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Structural determinants of phorbol ester binding in synaptosomes: pharmacokinetics and pharmacodynamics

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Abstract

The present study used structurally distinct phorbol esters to investigate the relationship between their pharmacokinetics of binding to protein kinase C (PKC) in rat brain cortex synaptosomes, their affinity for PKC in synaptosomes and ability to enhance noradrenaline release from rat brain cortex. Affinity binding studies using [^{3}H]phorbol 12,13-dibutyrate (PDB) yielded a rank order of potency for the phorbol esters in binding to synaptosomal PKC of phorbol 12-myristate-13-acetate (PMA) > deoxyphorbol 13-tetradecanoate (dPT) = PDB ≫ 12-deoxyphorbol 13-acetate (dPA) = phorbol 12,13-diacetate (PDA). In intact synaptosomes PDB, dPA and PDA rapidly displaced bound [^{3}H]PDB whereas PMA and dPT were comparatively slow. However, the displacement rates for all the phorbol esters were equally rapid in synaptosomal membranes or synaptosomes permeabilised with *Staphylococcus* α-toxin. These results suggest that the lipophilic phorbol esters (dPT and PMA) are slower to displace [^{3}H]PDB binding because they are hindered by the plasma membrane. In brain cortex slices it was found that the rate of displacement of [^{3}H]PDB binding was closely correlated with the degree of elevation of transmitter noradrenaline release. Thus kinetic characteristics may determine biological responses and this may be particularly evident in events which occur rapidly or where there is fast counter-regulation.

Keywords: Phorbol ester; Noradrenaline release; Synaptosome; Protein kinase C

1. Introduction

The protein kinase C (PKC) family of enzymes which are activated principally by diacylglycerol derived from phosphoinositide or phosphatidylcholine metabolism (Nishizuka, 1992) can also be activated by phorbol esters (Castagna et al., 1982; Sharkey et al., 1984). Despite the acceptance of PKC as the major site of action of phorbol esters, these drugs have a marked heterogeneity in biological response particularly in intact cellular systems. Thus some phorbol esters are tumour promoting whilst others act as anti-tumour agents (Zayed et al., 1984; Szallasi et al., 1993) and the potency for various biological effects seems inconsistent. For example the 12-deoxyphorbol esters are much weaker tumour promoters than would be expected from their skin irritant activity actions (Hergenhahn et al., 1974) and in vascular smooth muscle, phorbol 12-myristate-13-acetate (PMA) is less potent in altering contraction than phorbol 12,13-diacetate (PDA; Sasaguri and Watson, 1990) even though PMA has a far higher affinity (Kazanietz et al., 1992). Attempts have been made to explain this biological diversity through the differential activation of the various PKC isozymes by different phorbol esters (Ryves et al., 1991), differences in the ability of various phorbol esters to insert PKC into membranes (Kazanietz et al., 1992) and different PKC receptors for phorbol esters (Dunn and Blumberg, 1983).

In a recent study we compared a series of 13 phorbol esters and 12-deoxyphorbol esters on their ability to elevate electrically evoked noradrenaline and dopamine release from rat brain cortex slices in vitro (Kotsonis and Majewski, 1996). The potency and efficacy of phorbol esters in enhancing transmitter release was not related to their reported affinities for PKC, their potency on isolated enzymes or their ability to insert PKC into membranes (see Kotsonis and Majewski, 1996). What was observed, how-

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ever, was that when the ester substituents were long chains such as PMA or 12-deoxyphorbol 13-tetradecanoate (dPT) then the effects on transmitter release were poor even though these compounds are reported to be potent PKC activators In contrast, those phorbol esters with short chain ester groups such as PDA and 12-deoxyphorbol 13-acetate (dPA) were more potent than expected. One possible explanation for these results is that the PKC associated with transmitter release is compartmentalised at the nerve terminal and the more lipophilic phorbol esters (PMA, dPT) have limited access to this pool of PKC, perhaps being sequestered by the plasma membrane or other lipid structures whereas short chain ester substituted phorbol esters rapidly reach all pools of PKC.

To better understand whether the actions of phorbol esters in neural tissue were dependent on accessing intraneuronal pools of PKC, we decided to monitor the kinetics of phorbol ester binding in synaptosomes purified from rat brain cortex. We examined the ability of 5 phorbol esters with different ester substituents to displace [^{3}H]4β-phorbol 12,13-dibutyrate (PDB) binding from rat brain synaptosomes under three conditions: intact synaptosomes, synaptosomal membranes and in α-toxin permeabilised synaptosomes. This latter model should allow access of phorbol esters into the interior of the synaptosomes without losing the intracellular proteins as the pores created allow passage of molecules up to about 1000 Da (Bhakdi and Tranum-Jensen, 1991).

2. Materials and methods

2.1. Rat cortex synaptosomes

Rat cortex synaptosomes were prepared according to the method of Dunkley et al. (1986). Two male Sprague–Dawley rats (approximately 200 g) were decapitated and the brains removed and placed in ice-cold buffer (0.32 M sucrose/5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid)/1 mM EGTA, pH 7.4). The cerebellum was removed and the brains separated into hemispheres, and the white matter removed. The rat cortices were gently homogenised in 10 ml ice-cold sucrose/HEPES buffer using a Teflon/glass homogeniser. The suspension was centrifuged at $1000 \times g$ for 10 min at 4°C. The supernatant was collected and centrifuged at $15{,}000 \times g$ for 30 min at 4°C. The P_2 pellet was resuspended in sucrose/HEPES buffer and loaded onto a discontinuous Percoll/sucrose gradient, consisting of Percoll concentrations of 3, 10, 15 and 23%. This was centrifuged at $25{,}000 \times g$ for 8 min at 4°C. Previous studies by Dunkley et al. (1986) have shown that synaptosomes are enriched in the resulting fractions 3 and 4, corresponding to the interfaces between 10–15% Percoll and 15–23% Percoll. The material from these fractions was removed and pooled, diluted with 40 ml ice-cold physiological saline solution A (PSSA: composition (in mM): NaCl 131; KCl 4.7; KH_2PO_4 1.03; glucose 11.1; HEPES 9.7; $CaCl_2$ 1.3; $MgSO_4$ 1.2, disodium EDTA 0.067 and 0.2% (v/v) DMSO, pH = 7.4) and centrifuged at $15{,}000 \times g$ for 30 min. The resulting pellet was resuspended in PSSA to give a final protein concentration of approximately 1 mg ml^{-1}.

2.2. Permeabilised synaptosomes

Synaptosomes were prepared as described above. However, they were then further prepared in different salt solutions to mimic the intracellular ion composition and provide an energy source as is usual in permeabilised preparations (Bader et al., 1986). After density gradient centrifugation the pooled fractions 3 and 4 were diluted in K^+–PIPES buffer (concentrations in mM: KOH 150; PIPES 10; EGTA 5; pH adjusted to 7.2 with propionic acid) and centrifuged at $15{,}000 \times g$ for 30 min. The synaptosomes were resuspended in 2 ml K^+–PIPES buffer and incubated with 50 U ml^{-1} of *Staphylococcus* α-toxin at 37°C for 30 min. At the end of this period the synaptosomes were centrifuged at $15{,}000 \times g$ for 5 min and resuspended in ice-cold 1 ml toxin salt solution (TSS) (mM: KOH 120; $MgCl_2$ 8.93; Na_2ATP 4; phosphocreatine 10; EGTA 1; PIPES 20; $CaCl_2$ 554 nM; DMSO 0.2%; pH adjusted to 7.2 with propionic acid). The permeabilised synaptosomes were washed once in TSS before use (see below).

2.3. Rat cortex synaptosomal membranes

Synaptosomes were prepared as described above. After the density gradient centrifugation, the pooled fractions 3 and 4 were diluted with 40 ml sucrose/HEPES buffer and centrifuged at $15{,}000 \times g$ for 30 min. The pellet was resuspended in 2 ml sucrose/HEPES and diluted with 15 ml 6 mM TRIS–HCl (pH 8.1). This stood on ice for 1.5 h before being centrifuged at $30{,}000 \times g$ for 15 min. The resulting pellet was resuspended in 50 mM TRIS buffer (TRIS–HCl containing 0.2% DMSO, pH = 7.4) to give a final protein concentration of approximately 1 mg ml^{-1}.

2.4. Binding studies

All binding studies were performed in duplicate or triplicate samples from each preparation and the mean value calculated from at least three different preparations. Non-specific binding of [^{3}H]PDB was determined for each time-point in the presence of 30 μM PDB.

In saturation-binding studies 100 μl of synaptosomal membranes were incubated with 200 μl TRIS buffer and 100 μl aliquots of varying concentrations of [^{3}H]PDB for 30 min at 25°C, containing a total of 1% bovine serum albumin.

In concentration–displacement studies, 100 μl of synaptosomal membranes were incubated with 100 μl TRIS buffer, 100 μl, 80 nM [^{3}H]PDB and 100 μl of the displacing ligand at varying concentrations for 30 min at 25°C.

In studies examining the kinetics of displacement of [^{3}H]PDB from synaptosomes by various phorbol esters, 100 μl of synaptosomes were incubated with 100 μl PSSA and 100 μl, 80 nM [^{3}H]PDB (final specific activity = 1.1 Ci mmol^{-1} in PSSA) at 25°C for 15 min. One hundred microliters of the displacing ligand (in PSSA) was then added to give the appropriate final concentration. Studies using permeabilised synaptosomes or synaptosomal membranes were carried out as described above with the exception that PSSA was replaced with TSS or TRIS buffer, respectively.

In all studies, the reaction was terminated by rapid vacuum filtration and the synaptosomes were collected on Whatman GF/C filter paper which had been pre-soaked in 0.3% (v/v) polyethyleneimine. The filters were washed twice with 4 ml ice-cold TRIS buffer and the paper placed in 3 ml ReadyProtein scintillation fluid (Beckman, Melbourne, Australia), left overnight at 4°C and subjected to liquid scintillation counting the following day. Corrections for counting efficiency were made by external standardisation and results are expressed as disintegrations per minute (d min^{-1}).

2.5. Noradrenaline release from rat cortex

Rat brains were placed in ice-cold physiological saline solution B (PSSB: (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.03, $NaHCO_3$ 25, D-(+)-glucose 11.1, $MgSO_4$ 1.2, $CaCl_2$ 1.3, ascorbic acid 0.14 and disodium EDTA 0.067) previously gassed with a mixture of 5% CO_2 and 95% O_2. Slices from cerebral cortex (400 μm in thickness) were obtained with a Campden vibroslice. Slices were incubated in PSSB containing [^{3}H]noradrenaline (10 μCi ml^{-1}, 0.2 μM), maintained at 37°C and gassed with the mixture described above, for 20 min. Following incubation, the slices were rinsed, transferred to flow cells and continuously superfused at 0.5 ml min^{-1} with PSSB which had been pre-gassed with 5% CO_2/95% O_2 and maintained at 37°C. The slices were superfused for 60 min before sample collection began. After 30 min of this washing period, an electrical priming stimulation was delivered through a pair of parallel platinum electrodes (34 V cm^{-1}, 22 mA, square wave pulses of 2-ms duration, 1 Hz for 60 s). After the washing period the superfusate was collected over 5 min periods for the following 150 min. At 10, 55, 95 and 135 min after the commencement of the collection period, the cortical slices were stimulated (each at 1 Hz for 60 s, S_1–S_4). Phorbol esters, where present, were added to the superfusate 35 min prior to the second stimulation period at a concentration of 3 μM. At the completion of the experiment the cortical slices were 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 ml Picofluor-40 (Packard Instruments) 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}).

In calculating the results for noradrenaline release experiments, 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 outflow from the radioactive content of each of the two 5-min samples collected immediately after the commencement of each stimulation. These values were 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.

2.6. Statistics

Data obtained from kinetic binding experiments were analysed using computer program KINETIC/EBDA/LIGAND (Elsevier Biosoft, Cambridge,UK) and non-linear curve fitting contained in Deltagraph 3 (Deltapoint, Monterey, CA, USA). Data obtained from saturation and concentration–displacement binding experiments were analysed using the GraphPad Prism (San Diego, CA, USA) programs. The values are given as mean and standard error of the mean (S.E.M.). The *n* indicates the number of synaptosomal preparations used for binding experiments, or the number of slices used in noradrenaline release experiments; each synaptosomal preparation was derived from different rat brains, cortex slices came from different animals. The results from release experiments were analysed using Sheffè's test after a two-way analysis of variance (ANOVA). Values of $P < 0.05$ were taken as indicating statistical significance. The statistical package used was GB-Stat (Dynamic Microsystems, Silver Spring, USA).

2.7. Radiochemicals and drugs

Drugs used were: [20-^{3}H(*N*)]PDB (specific activity 19.6 Ci mmol^{-1}, (−)-[ring-2,5,6-^{3}H]noradrenaline (specific activity 43.7 Ci mmol^{-1}) (both DuPont NEN Products; Boston, USA); PDB, PMA, PDA, dPA, dPT (LC Laboratories, Woburn, USA); *Staphylococcus aureus* α-toxin (Calbiochem, USA). Stock solutions of the phorbol esters were dissolved in DMSO and stored at −20°C. On the day of the experiment they were diluted in the appropriate solution. Appropriate vehicle experiments were carried out where required.

3. Results

3.1. Binding of [^{3}H]PDB to synaptosomal membranes

The time-course of association of [^{3}H]PDB (20 nM) to rat cortex synaptosomal membranes was examined. [^{3}H]PDB bound rapidly to synaptosomal membranes at 25°C, reaching equilibrium within 15 min and binding did not decline significantly over the following 45 min (not shown). The initial rate of association was too rapid to allow an association constant to be calculated, with nearly 35% of total [^{3}H]PDB binding occurring within 5 s following addition to the preparation. In saturation-binding studies 30-min contact time was used and it was found that specific binding of [^{3}H]PDB to rat cortex synaptosomal membranes was saturable (Fig. 1A), and at equilibrium non-specific binding constituted, at the highest concentration of PDB used, approximately 35% of total binding (not shown). Analysis of the Scatchard plot of the saturation binding curve suggested a single population of [^{3}H]PDB binding sites with a K_d of 19.9 ± 5.3 nM and a B_{max} of 13.1 ± 0.3 pmol mg^{-1} protein (Fig. 1A).

Five phorbol esters, PDB, PMA, PDA, dPA and dPT were tested for their ability to displace [^{3}H]PDB binding in synaptosomal membranes (Fig. 1B). These studies were conducted with 30-min contact time. There were marked differences in the affinities of the phorbol esters for the [^{3}H]PDB binding site, the rank order of potency being PMA > dPT = PDB ≫ dPA = PDA (Fig. 1B, Table 1). After correction for ligand concentration using the Cheng–Prusoff equation, the K_i calculated for PDB was (61.6 ± 1.9 nM). Analysis of the concentration–displacement curves with GraphPad Prism suggested that each of the phorbol esters tested bound to a single population of binding sites (Fig. 1B). However, the Hill coefficient for PMA and dPT was significantly different from 1 indicating that there may be cooperativity (see Table 1).

Table 1
Displacement data for [^{3}H]PDB binding in synaptosomal membranes. The IC_{50} and K_i values for the [^{3}H]PDB binding site for each phorbol ester calculated from data shown in Fig. 1. Data shown as mean ± S.E.M. or with confidence limits as appropriate. The rank order of potency which was statistically significant (Mann–Whitney rank sum test, $P < 0.05$) was PMA > dPT = PDB ≫ dPA = PDA

Drug	IC_{50} (nM)	K_i (nM)	(−) Hill coefficient (95% confidence limits)
PDB	123.0 ± 3.8	61.6 ± 1.9	1.3 (1.9–0.7)
dPA	1100.0 ± 132.0	549.0 ± 66.2	1.1 (1.4–0.8)
PDA	1460.0 ± 201.0	730.0 ± 100.0	1.5 (1.8–1.1)
PMA	5.2 ± 0.7	2.6 ± 0.3	1.5 (1.8–1.1)
dPT	53.5 ± 5.6	26.7 ± 2.8	1.3 (1.7–1.0)

Phorbol esters were used at a concentration of 3 μM to examine the rate at which they displaced [^{3}H]PDB binding from synaptosomal membranes (Fig. 2). The phorbol ester with lowest affinity (dPA) did not fully displace bound [^{3}H]PDB at equilibrium (Fig. 2A). The time–displacement curve for all the phorbol esters was best described by a two-site model. The initial rates of displacement were similar for all the phorbol esters (Fig. 2A) and the amount

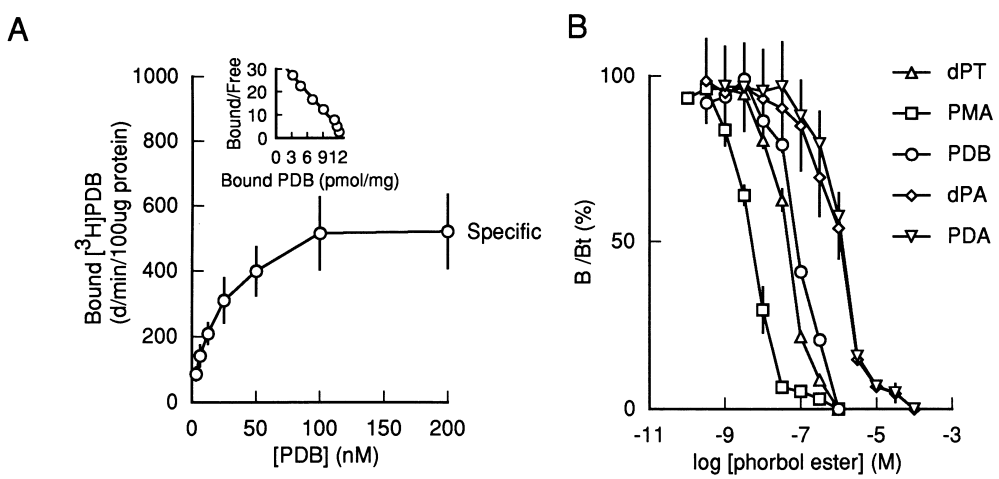

Fig. 1. Binding of [^{3}H]PDB to synaptosomal membranes derived from rat brain cortex. (Panel A) Saturation binding: membranes were incubated at 25°C for 30 min with the concentration of [^{3}H]PDB indicated and the reaction terminated by rapid vacuum filtration. The *y*-axis represents [^{3}H]PDB bound specifically in disintegrations per minute (d min^{-1}) per 100 μg/protein. The contact time was 30 min. The non-specific binding of [^{3}H]PDB was determined for each time-point in the presence of 30 μM and subtracted from total binding to reveal specific binding. Points represent the mean ± S.E.M. for six separate membrane preparations, with two replicates made in each preparation. The inset shows the Scatchard transformation of the curve for specific binding. (Panel B) Displacement of specific [^{3}H]PDB binding (20 nM) from rat cortex synaptosomal membranes by varying concentrations of dPT, PMA, PDB, dPA and PDA. In each experiment specific binding of [^{3}H]PDB in the presence of the displacing ligand was expressed as a percentage of the total specific binding in the absence of competing ligand. The non-specific binding of [^{3}H]PDB was determined in the presence of 30 μM and subtracted from total binding to reveal specific binding. Points represent the mean ± S.E.M. for three–six separate membrane preparations, with two replicates in each preparation. See Table 1 for K_i data.

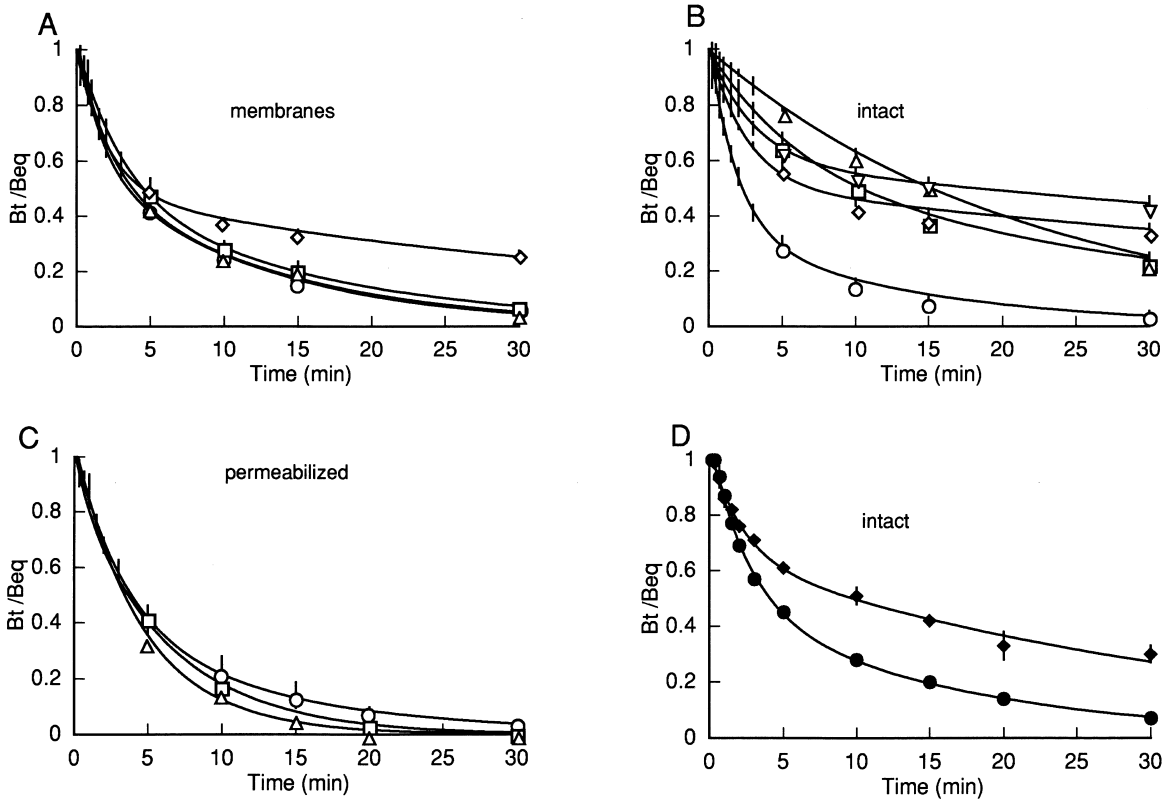

Fig. 2. Time-course of displacement of [^{3}H]PDB from rat cortex synaptosomes membranes in the presence of 3 μM dPT (△), PMA (□), PDB (○), dPA (◇) and PDA (▽). There were three preparations. (Panel A) Synaptosomal membranes. (Panel B) Intact synaptosomes. (Panel C) α-toxin permeabilised synaptosomes. (Panel D) Intact synaptosomes with 10 μM dPA (●) and PDA (♦). In all cases preparations were incubated with 20 nM [^{3}H]PDB for 15 min at 25°C, then the displacing drug was added and the reaction terminated at the indicated time by rapid vacuum filtration. At each time point specific binding of [^{3}H]PDB in the presence of the displacing ligand (Bt) was expressed as a fraction of the total specific binding prior to the ligand being added (Beq). Non-specific binding was determined in the presence of 30 μM PDB. Points represent the mean ± S.E.M. for three separate membrane preparations, with three determinations made in each preparation.

of bound [^{3}H]PDB displaced after 2 min was similar for all the phorbols (Fig. 3).

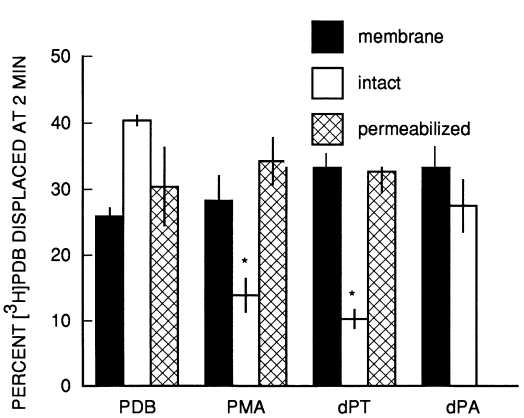

Fig. 3. Comparison of amount of displacement of [^{3}H]PDB in the presence of dPT, PMA, PDB and dPA each with 2-min contact in rat cortex synaptosomal membranes; intact synaptosomes and α-toxin permeabilised synaptosomes. Data taken from Fig. 2. * Significantly different from membrane and permeabilised preparations, $P < 0.05$, Student's *t*-test.

3.2. Binding of [^{3}H]PDB in intact synaptosomes

In intact synaptosomes, binding studies were conducted in a modified physiological salt solution to maintain the integrity of the synaptosomes. Binding of [^{3}H]PDB reached equilibrium within 15 min and binding did not decline significantly over the following 45 min (not shown). PDB (3 μM) rapidly displaced [^{3}H]PDB (20 nM) from rat cortex synaptosomes (Fig. 2B). dPA and PDA (both 3 μM) were marginally less rapid displacers of [^{3}H]PDB binding than PDB from synaptosomes (Fig. 2B). PMA and dPT (both 3 μM) were significantly slower displacers of [^{3}H]PDB binding than PDB, dPA and PDA (Fig. 2B). PMA and dPT also displaced less [^{3}H]PDB over the first 2 min of exposure than the other phorbol esters (Fig. 3). As in synaptosomal membranes, at 3 μM the phorbol esters with lowest affinity (dPA and PDA) did not fully displace bound [^{3}H]PDB over 30 min (Fig. 2B). Higher concentrations of dPA and PDA (10 μM) did cause significantly greater displacement of dPT and PMA over 30 min, but the initial rate of displacement was not different from that observed with 3 μM (Fig. 2D). PMA and dPT fully

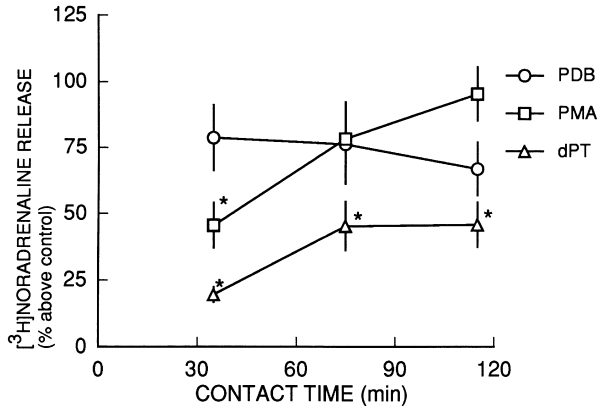

Fig. 4. The time-course of the effects of PDB (○, 3 μM), PMA (□, 3 μM) and dPT (△, 3 μM) on the fractional S-I outflow of radioactivity from rat cortex slices pre-incubated with [^{3}H]noradrenaline. There were four periods of electrical stimulation (each 1 Hz for 60 s,) and phorbol esters were present during the second, third and fourth stimulation period which were 40 min apart. For each stimulation period (2–4) the fractional release (FR) was expressed as a ratio of that in the first stimulation (FR_x/FR_1) and normalised such that phorbol ester-free controls = 100. Each symbol represents the mean ± S.E.M. * Represents a significant difference from PDB initial effect at $t = (P < 0.05$, Sheffè's test after two-way ANOVA).

displaced [^{3}H]PDB binding after 60 min exposure (amount [^{3}H]PDB displaced after 60 min: PMA 96.0 ± 4.2%, $n = 3$; dPT 90.8 ± 6.8%, $n = 3$, see also Fig. 2B). A dual exponential rate of displacement, suggesting a two-site model best fitted the time–displacement curve obtained with all the phorbol esters tested in synaptosomes apart from dPT, whose displacement curve is best described by a single exponential rate.

Displacement experiments were also performed in synaptosomes permeabilised with *Staphylococcus* α-toxin. In these studies the rates of [^{3}H]PDB displacement by PDB, dPT and PMA were not significantly different, and similar to the rates observed in synaptosomal membranes (Fig. 2C). The amount of [^{3}H]PDB binding displaced over the first 2 min of exposure was similar for all three phorbol esters, and similar to the amount displaced by these compounds in synaptosomal membranes (Fig. 3).

3.3. Phorbol esters and [^{3}H]noradrenaline release

These studies examined the time-course of the effect of phorbol esters on electrical S-I outflow of [^{3}H]noradrenaline from slices of rat brain cortex. PDB caused a rapid facilitation of (S-I) outflow of [^{3}H]noradrenaline which was maximal by 35 min whereas both PMA and dPT required 90 min for their maximal effect to be apparent (Fig. 4). The maximal effect of dPT was less than that of both PMA and PDB (Fig. 4).

3.4. Relationship between transmitter release and binding rate constants and affinity

We used the data on noradrenaline release in rat cortex slices incubated with [^{3}H]noradrenaline (Kotsonis and Majewski, 1996). In these experiments the stimulation frequency was the same as the present study (1 Hz for 60 s) and the concentrations of the phorbol esters was 3 μM as in the present study. Correlations were made for the five phorbol esters tested between release efficacy and the rate at which the displaced [^{3}H]PDB binds with intact synaptosomes. In this case there was a strong positive correlation ($r^2 = 0.87$; Fig. 5A). On the other hand there was no positive relationship between affinity (K_i) and effect on noradrenaline release ($r^2 = 0.42$; Fig. 5B).

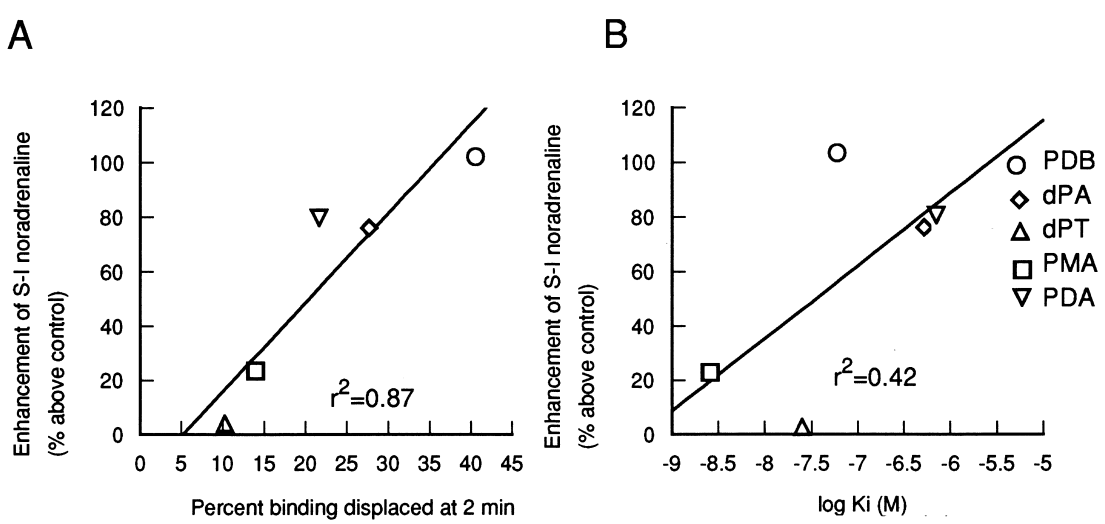

Fig. 5. Relationship between elevation of noradrenaline release and phorbol ester affinity and rate of displacement of binding. The noradrenaline release data are taken from (Kotsonis and Majewski, 1996) with the affinity data of the phorbol esters (*x*-axis) taken from Fig. 1B and the percentage of [^{3}H]PDB displaced in 2 min in intact synaptosomes by the various phorbol esters, from the data in Fig. 3A. The line drawn is the regression fit.

4. Discussion

The present study examined phorbol ester binding in rat cortex synaptosomes using the ligand [^{3}H]PDB which binds to PKC at the same site as diacylglycerol, the endogenous PKC activator (Castagna et al., 1982; Sharkey et al., 1984). The synaptosomal preparation is probably heterogeneous with respect to PKC isozymes with at least PKC α, β and γ being found in rat cortex synaptosomes (Oda et al., 1991; Tanaka, 1991). Nevertheless, our data suggest a single population of [^{3}H]PDB binding sites in synaptosomal membranes. The affinity of [^{3}H]PDB for this site (19.9 nM) was comparable with that reported in other tissues (Dunphy et al., 1981). The aim of our study was to compare the effects of a series of phorbol esters on [^{3}H]PDB binding. In addition to PDB, we studied four compounds where the lipophilicity of the ester substituents were different: the deoxyphorbols dPT and dPA which differ in the size of the 13 ester substituents (tetradecanoate vs. acetate) and PMA and PDA which differ in the size of the 12 ester substituents (myristate vs. acetate). The rank order of potency for displacement of [^{3}H]PDB binding in synaptosomal membranes was: PMA > dPT = PDB ≫ dPA = PDA and this is similar to that reported in other studies with purified PKC isozymes (Kazanietz et al., 1992, 1993; Dimitrijevic et al., 1995), although no information is available for dPT. It should be noted that these compounds have no marked selectivity for any of the isoenzymes in the cPKC or nPKC families (Kazanietz et al., 1992, 1993; Dimitrijevic et al., 1995) again there is no information for dPT.

The time-course of the displacement of [^{3}H]PDB binding in synaptosomal membranes was also studied and all of the compounds showed a rapid displacement of binding suggesting that they all access the PKC to a similar extent. Although the initial rate of displacement of binding was similar for all compounds in the membranes, dPA and PDA did not displace all of the binding at a concentration of 3 μM which is consistent with their low affinity. PDB, dPA and PDA showed similar rapid rates of displacement between intact synaptosomes and membranes. However, PMA and more strikingly dPT were markedly slower in displacing [^{3}H]PDB binding in intact synaptosomes compared to the other compounds and compared to their effects in membranes. We suggest that the synaptosomal membrane presents a barrier for PMA and dPT to access PKC and that this probably relates to their greater lipophilicity compared to PDB, PDA and dPA. We also used α-toxin in intact synaptosomes to create small pores to allow passage of molecules up to 1000 D (Bhakdi and Tranum-Jensen, 1991). In this case there were no differences in the rate of displacement of [^{3}H]PDB binding between PDB, dPT and PMA which is in accord with the plasma membrane acting as a barrier for dPT and PMA.

The weight of evidence suggests that PKC recognises the membrane dissolved form of the phorbol ester (Sharkey and Blumberg, 1985) which may suggest that the slower effects of dPT and PMA in intact synaptosomes may be because they may have difficulty in transferring from the aqueous phase to the membrane lipid as has been reported for phorbol 12,13 dimyristate in micelle systems (Sharkey and Blumberg, 1985). This is unlikely since in the membrane systems and permeabilised synaptosomes where such a transfer is also required both compounds had rates of initial displacement similar to PDB. An alternative explanation is that lipophilic compounds may be sequestered in the membrane and slowly reach distal structures on the internal surface of the intact synaptosome plasma membrane or deeper within the synaptosome. There are suggestions that PKC isozymes may be compartmentalised within cells (Mochly-Rosen et al., 1991; Leach et al., 1992; Wetsel et al., 1992; Buchner, 1995) including the cytoskeletal structures of synaptosomes (Tanaka, 1991). PMA washes out of cell membranes less effectively than PDB (Szallasi et al., 1994) and presumably this indicates that PMA is also slower to enter the cell cytoplasm and to partition into other internal structures.

The impetus for the current study was to try and explain why potent phorbol esters such as dPT and PMA were less effective in elevating transmitter release in rat cortex than those with low affinity for PKC such as PDA and dPA (Kotsonis and Majewski, 1996). The present study suggests that even though PDA and dPA may have 100 fold less potency in displacing [^{3}H]PDB binding than PMA and dPT, that they act extremely rapidly in intact synaptosomes presumably because the ester substituents are less lipophilic and they are not restricted by the plasma membrane. The rate of activation of PKC may be important because the phosphorylation of PKC substrates appears tightly counter-regulated by phosphatases. For example in rat cortex synaptosomal membranes B50 is a PKC target thought to be involved in transmitter release (Decker et al., 1989). Contact with PDB for 30 s results in maximal phosphorylation of B50 with longer contact times showing less phosphorylation because of endogenous phosphatase action (Dokas et al., 1990). With a slow rate of activation such as with dPT in the present study, phosphatases may more effectively limit phosphorylation.

In the study of Kotsonis and Majewski (1996) the contact time with the phorbol esters varied as between 15 and 45 min due to the study design which used cumulative concentration–response curves making strict kinetic analysis difficult. Nevertheless, the effect of phorbol esters on transmitter release (data from Kotsonis and Majewski, 1996) is extremely well correlated with their initial rate of displacement of [^{3}H]PDB binding in the present study in intact synaptosomes and is not related to affinity. In the present study, we also carried out a long term incubation with three phorbol esters and at 15-min contact the order of enhancement of noradrenaline release potency PDB > PMA > dPT was similar to the predictions from displacement of [^{3}H]PDB binding, although with longer contact

time PMA did have an increased effect on noradrenaline release. However, the slowest displacer of [^{3}H]PDB binding in intact synaptosomes, dPT, only elevated transmitter release to 50% of what PDB did even after 90 min contact. It should be noted that 60 min was sufficient time for dPT to displace all [^{3}H]PDB binding suggesting that it was not an access problem. This reinforces the view that for transmitter release that the rate of activation of PKC is important.

There are many reports of anomalous behaviour of phorbol esters in intact biological systems where rank orders of potency of phorbol esters do not fit with known potency to activate PKC in enzyme studies (see Section 1). In some systems where there is fast signalling, the initial rate of activation of PKC may be important for the biological effect due to rapid counter regulation. On the other hand with slower events such as tumour formation the PKC targets may not be so quickly counter-regulated and therefore the biological effect is more closely related to affinity for the enzyme.

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