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Protein kinase C involvement in maintenance and modulation of noradrenaline release in the mouse brain cortex

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1 The role of protein kinase C in the modulation of noradrenaline release was investigated in mouse cortical slices which were pre-incubated with [^{3}H]-noradrenaline. The aim was to investigate the hypothesis that protein kinase C is activated during high levels of transmitter release to maintain transmitter output.

2 The protein kinase C activators, phorbol myristate acetate (0.01 – 0.3 μM) and to a greater extent 4β-phorbol 12,13-dibutyrate (0.01 – 0.3 μM) significantly enhanced stimulation-induced noradrenaline release whereas 4α-phorbol 12,13-dibutyrate (0.1 μM) which does not activate protein kinase C was without effect. The effect of the protein kinase C activator, phorbol myristate acetate, on noradrenaline release was attenuated by the protein kinase C inhibitor, polymyxin B (21 μM) which by itself inhibited stimulation-induced noradrenaline release.

3 Protein kinase C was down-regulated by 10 h exposure of the cortical slices to 4β-phorbol 12,13-dibutyrate (1 μM). In this case the facilitatory effect of 4β-phorbol 12,13-dibutyrate (0.1 μM) on noradrenaline release was abolished as was the inhibitory effect produced by polymyxin B. This indicates that polymyxin B was acting selectively at protein kinase C.

4 The inhibitory effect of polymyxin B on noradrenaline release, when expressed as a percentage of the appropriate frequency control, was constant at 1, 5 and 10 Hz. Furthermore, the ratio of release at 5 Hz to that at 10 Hz was not altered by protein kinase C down-regulation, indicating that there is no additional effect of protein kinase C at higher stimulation frequencies.

5 When transmitter release was elevated by blocking α_2-adrenoceptor auto-inhibition with idazoxan (0.1 μM) or K^+ channels with tetraethylammonium (300 μM), the elevation in transmitter release was significantly attenuated by protein kinase C down-regulation, suggesting an involvement of protein kinase C.

6 We conclude that protein kinase C is involved in the modulation of noradrenaline release over a wide range of stimulation frequencies, in addition to a role when noradrenaline release is elevated by presynaptic mechanisms.

Keywords: Protein kinase C; noradrenaline release; mouse cortex; prejunctional modulation; phorbol ester; polymyxin B

Introduction

The protein kinase C family of enzymes are activated by diacylglycerol and/or arachidonic acid and metabolites such as lipoxins which are formed through various pathways after breakdown of membrane phospholipids by phospholipases (Stabel & Parker, 1991; Nishizuka, 1992). It has been postulated that protein kinase C within nerve terminals may be involved in the modulation of transmitter release. The first line of evidence is that some proteins phosphorylated by protein kinase C may be involved in various stages of the release process, for example B-50 (Dekker *et al.*, 1989), annexins (Johnstone *et al.*, 1992; Wang & Creutz, 1992) and membrane ion channels (Shearman *et al.*, 1989). Secondly, activators of protein kinase C such as phorbol esters enhance transmitter release from a variety of neurone types. For example, in noradrenergic neurones in the peripheral and the central nervous system, phorbol esters enhance stimulation-induced noradrenaline release in several tissues including mouse atria (Musgrave & Majewski, 1989), rat atria (Ishac & De Luca, 1988), rat hippocampus (Versteeg & Florijn, 1987; Allgaier *et al.*, 1991), rat tail artery (Bucher *et al.*, 1991), canine saphenous vein (Takata *et al.*, 1991), rat brain synaptosomes (Nichols *et al.*, 1987; Oda *et al.*, 1991), guinea-pig brain synaptosomes (Shuntoh *et al.*, 1988), rabbit hippocampus (Allgaier & Hertting, 1986; Allgaier *et al.*, 1986; 1987; Huang *et al.*, 1988), rat amygdala (Versteeg & Ulenkate, 1987), rat salivary gland (Wakade *et al.*, 1985), rat sinus node (Shuntoh & Tanaka, 1986), guinea-pig atria (Brasch, 1991) and guinea-pig perfused heart (Haas *et al.*, 1990). Finally, inhibitory effects of protein kinase C inhibitors on action-potential evoked noradrenaline release have been often observed (e.g. Allgaier & Hertting, 1986; Ishac & De Luca, 1988).

Although a physiological role for protein kinase C in noradrenaline release is critically dependent on inhibitor studies, protein kinase C inhibitors are notoriously non-selective (Schächtele *et al.*, 1989; Rüegg & Burgess, 1989) making firm conclusions difficult. Furthermore, in some cases inhibitory effects of protein kinase C inhibitors on noradrenaline release are seen only in concentrations in excess of that required to block the facilitatory actions of phorbol esters (e.g. Daschmann *et al.*, 1988; Musgrave & Majewski, 1989), which raises questions about the mechanism involved.

In mouse atria the protein kinase C inhibitor, polymyxin B, at a low concentration, did not inhibit transmitter release at 5 Hz stimulation but did have a significant inhibitory effect at 10 Hz (Musgrave & Majewski, 1989). This rules out non-specific depression of transmitter release by polymyxin B. Furthermore, polymyxin B as well as down-regulation of protein kinase C depressed high output transmitter release regardless of whether this was elicited by high frequency stimulation or by activation of either presynaptic receptors (angiotensin II) or second messenger systems (8-bromocyclic AMP) or blockade of presynaptic α-adrenoceptors (idazoxan, phentolamine) or blockade of presynaptic K^+ channels (tetraethylammonium) (Musgrave & Majewski, 1989; Foucart *et al.*, 1991). It was

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suggested that during high levels of noradrenaline release, protein kinase C is endogenously activated to maintain transmitter output and this is the basis of the protein kinase C/high output noradrenaline release hypothesis (Musgrave & Majewski, 1989; Foucart *et al.*, 1991).

The aim of the present study was to investigate the protein kinase C/high output noradrenaline release hypothesis in mouse brain cortex. We used three separate ways of enhancing noradrenaline release: blockade of repolarizing K^+ channels with tetraethylammonium (Armstrong & Binstock, 1965), blocking auto-inhibition through presynaptic α_2-adrenoceptors with idazoxan (Starke *et al.*, 1989) and using high frequency (10 Hz) nerve stimulation. Disruptions to the proposed protein kinase C involvement were assessed by using polymyxin B, a protein kinase C inhibitor (IC_{50} 4 to 8 μM for protein kinase C, Mazzei *et al.*, 1982; Kuo *et al.*, 1983; cf. IC_{50} protein kinase A, protein kinase G > 100 μM, Mazzei *et al.*, 1982; Schächtele *et al.*, 1989), and down-regulation of protein kinase C by prolonged treatment with the protein kinase C activator 4β-phorbol 12,13-dibutyrate (Matthies *et al.*, 1987; Foucart *et al.*, 1991). The selectivity of polymyxin B was also tested by examining its effects on transmitter release in brain slices in which protein kinase C had been down-regulated.

Methods

Noradrenaline release from mouse cerebral cortex

Outbred male Swiss White mice (17–25 g) were decapitated and the brains rapidly excised. Slices from the cerebral cortex (400 μm thick) were obtained with a Campden vibroslice and were incubated in a Krebs-Henseleit solution maintained at 37°C and bubbled with a mixture of 5% CO_2 and 95% O_2 containing [^{3}H]-noradrenaline (10 μCi ml^{-1}, 0.1 μM) for 20 min. Following incubation, the slices were rinsed, transferred to flow cells (4 cells per bank with electrodes connected in series) and continuously superfused at 1 ml min^{-1} with [^{3}H]-noradrenaline-free Krebs-Henseleit solution maintained at 37°C. The slices were superfused for 45 min before sample collection began. This was the washing period. After 30 min of washing, an electrical priming stimulation was delivered through a pair of parallel platinum electrodes placed either side of the brain slice (field strength 6 V/cell, 22 mA, square wave pulses at a frequency of 5 Hz with 2 ms pulse duration over 60 s). After the washing period was completed, the collection period began in which superfusate fractions were collected over consecutive 3 min periods for a total of 69 min. At 9 and 54 min after the start of the collection period, the cortical slices were stimulated (each at 5 Hz for 60 s, S_1 and S_2). In some experiments S_2 was at either 1 Hz or 10 Hz for 60 s. The change in frequency at S_2 (1, 5 or 10 Hz) simultaneously changes the number of pulses (60, 300 and 600 pulses respectively). Since this is not relevant for the interpretation, only the change in frequency is indicated below. The effect of drugs on the electical stimulation-induced outflow of radioactivity was determined by adding them to the superfusate solution 15 min before the second stimulation with the exception of the protein kinase C inhibitor, polymyxin B, as well as idazoxan, which were added 30 min before the second stimulation. At the completion of the experiments the cortical slices were removed from the flow cells and placed in 1 ml Soluene (Packard Instruments) for 24 h to solubilize completely the tissue. The radioactivity present in the superfusate solution and brain slices were determined by liquid scintillation counting after the solutions were mixed with 6 ml Picofluor 40 (Packard Instruments). Corrections for counting efficiency were made by atuomatic external standardization.

Long-term treatment with phorbol esters

Cortical brain slices from the mouse were prepared as described previously. The slices were placed in 50 ml Krebs-Henseleit medium containing bovine serum albumin (5 g l^{-1}) in an open Petri dish and maintained at 37°C in a tissue culture incubator for 10 h. The atmosphere of the incubator was a mixture of 95% O_2 and 5% CO_2. Phorbol ester or vehicle, dimethyl sulphoxide (DMSO) was added to the culture medium and equilibrated in the incubator for 30 min before the cortical brain slices were added. At the end of the 10 h incubation, the brain slices were removed from the culture medium and washed in 100 ml Krebs-Henseleit solution. The slices were then incubated with [^{3}H]-noradrenaline (10 μCi ml^{-1}, 0.1 μM) in Krebs-Henseleit medium at 37°C for 20 min and then followed the identical protocol as described above.

Calculation of results

The resting (spontaneous) outflow of radioactivity for each stimulation period was taken as the mean radioactive content of the bathing solution during the 3 min period immediately before and the 3 min period commencing 9 min after the start of the respective stimulation. The stimulation induced (S-I) component of the outflow of radioactivity was calculated by subtracting the mean spontaneous radioactive outflow from the radioactive content of each of the three 3 min samples collected immediately after the start of the first stimulation (S_1) and each of the three 3 min samples collected immediately after the start of the second (S_2). This value was then expressed as a fraction of the radioactivity present in the tissue at the onset of stimulation (the fractional S-I outflow FR). Drug effects on the S-I outflow of radioactivity were evaluated by comparing the ratio FR_2/FR_1. For greater clarity, in the figures the FR_2/FRM_1 ratios were normalised such that control represents 100 arbitrary units.

Statistics

The values are given as mean and standard error of the mean (s.e.mean), *n* indicates the number of experiments; within each experimental group, the slices came from different animals. The results were analysed by *a priori* Student's unpaired *t* tests after one-way analysis of variance or two-way analysis of variance as indicated in the text; *t* tests were modified according to the Bonferroni method when multiple results were compared to a single control. For unplanned multiple comparisons Tukey's test was used after analysis of variance. 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. Where appropriate two-way analysis of variance was also carried out to determine whether there was an interaction between manipulations to enhance noradrenaline release (A) and polymyxin B or protein kinase C 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. This indicates whether the effect of the manipulation (A) was significantly altered in the presence of a given anti-protein kinase C measure (B) and correspondingly whether the effect of the anti-protein kinase C measure (B) was significantly altered in the presence of manipulation (A) (see Snedecor & Cochran, 1974).

Materials

The Krebs-Henseleit solution contained (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.

Drugs

Drugs used were (−)-[ring-2,5,6-^{3}H]-noradrenaline (DuPont NEN Products; Boston, U.S.A.) and (±)-idazoxan HCl (Reckitt and Colman, Hull, U.K.). Bovine serum albumin was obtained from the Commonwealth Serum Laboratories (Parkville, Australia). Tetraethylammonium chloride, tetrodotoxin,

phorbol 12-myristate 13-acetate, 4β-phorbol 12,13-dibutyrate and polymyxin B sulphate were obtained from Sigma (St Louis, U.S.A.). 4α-phorbol 12,13-dibutyrate was obtained from LC Laboratories (Woburn, U.S.A.). Stock solutions of phorbol esters were made up in dimethyl sulphoxide (DMSO) and stored at −20°C. The other drugs were initially dissolved in Krebs-Henseleit solution before being diluted in Krebs-Henseleit solution except for idazoxan, which was initially dissolved in deionized water. Where appropriate, contemporaneous control experiments were carried out in the presence of the appropriate vehicle. None of the vehicles had a significant effect on the S-I outflow.

Results

[^{3}H]-noradrenaline was incorporated into the noradrenergic transmitter stores of mouse cerebral cortical slices and electrical stimulation-induced (S-I) outflow of radioactivity from the brain slices in response to two periods of field stimulation (S_1 and S_2, both at 5 Hz, 60 s) was used as an index of noradrenaline release. Tetrodotoxin (0.3 μM) added for the second stimulation (S_2) and the removal of calcium from the bathing fluid (after adding the Ca^{2+} chelator EGTA, 0.1 mM) both almost abolished the fractional S-I outflow of radioactivity in the second stimulation (control $FR_2/FR_1 = 1.16 \pm 0.06$, $n = 10$, tetrodotoxin $FR_2/FR_1 = 0.09 \pm 0.07$, $n = 3$; zero Ca^{2+} $FR_2/FR_1 = 0.05 \pm 0.02$, $n = 4$, $P < 0.05$, Student's t test with Bonferroni correction). This indicates that the release followed the normal pattern for action-potential-evoked noradrenaline release. In this and all series described below, none of the drugs affected the resting outflow of radioactivity except where specifically indicated.

Effect of protein kinase C activators and inhibitors: series 1

The protein kinase C activator, 4β-phorbol 12,13-dibutyrate (4βPDB; 0.01, 0.03, 0.1 and 0.3 μM) enhanced the fractional S-I outflow of radioactivity in a concentration-dependent manner (Figure 1). The inactive isomer, 4α-phorbol 12,13-dibutyrate (4αPDB, 0.1 μM), had no effect on the fractional S-I outflow of radioactivity (Figure 1). The protein kinase C inhibitor, polymyxin B (21 μM) attenuated the facilitatory effect of 4βPDB (0.03 but not 0.1 μM) to a greater extent than it attenuated the S-I outflow in the absence of the respective concentration of 4βPDB ($P < 0.05$, Figure 1) although in percentage terms the inhibition produced by polymyxin B was not different in the absence (32.8 ± 7.9%) or presence of 0.03 μM 4βPDB (42.3 ± 4.2%, $P > 0.05$, Student's t test). Results with another phorbol ester, phorbol myristate acetate (PMA) more clearly show an attenuation of phorbol ester effects by polymyxin B. Thus, PMA (0.1 and 0.3 μM) also enhanced the fractional S-I outflow of radioactivity (Figure 2) and in the presence of polymyxin B (21 μM), PMA had no effect on the fractional S-I outflow of radioactivity ($P > 0.05$, Figure 2). It should be noted that the maximal enhancement by PMA was significantly less than that produced by 4βPDB ($P < 0.05$, Student's t test, compare Figures 1 and 2).

Effect of idazoxan and tetraethylammonium: series 1

The α_2-adrenoceptor antagonist, idazoxan (0.1 μM), significantly enhanced the fractional S-I outflow of radioactivity as did the K^+ channel antagonist, tetraethylammonium (300 μM) (Figure 3). Polymyxin B had a greater inhibitory effect in the presence of both idazoxan and tetraethylammonium ($P < 0.05$, Figure 3). This may be due to the increased S-I outflow with these drugs and when the effect of polymyxin B was expressed as a percentage inhibition of the release for control (34 ± 4%), tetraethylammonium (39 ± 4%) and idazoxan (39 ± 3%) respectively, the inhibition was not different between the three conditions ($P > 0.05$, Tukey's test).

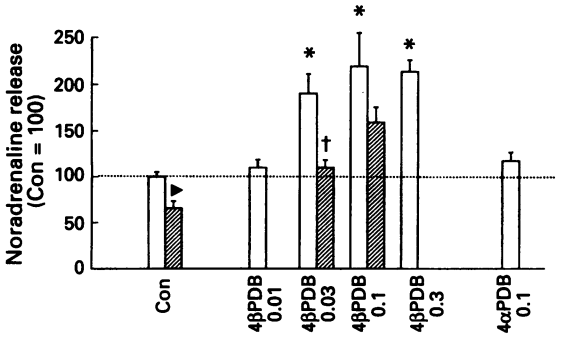

Figure 1 The effect of 4β-phorbol 12,13-dibutyrate on the fractional stimulation-induced (S-I) outflow of radioactivity from mouse cortex slices incubated with [^{3}H]-noradrenaline. There were two periods of test stimulation (each at 5 Hz for 60 s) and drugs (4β-phorbol 12,13-dibutyrate, 4βPDB, 0.01–0.3 μM; 4α-phorbol 12,13-dibutyrate, 4αPDB, 0.1 μM and polymyxin B, 21 μM) were present during the second stimulation. The fractional S-I outflow in the second stimulation was calculated as a fraction of the first. All results were normalized such that control, (Con) = 100. The FR_2/FR_1 ratio for Con was 1.19 ± 0.06, $n = 14$. Means and s.e.mean are shown. The number of experiments was between 4 and 14 for each group. The open columns are experiments in the absence of polymyxin B and the hatched columns are in the presence of polymyxin B. *Represents a significant difference from control ($P < 0.05$, Student's t test with Bonferroni correction). ▶Represents a significant inhibitory effect of polymyxin B versus its respective control ($P < 0.05$, Student's t test with Bonferroni correction). †Polymyxin B reduced the S-I outflow to a greater extent in the presence of 4βPDB compared to the reduction of S-I outflow by polymyxin B in the absence of 4βPDB ($P < 0.05$, interaction term, two-way analysis of variance).

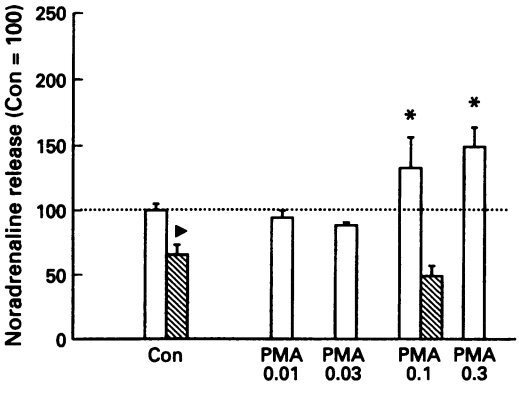

Figure 2 The effect of phorbol myristate acetate (PMA) on the fractional stimulation-induced outflow (S-I) of radioactivity from mouse cortex slices incubated with [^{3}H]-noradrenaline. There were two periods of test stimulation (each at 5 Hz for 60 s) and drugs (PMA, 0.01–0.3 μM; polymyxin B, 21 μM) were present during the second stimulation. The fractional S-I outflow in the second stimulation was calculated as a fraction of the first. All results were normalized such that control, (Con) = 100. The FR_2/FR_1 ratio for Con was 1.19 ± 0.06, $n = 14$. Means and s.e.mean are shown. The number of experiments was between 4 and 14 for each group. The open columns are experiments in the absence of polymyxin B and the hatched columns are in the presence of polymyxin B. *Represents a significant difference from control ($P < 0.05$, Student's t test with Bonferroni correction). ▶Represents a significant inhibitory effect of polymyxin B versus its respective control ($P < 0.05$, Student's t test with Bonferroni correction). In the presence of polymyxin B, PMA (0.1 μM) did not elevate release above that for polymyxin B alone ($P > 0.05$, Student's t test).

Effect of long-term treatment with phorbol esters: series 2

In a separate series of experiments (series 2), in order to down-regulate protein kinase C, brain slices were incubated in Krebs-Henseleit medium with albumin containing either vehicle (0.05% DMSO) or 4βPDB (1 μM) for 10 h before being incubated with [^{3}H]-noradrenaline. The vehicle treatment did not affect the fractional S-I outflow of radioactivity or resting outflow when compared to those measurements in freshly excised atria (compare vehicle treated and untreated, series 2, Table 1). Compared to vehicle-treated tissues, 4βPDB pre-incubation significantly increased the fractional S-I outflow of radioactivity but did not affect the fractional resting outflow of radioactivity (Table 1). These experiments should not be directly compared to the experiments above in freshly excised tissues (series 1 or series 3) since different flow cell designs were used which gave different absolute release values (see Table 1).

In mouse cerebral cortical slices which were pre-incubated for 10 h in Krebs-Henseleit medium with vehicle (DMSO 0.05%) before incubation with [^{3}H]-noradrenaline, 4βPDB (0.1 μM) enhanced the fractional S-I outflow of radioactivity (Figure 4). However, when the brain slices were pre-incubated for 10 h in Krebs-Henseleit medium with 4βPDB (1 μM), subsequent applications of 4βPDB (0.1 μM) failed to enhance the fractional S-I outflow of radioactivity (Figure 4). Shorter

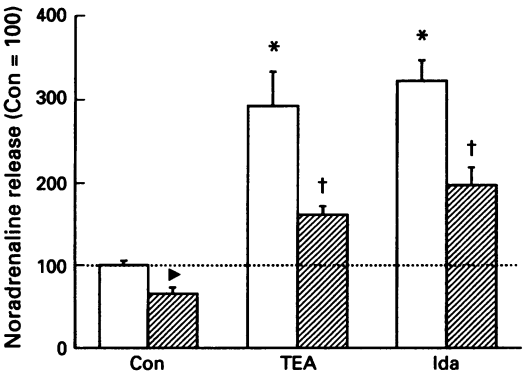

Figure 3 The effect of tetraethylammonium and idazoxan on the fractional stimulation-induced (S-I) outflow of radioactivity from mouse cortex slices incubated with [^{3}H]-noradrenaline. There were two periods of test stimulation (each at 5 Hz for 60 s) and drugs (tetraethylammonium, TEA, 300 μM; idazoxan, Ida, 0.1 μM; polymyxin B, 21 μM) were added for the second stimulation. The fractional S-I outflow in the second stimulation was calculated as a fraction of the first. All results were normalized such that control, (Con) = 100. The FR_2/FR_1 ratio for Con was 1.16 ± 0.06, n = 10. Means and s.e.mean are shown. The number of experiments was between 4 and 10 for each group. The open columns are experiments in the absence of polymyxin B and the hatched columns are in the presence of polymyxin B. *Represents a significant difference from control (P < 0.05, Student's t test with Bonferroni correction). ▶Represents a significant inhibitory effect of polymyxin B versus its respective control (P < 0.05, Student's t test with Bonferroni correction). †Polymyxin B reduced S-I outflow to a greater extent in the presence of TEA or Ida compared to the effect of polymyxin B in the absence of these drugs (P < 0.05, interaction term, two-way analysis of variance).

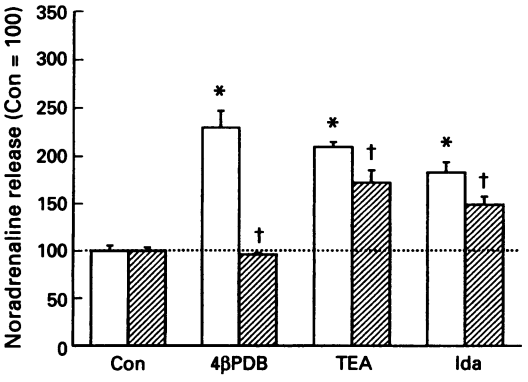

Figure 4 The influence of pretreatment with 4β-phorbol 12,13-dibutyrate (4βPDB, 1 μM for 10 h) on the fractional stimulation-induced (S-I) outflow of radioactivity from mouse cortex slices subsequently incubated with [^{3}H]-noradrenaline: interaction with 4βPDB, tetraethylammonium and idazoxan. There were two periods of test stimulation (each at 5 Hz for 60 s) and drugs (4βPDB 0.1 μM; tetraethylammonium, TEA, 300 μM; idazoxan, Ida, 0.1 μM) were present during the second stimulation where indicated. The fractional S-I outflow in the second stimulation was calculated as a fraction of the first. All results were normalized such that control (Con) = 100 for each group. For vehicle-treated slices (open columns), the FR_2/FR_1 ratio for Con was 1.12 ± 0.06, n = 5. For 4βPDB treated slices (down-regulation) the FR_2/FR_1 ratio for Con was 1.02 ± 0.04, n = 5. Means and s.e.mean are shown. The number of experiments was between 4 and 10 for each group. The open columns are experiments in vehicle-treated slices and the hatched columns are in 4βPDB-treated slices. *Represents a significant difference from control (P < 0.05, Student's t test with Bonferroni correction). †4βPDB pretreatment significantly reduced the effects of 4βPDB, TEA and Ida on the fractional S-I outflow of radioactivity compared to the effects of these drugs in vehicle treated slices (P < 0.05, interaction term, two-way analysis of variance).

Table 1 The fractional resting (R_1) and stimulation-induced (FR_1) outflow of radioactivity from mouse cortical slices incubated with [^{3}H]-noradrenaline associated with the first stimulation period (S_1)

	FR_1	R_1
Series 1		
untreated, n = 148	0.0116 ± 0.0007	0.0057 ± 0.0002
Series 2		
untreated, n = 20	0.0221 ± 0.0029	0.0087 ± 0.0009
vehicle-treated, n = 25	0.0208 ± 0.0017	0.0094 ± 0.0004
4βPDB-treated, n = 24	0.0313 ± 0.0028*	0.0098 ± 0.0003
Series 3		
untreated, n = 40	0.0408 ± 0.0023	0.0077 ± 0.006
vehicle-treated, n = 30	0.0411 ± 0.0034	0.0108 ± 0.0006
4βPDB-treated, n = 30	0.0609 ± 0.0029*	0.0134 ± 0.0008*

There were separate series of experiments and various treatments as described in the Results. Series 1 (data presented in Figures 1,2 and 3) used a different flowcell design from series 2 (data presented in Figure 4) and series 3 (data presented in Figures 5 and 6), and series 3 was performed 18 months after series 1 and 2. R_1 represents the outflow of radioactivity over a 3 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 (5 Hz for 60 s) expressed as a fraction of tissue radioactivity. * Represents significant difference from vehicle-treated slices, P < 0.05, Student's t test. 4βPDB = 4 β-phorbol 12,13-dibutyrate.

periods of pre-incubation (8 h) only partially reduced the effect of 4βPDB (0.1 μM), whilst longer preincubations (12 h) led to non-viable brain slices (not shown).

The ability of tetraethylammonium (300 μM) to enhance the fractional S-I outflow of radioactivity was reduced in 4βPDB pe-incubated brain slices when compared with vehicle pre-incubated brain slices ($P<0.05$, Figure 4).

Stimulation frequency: series 3

This was a separate experimental series conducted 18 months after series 1 and 2 (see Table 1 for comparisons of fractional S-I outflow and resting outflow), where the effects of polymyxin B (21 μM) on noradrenaline release elicited by 1, 5 and 10 Hz frequencies of electrical stimulation were tested (each for 60 s, i.e. 60, 300 and 600 pulses respectively, Figure 5). In these experiments the first stimulation was held at 5 Hz and the second stimulation varied. In each case polymyxin B in the second stimulation, decreased the fractional S-I outflow of radioactivity (Figure 5) and although the effect of polymyxin B was less at the 1 Hz stimulation than at 5 Hz stimulation, this may be due to the decreased S-I outflow. When the effect of polymyxin B at each frequency was expressed as a percentage inhibition of the release for 1 Hz (49±9%), 5 Hz (48±8%) and 10 Hz (34±10%) respectively, the inhibition was not different between the three conditions ($P>0.05$, Tukey's test).

Down-regulation of protein kinase C was achieved as previously described for series 2 by 10 h treatment with 4βPDB (1 μM). As in series 2, the vehicle treatment did not affect the fractional S-I outflow of radioactivity or resting outflow when compared to measurements in freshly excised atria (Table 1, series 3: compare vehicle-treated and untreated). Furthermore, compared to vehicle-treated tissues, 4βPDB pre-incubation significantly increased the fractional S-I outflow of radioactivity and also affected the fractional resting outflow of radioactivity (Table 1). With vehicle treatment for 10 h, the inhibitory effect of polymyxin B (21 μM) at 5 and 10 Hz (Figure 6) was significantly reduced compared to freshly excised atria (compare with Figure 5, $P<0.05$). With 4βPDB treatment for 10 h, polymyxin B (21 μM) no longer inhibited the fractional S-I outflow of radioactivity at either 5 Hz or 10 Hz stimulation ($P>0.05$, Figure 6).

The frequency relationship of noradrenaline release was not altered by protein kinase C down-regulation since the ratio of release induced by 5 Hz, 60 s stimulation to that induced by the 10 Hz, 60 s stimulation was not significantly different between vehicle (10 Hz/5 Hz = 1.9±0.1) or 4βPDB treated cortical slices (10 Hz/5 Hz = 1.8±0.1) ($P>0.05$, Figure 6).

Discussion

For noradrenergic nerves of mouse atria, it has been suggested that protein kinase C has a direct role in transmitter release only when a substantial output of transmitter release is induced and that in this situation the endogenous activation of protein kinase C helps maintain transmitter output (Musgrave & Majewski, 1989; Foucart *et al.*, 1991). The evidence for this hypothesis is that when S-I noradrenaline release in mouse atria was elevated by tetraethylammonium, the α_2-adrenoceptor antagonist, idazoxan, 8-bromo cyclic AMP or high frequency (10 Hz) stimulation, then an inhibitory effect of noradrenaline release by the protein kinase C inhibitor polymyxin B (21 μM) was revealed (Musgrave & Majewski, 1989). At low frequency stimulation (5 Hz), polymyxin B had no effect on noradrenaline release. Protein kinase C down-regulation by prolonged incubation of the mouse atria with 4β-phorbol 12,13-dibutyrate also suggested that endogenous activation of protein kinase C was involved in maintaining high output transmitter release (Foucart *et al.*, 1991). This protein kinase C–high output release hypothesis was further investigated in the present study in the mouse brain cortex.

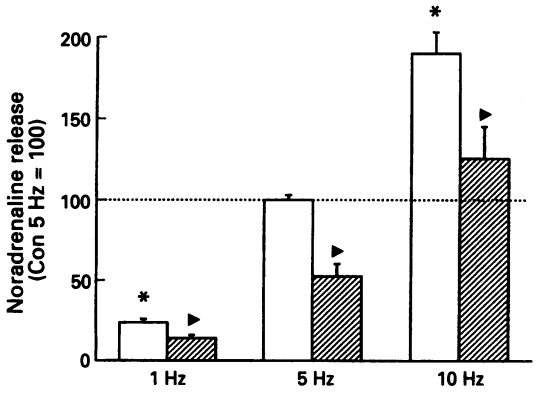

Figure 5 The effect of polymyxin B (21 μM) on the fractional stimulation-induced (S-I) outflow of radioactivity from mouse cortex slices incubated with [^{3}H]-noradrenaline at various stimulation frequencies. There were two periods of test stimulation (5 Hz for 60 s for S_1 and at 1, 5 or 10 Hz for 60 s for S_2) and polymyxin B (21 μM) was present during the second stimulation where indicated. The open columns are experiments in the absence of polymyxin B and the hatched columns are in the presence of polymyxin B. The fractional S-I outflow in the second stimulation was calculated as a fraction of the first. All results were normalized such that control 5 Hz = 100. The FR_2/FR_1 ratio for Con was 0.97±0.03, $n=7$. Means and s.e.mean are shown. The number of experiments was between 5 and 8 for each group. *Represents a significant difference from control 5 Hz ($P<0.05$, Student's t test with Bonferroni correction). ▶Represents a significant inhibitory effect of polymyxin B versus its respective frequency control ($P<0.05$, Student's t test with Bonferroni correction). The inhibitory effect of polymyxin B at 1 Hz was significantly different from that at 5 Hz ($P<0.05$, interaction term, two-way analysis of variance).

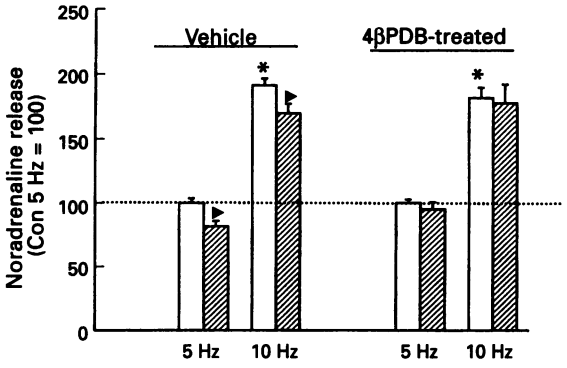

Figure 6 The influence of pretreatment with 4β-phorbol 12,13-dibutyrate (4βPDB, 1 μM for 10 h) on the fractional stimulation-induced (S-I) outflow of radioactivity from mouse cortex slices subsequently incubated with [^{3}H]-noradrenaline: interaction with polymyxin B and stimulation frequency. There were two periods of test stimulation (5 Hz for 60 s for S_1 and at either 5 or 10 Hz for 60 s for S_2, i.e. 300 or 600 pulses) and polymyxin B (21 μM) was added for the second stimulation where indicated. The fractional S-I outflow in the second stimulation was calculated as a fraction of the first. All results were normalized such that control 5 Hz, (open columns) = 100 for each group. For vehicle-treated slices, the FR_2/FR_1 ratio for control 5 Hz was 1.08±0.04, $n=10$. For 4βPDB treated slices (down-regulation) the FR_2/FR_1 ratio for control 5 Hz was 0.97±0.04, $n=7$. Means and s.e.mean are shown. The number of experiments was between 6 and 10 for each group. The open columns are experiments in the absence of polymyxin B and the hatched columns are in the presence of polymyxin B. *Represents a significant difference from control 5 Hz ($P<0.05$, Student's t test with Bonferroni correction). ▶Represents a significant inhibitory effect of polymyxin B versus its respective frequency control ($P<0.05$, Student's t test with Bonferroni correction). After 4βPDB treatment, polymyxin B did not reduce the fractional S-I outflow of radioactivity ($P>0.05$ Student's t test).

Electrical field stimulation of mouse cortical slices, pre-incubated with [^{3}H]-noradrenaline, elicited a reproducible outflow of radioactivity. The stimulation-induced (S-I) outflow of radioactivity was abolished by tetrodotoxin and was not observed in the absence of extracellular Ca^{2+}, consistent with neuronal release of noradrenaline as in previous studies in brain slices. Protein kinase C can modulate noradrenaline release in this tissue since phorbol esters which activate protein kinase C, phorbol myristate acetate (PMA) and 4β-phorbol 12,13-dibutyrate (4βPDB) enhanced the S-I release of noradrenaline, whereas the inactive isomer, 4α-phorbol 12,13-dibutyrate (4αPDB) did not (Figures 1 and 2). This is similar to other tissues (Wakade *et al.*, 1985; Allgaier & Hertting, 1986; Ishac & De Luca, 1988; Musgrave & Majewski, 1989; Foucart *et al.*, 1991).

Protein kinase C can be down-regulated by prolonged exposure to phorbol esters (Matthies *et al.*, 1987; Adams & Gullick, 1989; Bader *et al.*, 1989; Burgess *et al.*, 1989 Foucart *et al.*, 1991). In the present study after 10 h treatment of the cortical slices with 4βPDB (1 μM) the slices were washed and loaded with [^{3}H]-noradrenaline and subsequent application of 4βPDB failed to enhance S-I noradrenaline release, indicating that protein kinase C was no longer functional (Figure 4). Absolute fractional S-I noradrenaline release was also elevated by the 10 h 4βPDB pretreatment (Table 1). This might indicate that 4βPDB in the treatment medium was not washed out before the release experiment was started and that the lack of effect of subsequent application of 4βPDB was because the protein kinase C was already activated. However, since the protein kinase C inhibitor polymyxin B (21 μM) failed to inhibit S-I noradrenaline release in 4βPDB-treated brain slices (Figure 6), the above interpretation is ruled out. It should be noted that inhibitory effects of polymyxin B were slightly reduced in vehicle-treated cortical slices when compared to freshly excised brain slices (Figure 6, vehicle, versus Figure 5). This may be because key proteins may have been depleted from the nerve endings during the 10 h treatment and were not able to be replaced due to the separation of the cell bodies from the axonal projections. In mouse atria (Foucart *et al.*, 1991) a similar abolition of the facilitatory effects of both PMA and 4βPDB was seen after 10 h treatment with 4βPDB (1 μM). The present results suggest a physiological role for protein kinase C in the modulation of noradrenaline release in mouse cortex. Although this is based mainly on the inhibitory action of polymyxin B, this drug does appear to be acting selectively as its inhibitory effect on noradrenaline release is not seen if protein kinase C is down-regulated. However, in the mouse atria study (Foucart *et al.*, 1991) the inhibitory effect of a high concentration of polymyxin B (70 μM) on noradrenaline release was not attenuated by 4βPDB pretreatment which indicates that at higher concentrations than that used in the present study, non-specific effects of polymyxin B must be considered.

In freshly excised mouse cortex slices, polymyxin B (21 μM) decreased S-I noradrenaline release and the percentage inhibitory effect on S-I noradrenaline release was unaltered between 1, 5 and 10 Hz (Figure 5). Furthermore, the frequency (or pulse number; see Methods), noradrenaline release relationship was not altered by protein kinase C down-regulation since the ratio of release induced by 5 Hz stimulation to that induced by the 10 Hz stimulation was not significantly altered (Figure 6). This suggests that protein kinase C is involved in maintaining transmitter release across a wide range of stimulation conditions in mouse cortex and that there is no apparent additional activation at higher output transmitter release. This directly contrasts with the results in mouse atria where at low frequency (3 Hz) stimulation polymyxin B and protein kinase C down-regulation did not inhibit S-I release of noradrenaline but at high stimulation frequency they did (Musgrave & Majewski, 1989; Foucart *et al.*, 1991).

In the present study we also elevated noradrenaline release using the α_2-adrenoceptor antagonist, idazoxan, which enhanced noradrenaline release presumably by blocking α_2-adrenoceptor auto-inhibition (Starke *et al.*, 1989) and tetraethylammonium, which blocks outward repolarizing K^+ currents, prolonging depolarization of the nerve terminal and thus allowing a greater influx of Ca^{2+} (Armstrong & Binstock, 1965). The enhanced release produced by idazoxan and tetraethylammonium was significantly attenuated by polymyxin B (Figure 3). However, the percentage reduction of release by polymyxin B was not significantly different from that seen in the absence of idazoxan or tetraethylammonium, making it unclear if there was an additional activation of protein kinase C to maintain the high level of transmitter release. Using an alternative experimental approach, the facilitatory effects of idazoxan and tetraethylammonium were significantly less in protein kinase C down-regulated slices (Figure 4) which supports the view that protein kinase C is involved in maintaining the high levels of transmitter release with these drugs. This agrees with previous data in mouse atria where the facilitatory effect of α-adrenoceptor antagonists was attenuated by protein kinase C inhibition (idazoxan, Musgrave & Majewski, 1989) and down-regulation (phentolamine, Foucart *et al.*, 1991). This attenuation of the effects of α-adrenoceptor antagonists does not seem to occur at the level of the presynaptic α_2-adrenoceptor since in mouse atria, protein kinase C inhibition and down regulation did not affect the inhibitory effect on noradrenaline release of the α_2-adrenoceptor agonist, clonidine (Musgrave & Majewski, 1989; Foucart *et al.*, 1991).

Our experiments in mouse atria suggested that protein kinase C was activated in the noradrenergic neurones to maintain transmitter output only under conditions where noradrenaline release was markedly elevated (Musgrave & Majewski, 1989; Foucart *et al.*, 1991). The most striking difference of the present study from that in mouse atria is that endogenous protein kinase C modulation of noradrenergic transmission in the mouse cortex appears to operate over a wide range of frequencies (1 – 10 Hz) whereas in mouse atria it was limited to effects at high frequency (10 Hz) only. This may merely be an indication of different starting points where protein kinase C starts to be endogenously active during nerve stimulation. On the other hand there appears to be an additional activation of protein kinase C when noradrenaline release is enchanced by either idazoxan or tetraethylammonium and this is similar for both mouse atria and mouse cortex. It is interesting to note in this regard that studies in chick sympathetic neurones (Wakade *et al.*, 1991) suggest that depolarizing stimuli may lead to the production of the endogenous protein kinase C activator, diacylglycerol through phospholipase D cleavage of membrane phosphatidylcholine, whereas receptor activation leads to the production of diacylglycerol through phospholipase C cleavage of membrane phosphatidylinositol bisphosphate. Thus it is possible that protein kinase C may have multiple effects in the modulation of noradrenaline release, with a distinct activation route for enhancing noradrenaline release by electrical stimulation and another when release is elevated by modulatory drugs. The precise mechanism through which protein kinase C is activated in mammalian noradrenergic nerve terminals remains to be elucidated.

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