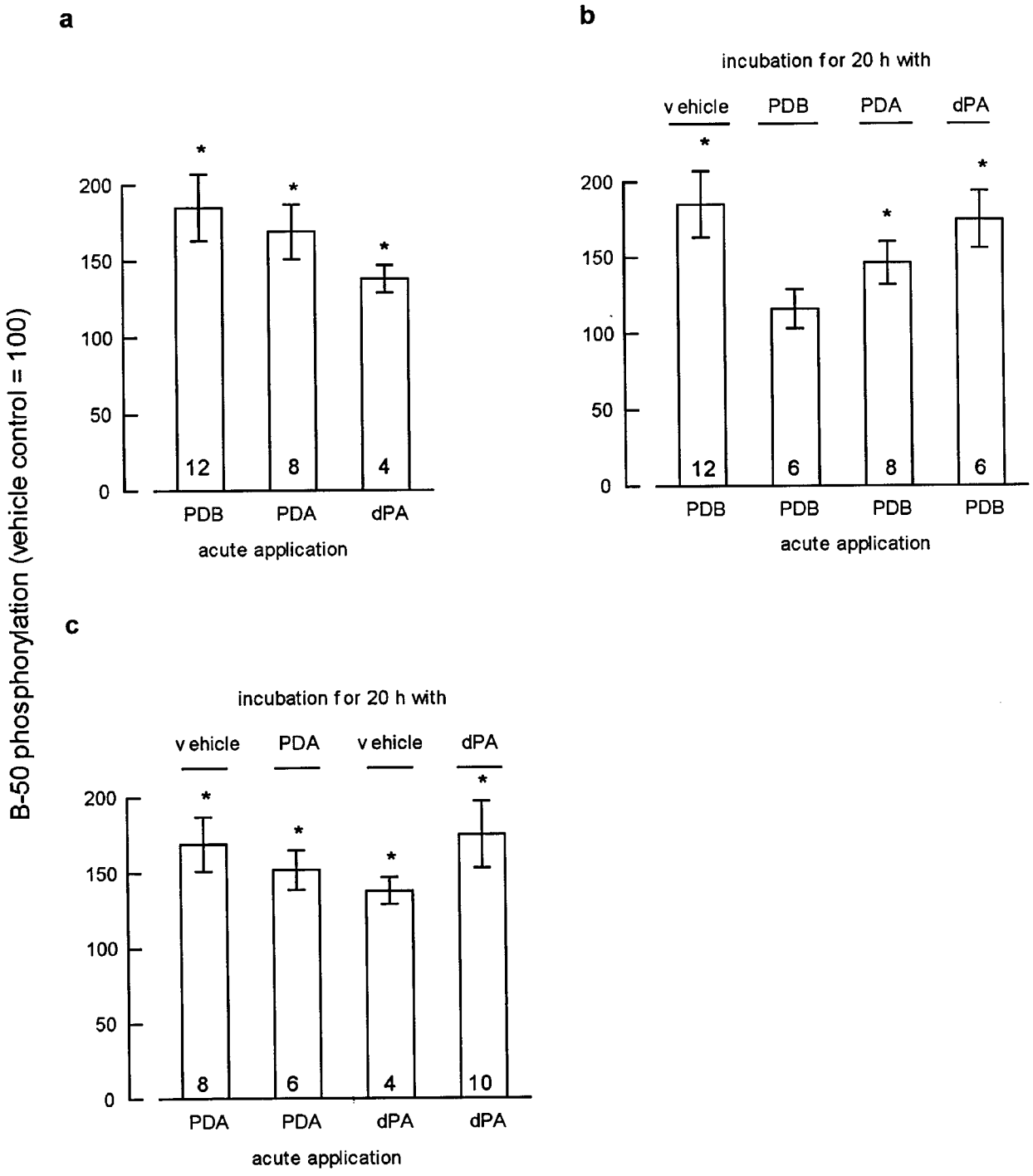

Figure 7 (a) Effects of acute application of PDB (3 μM), PDA (10 μM) and dPA (10 μM) on B-50 phosphorylation in tissues incubated for 20 h with vehicle. (Acute vehicle control = 100). (b) Effects of acute application with PDB on B-50 phosphorylation in tissues incubated for 20 h with vehicle, PDB, PDA or dPA. (Acute vehicle control = 100). (c) Effects of acute application with PDA or dPA on B-50 phosphorylation in tissues incubated for 20 h with PDA or dPA. (Acute vehicle application = 100). The number of animals used in each group is indicated at the base of each column. *Represents a significant increase as compared to acute vehicle application of tissues with the same incubation ($P < 0.05$, Student's *t*-test, comparison *versus* constant).

Perrett, 1986; Dekker *et al*., 1989). In slices incubated with vehicle for 20 h the subsequent acute application of PDB, PDA and dPA enhanced S-I noradrenaline release in a concentration dependent manner. The level of facilitation produced by these agents, the EC_{40} (dose producing 40% facilitation) and the rank order potency was similar to that observed previously in freshly excised rat brain cortical slices without 20 h incubation (see Kotsonis & Majewski, 1996). Taken together these observations suggest that the tissue slices were in fact viable and functional following 20 h incubation.

When slices were incubated for 20 h with PDB, the facilitatory effect on noradrenaline release after subsequent

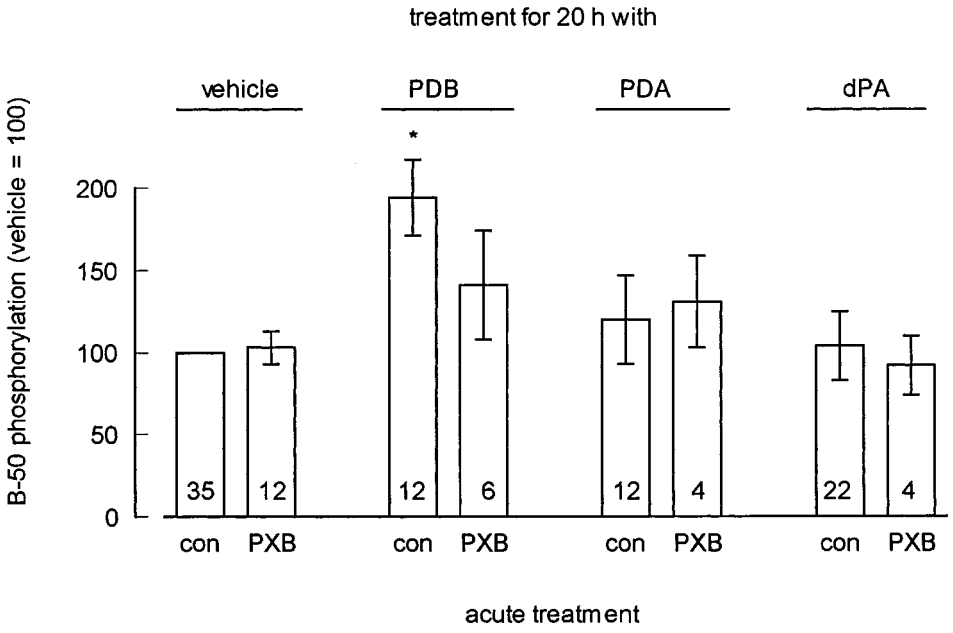

Figure 8 Effects of acute application of polymyxin B (3 μM) on B-50 phosphorylation in tissues incubated for 20 h with vehicle, PDB, PDA and dPA and influence of incubation with PDB, PDA and dPA on acute vehicle application. (Acute vehicle application in vehicle incubated tissues = 100). The number of animals used in each group is indicated at the base of each column. * Represents a significant increase as compared to acute vehicle treatment of tissues pre-treatment with vehicle for 20 h ($P<0.05$, Student's *t*-test, comparison *versus* 100).

acute application of PDB and dPA was abolished which is in agreement with previous studies in mouse cortex using PDB (Foucart *et al*., 1991). This is not due to residual PDB from the incubation pre-empting activation of PKC, since inhibitory effects of PKC inhibitors on noradrenaline release are also abolished (Schroeder *et al*., 1995). The effects of PDB appeared to be specific for PKC since the inactive 4α-isomer of PDB does not cause any diminution of responsiveness (e.g. Foucart *et al*., 1991). The most likely explanation of this effect is that prolonged exposure to PDB induced PKD down-regulation.

In the present study we obtained biochemical verification of PKC down-regulation by examining the phosphorylation of the PKC substrate, B-50, which has been proposed to be the protein which mediates PKC effects on transmitter release (Coggins & Zwiers, 1989; Dekker *et al*., 1989). In this case in rat cortex synaptosomal membranes, B-50 phosphorylation was increased by acute application of PDB, however acute PDB had no effect on synaptosomes from cortex slices previously incubated with PDB for 20 h. As a further index of PKC down-regulation we also measured PKCα immunoreactivity and it was reduced following PDB treatment. These effects on B-50 are in agreement with other studies where prolonged incubation with phorbol esters results in a loss of detectable PKC enzyme protein and kinase activity (Ballester & Rosen, 1985; Adams & Gullick, 1989; Matthies *et al*., 1987; Lindner *et al*., 1991) indicating down-regulation.

It should be noted that the basal phosphorylation of B-50 was increased by the 20 h PDB (1 μM) incubation. Although the reason for this is unclear, it is unlikely that it indicates possible residual activation of PKC following the incubation, as the PKC inhibitor polymyxin B did not significantly decrease the basal phosphorylation. The most likely explanation is that other kinases are more active in our system after the PDB treatment. Preliminary experiments with inhibitors of CAM kinase II and protein kinase A and G also did not reduce the phosphorylation thus the nature of the basal phosphorylation is unknown.

The question arises whether other phorbol esters such as dPA or PDA also cause PKC down-regulation. In slices incubated with dPA (3 μM) for 20 h, the subsequent application of dPA or PDB enhanced the S-I noradrenaline release in a concentration-dependent manner analogous to vehicle incubated tissues. Similar results were obtained in tissues incubated with PDA (3 μM) for 20 h where acute PDA also enhanced S-I release. The lack of any diminution in responsiveness to dPA and PDA in slices incubated with either dPA or PDA markedly contrasts the findings with PDB incubation and strongly suggests that PKC is not down-regulated by PDA and dPA in cortex synaptosomes. These findings were further supported with Western blot analysis which showed that when slices were exposed to PDA for 20 h immunodetection of PKCα was not reduced. It is possible that the concentration of PDA and dPA (3 μM) was not adequate to produce down-regulation, however, these concentrations are equipotent with 1 μM PDB in facilitating transmitter release (see present study and Kotsonis & Majewski, 1996). It was also possible that dPA and PDA were preferentially metabolized in the incubation medium over 20 h such that they were no longer present in sufficient concentrations to cause PKC down-regulation. However, using HPLC and radiolabelled PDA and PDB these agents were found to be chemically intact following 20 h incubation with tissue slices ruling this out.

PKC is a family of multiple related isoenzymes (see Dekker & Parker, 1994) with the various PKC isoenzymes down-regulating at different rates, kinetics and sensitivities to PKC activator drugs (Roivainen & Messing, 1993; Huwiler *et al*.,

1994; Terzian *et al*., 1996; Stanwell *et al*., 1996; Turner *et al*., 1996). Therefore a possible explanation for the lack of effect of dPA and PDA in the present study is that dPA and PDA are acting on different isoenzymes which are less susceptible to down-regulation. Little is known about which isoenzyme(s) mediate the facilitatory effects of phorbol esters in central noradrenergic neurones although PKCα, PKCβ and PKCγ have been detected in rat cortex synaptosomes (a mixed population of cortical nerve terminals, Tanaka, 1991; Oda *et al*., 1991). *In vitro* binding data from rat cortical synaptosomes suggest that both PDB and dPA bind to the same purified classical (c)PKC and novel (n)PKC isoenzymes (Kazanietz *et al*., 1993). It is likely that dPA elevates transmitter release through PDB sensitive isoenzymes as we have previously reported that the facilitatory effect of dPA on S-I noradrenaline release in rat cortex slices was abolished by PDB incubation (20 h) (Kotsonis & Majewski, 1996). Thus when PKC is down-regulated by PDB then dPA and PDA have no effect on transmitter release thus those isozymes down-regulated by PDB must be those which PDA and dPA use to elevate transmitter release.

The process of down-regulation is thought to be associated with a conformational change of PKC, with subsequent proteolysis generating a Ca^{2+} and co-factor independent kinase activity causing degradation and consequently depletion of PKC from the cell (Shea *et al*., 1994; Cressman *et al*., 1995). The rank order of potency for displacement of [^{3}H]-PDB binding in rat cortex synaptosomal membranes was PDB$>>>$dPA$>$PDA (Murphy *et al*., 1999) and this is similar to that reported in other biological systems such as purified PKC isoenzymes (e.g. Dunn & Blumberg, 1983; Dimitrijevic *et al*., 1995). The ability to generate Ca^{2+} independent kinase activity is related to affinity for PKC, where potent activators (e.g. PDB) generate Ca^{2+} independent kinase activity and irreversibly insert PKC into phospholipid vesicles but low affinity phorbol esters (dPA, PDA) do not have this ability (Kazanietz *et al*., 1992). The present findings in intact cells appear to be consistent with this hypothesis. The paradox is that both dPA and PDA are more potent in enhancing transmitter release than predicted by their affinity for PKC compared to lipophilic phorbol esters such as phorbol myristate acetate (PMA) (Kotsonis & Majewski, 1996) yet functionally do not down-regulate their facilitatory actions on transmitter release. The increased relative potency in neurones may be because PDA and dPA are able to better access intraneuronal pools of PKC compared to PMA (Murphy *et al*., 1999).

The lack of effect of prolonged exposure of PDA and dPA in ablating phorbol ester induced transmitter release correlates with their lack of effect in ablating phorbol ester induced B-50 phosphorylation. Conversely, the ability of prolonged exposure to PDB to ablate phorbol ester induced transmitter release correlates with its effect in ablating phorbol ester induced B-50 phosphorylation. Whilst this agrees with the known effects of these agents in inducing Ca^{2+} independent kinase activity (see above) another possible explanation for the lack of effect of dPA and PDA in the present study is that dPA and PDA are acting on different isoenzymes to PDB which are less susceptible to down-regulation. This is unlikely because *in vitro* binding data from rat cortical synaptosomes suggest that both PDB and dPA bind to the same purified classical (c)PKC and novel (n)PKC isoenzymes (Kazanietz *et al*., 1993). Further, prolonged PDB exposure ablates dPA facilitation of transmitter release (Kotsonis & Majewski, 1996) suggesting that PDB may activate the same isozymes.

The inability of dPA and PDA to cause down-regulation adds to their unique character in being able to elevate action-potential induced transmitter release to an extent beyond predictions from their potency on PKC.

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