British Journal of Pharmacology (1997) 121, 1196–1202 © 1997 Stockton Press All rights reserved 0007–1188/97 $12.00

Noradrenaline release and the effect of endogenous activation of the phospholipase C/protein kinase C signalling pathway in rat atria

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1 It has been proposed that protein kinase C (PKC) in sympathetic nerves is activated during action-potential evoked release of noradrenaline and helps maintain transmitter output. We studied this phenomenon further in rat atria radiolabelled with [^{3}H]-noradrenaline.

2 Noradrenaline release was elevated by continuous electrical stimulation of the atria for 10 min at either 5 or 10 Hz. Two inhibitors of PKC, polymyxin B (21 μM) and Ro 318220 (3 μM), markedly inhibited the release of noradrenaline but only at the higher stimulation frequency.

3 Further experiments were conducted with 10 Hz stimulation but for shorter train durations. In this case polymyxin B inhibited noradrenaline release during a 10 or 15 s train of impulses but not during a 5 s train. This suggests that PKC effects are induced during the stimulation train by some process.

4 The diacylglycerol kinase inhibitor R59949 (10 μM), which prevents the breakdown of diacylglycerol, enhanced noradrenaline release elicited by stimulation at 10 Hz for 10 or 15 s. This effect was not seen if polymyxin B was present and suggests that diacylglycerol is the endogenous activator of PKC.

5 The source of the diacylglycerol may be through phospholipase C pathways, since the phospholipase C inhibitor U73122 (3 μM) inhibited noradrenaline release at 10 Hz for 10 s and the effect was not seen if polymyxin B was also present.

6 It is unlikely that phospholipase D is the source of diacylglycerol. Although the phospholipase D inhibitor wortmannin (1 μM) inhibited noradrenaline release, this effect was still observed in the presence of polymyxin B. Furthermore ethanol, which inhibits diacylglycerol formation by phospholipase D, had no effect on noradrenaline release.

7 We therefore suggest that during a train of high frequency pulses phospholipase C is activated and this results in the production of diacylglycerol which in turn activates PKC. This enables the neurones to maintain transmitter release at a high level.

Keywords: Noradrenaline; protein kinase C; phospholipase C; diacylglycerol; polymyxin B; R59949; U73122; Ro 318220; sympathetic nerve

Introduction

Several studies have implicated protein kinase C (PKC) in the modulation of neurotransmitter release, since drugs which activate PKC such as phorbol esters markedly enhance action-potential evoked transmitter release (e.g. Wakade *et al*., 1985; Allgaier & Hertting, 1986; Nichols *et al*., 1987; Musgrave *et al*., 1991). The question arises whether protein kinase C is activated endogenously by substances generated in the nerve terminal and thus forms part of a physiological mechanism to elevate transmitter release. Investigations into this hypothesis have mainly used inhibitors of PKC to elucidate a physiological role and for noradrenaline release there are many studies showing PKC inhibitors by themselves reducing noradrenaline release (rabbit hippocampus, Allgaier & Hertting, 1986; Allgaier *et al*., 1987; guinea-pig atria, Brasch, 1991; rat sinus node, Shuntoh & Tanaka, 1986; rat amygdala, Versteeg & Ulenkate, 1987; rat atria, Ishac & De Luca, 1988; mouse atria, Musgrave & Majewski, 1989; mouse cortex, Schroeder *et al*., 1995). The inhibitory effect of the PKC inhibitor polymyxin B (21 μM) on noradrenaline release in mouse cortex was absent after PKC down-regulation (Schroeder *et al*, 1995) which adds some weight to the argument that PKC is endogenously activated to elevate noradrenaline release. In mouse atria the inhibitory action of polymyxin B was frequency-dependent (Musgrave & Majewski, 1989) and it was suggested that PKC is endogenously activated during the release process to maintain transmitter output during high frequency stimulation (Musgrave & Majewski, 1989; Foucart *et al*., 1991).

If there is an endogenous activation of protein kinase C in sympathetic nerves this opens up the possibility of new pharmacological mechanisms to alter noradrenaline release. In general the PKC family of enzymes are thought to be activated principally by diacylglycerol derived from cleavage of membrane phospholipids by phospholipase C (PLC), although diacylglycerol derived from phospholipase D cleavage of phosphatidylcholine has also been found (see Liscovitch, 1992). The aim of the present study was to examine in more detail the proposition that PKC is activated during high frequency nerve stimulation to maintain transmitter output. In particular we used pharmacological probes, such as the diacylglycerol kinase inhibitor R59949, which blocks diacylglycerol breakdown (de Chaffoy de Courcelles *et al*., 1989) and the phospholipase C inhibitor U73122 (Bleasdale *et al*., 1990), to assess the endogenous route of activation of PKC.

Methods

Noradrenaline release from rat atrial strips

Male Sprague Dawley rats (140–300 g) were decapitated and the hearts rapidly excised. The right atrium was isolated and dissected free and incubated for 20 min in a physiological salt solution (PSS) containing [^{3}H]-noradrenaline (10 μCi ml^{-1}, 0.1 μM) which was maintained at 37°C and bubbled with a mixture of 5% CO_2 and 95% O_2. Following incubation, the

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atria was cut into strips which were rinsed and transferred to flow cells (1 strip per flow cell) and continuously superfused at 1 ml min^{-1} with [^{3}H]-noradrenaline-free PSS maintained at 37°C. The slices were superfused for 60 min before sample collection began. This was the washing period. After 24 min of washing, an electrical priming stimulation was delivered through a pair of parallel platinum electrodes located either side of the atrial strips; (the electrodes of two flow cells were connected in series) with an electronic stimulation (field strength 12 V cm^{-1}, 80 mA, square wave pulses at a frequency of 5 Hz with 1 ms pulse duration) over either 60 s (for the 10 min stimulation experiments, see below) or 30 s (for all other experiments).

Ten minutes stimulation experiments

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 51 min, following by sampling at 30 s intervals for a further 20 min. At 9 min after the start of the collection period, the atrial strips were stimulated (each at 5 Hz for 60 s, S_1). Forty five minutes after the start of S_1 a further stimulation was given, S_2 (at either 5 Hz or 10 Hz for 10 min). The effect of drugs on the electrical stimulation-induced outflow of radioactivity was determined by adding them to the superfusate solution 30 min before the second stimulation. At the completion of the experiments the atrial strips 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 atrial strip was determined by liquid scintillation counting after the solutions had been mixed with 4 ml Picofluor 40 (Packard Instruments). Corrections for counting efficiency were made by automatic external standardisation.

Five, ten and fifteen seconds stimulation experiments

After the washing period was completed, the collection period began in which superfusate fractions were collected over consecutive 2 min periods for a total of 60 min. At 9 min after the start of the collection period, the atrial strips were stimulated (each at 5 Hz for 10 s, S_1). Forty-five minutes after the start of S_1 a further stimulation was given S_2 (at 10 Hz for either 5, 10 or 15 s). The effect of drugs on the electrical stimulation-induced outflow of radioactivity was determined by adding them to the superfusate solution 30 min before the second stimulation. At the completion of the experiments the atrial strips were removed from the flow cells and superfusate samples and tissues were processed as above for determination of radioactive content.

Calculation of results

For the 10 min stimulation The resting (spontaneous) outflow of radioactivity for the first stimulation period (S_1) 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 first 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). This value was then expressed as a fraction of the radioactivity present in the tissue at the onset of stimulation. For the second stimulation which lasted 10 min, the S-I component was calculated by subtracting the spontaneous radioactive outflow of the sample immediately before stimulation commenced from each of the successive 20 samples (each of 30 s duration) and then expressing these values as a fraction of tissue radioactivity. The values depicted in Figure 1 represent the fractional S-I outflow of radioactivity in each 30 s sample in S_2 as a percentage of the fractional S-I outflow in S_1.

For the 5, 10 and 15 s stimulations For each stimulation cycle (S_1 and S_2) the resting (spontaneous) outflow of radioactivity was taken as the radioactive content of the bathing solution during the 2 min period immediately before the start of each 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 two 2 min samples collected immediately after the start of each stimulation. 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 fractional S-I outflow of radioactivity were evaluated by comparing the ratio FR_2 as a percentage of FR_1 for control and drug.

Statistics

The values are given as mean ± s.e.mean, n indicates the number of experiments (strips); within each experimental group, each strip came from different animals. Results were analysed either by analysis of variance with repeated measures, for Figure 1, or, in all other figures, non parametric analysis was done by the Kruskal Wallis test and Mann Whitney Rank sum test since comparison of the variances indicated non-homogeneity. 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.

Materials

The PSS contained (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.03, $NaHCO_3$ 25.0, D-glucose 11.1, $MgSO_4$ 0.82, $CaCl_2$ 1.8, 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.). Wortmannin, indomethacin, and polymyxin B sulphate were obtained from Sigma (St Louis, U.S.A.). R59949 (3-[2-[4[(4-fluorophenyl*o*methylene]-1-piperidinyl]ethyl]-2,3-dihydro-2-thioxi-4(1H)-quinazoline), U73122 (1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione), U73343 (1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidinedione) were obtained from RBI (Natick, U.S.A.), made up in dimethyl sulphoxide (DMSO) and used on the day of the experiment without storage. Other drugs dissolved in DMSO were wortmannin and Ro 318220 ([1-[3-(amidinothio)propyl-1H-indoyl-3-yl]-3-(1-methyl-1H-indoyl-3-yl)-maleimide-methane sulphate) which was kindly donated by Roche Pharmaceuticals, U.K. and also purchased from Calbiochem, U.S.A. Indomethacin was initially dissolved in 0.1 M Na_2CO_3. The remaining drugs were initially dissolved in physiological salt solution (PSS) before being further diluted in PSS. Where appropriate, contemporaneous control experiments were carried out in the presence of the appropriate vehicle which is mentioned in the appropriate figure legend.

Results

Stimulation for 10 min

Rat isolated atria were incubated with [^{3}H]-noradrenaline and the stimulation-induced (S-I) outflow of radioactivity during electrical stimulation for 10 min at either 5 or 10 Hz was measured. Onset of electrical stimulation at 5 Hz evoked a rapid stimulation-induced release of radioactivity which was well maintained over the 10 min (Figure 1). With stimulation at 10 Hz, the initial rise was more marked than at 5 Hz and not as well maintained (Figure 1). At the 10 Hz stimulation, the protein kinase inhibitors polymyxin B (21 μM) and Ro 318220 (3 μM) caused a decrease in the fractional S-I outflow

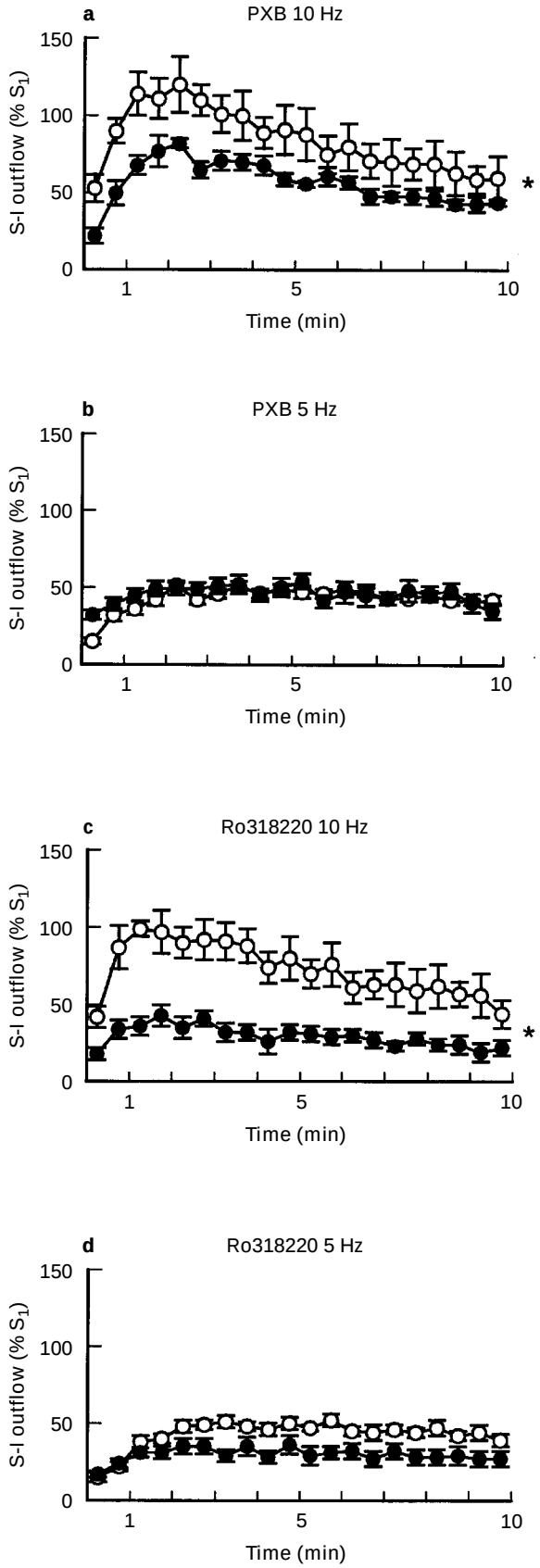

Figure 1 Effect of PKC inhibitors on release of radioactivity from rat atria strips preincubated with [^{3}H]-noradrenaline. In these experiments there were two periods of stimulation S_1 (5 Hz for 60 s) and S_2 (5 or 10 Hz for 10 min). The diagrams represent the fractional S-I outflow of radioactivity during S_2 in 30 s samples of bathing solution expressed as a percentage of the fractional S-I outflow in S_1 (for all experiments combined, the mean value of S_1 was $=2375\pm195$ d min^{-1}, $n=68$; resting outflow immediately before S_1 was 1207 ± 72 d min^{-1} and the tissue radioactivity was 306247 ± 24865 d min^{-1}). PKC inhibitors (a and b) polymyxin B (PXB) 21 μM or (c and d) Ro 318220 3 μM or vehicle (0.05% DMSO in the case of Ro 318220) were added 30 min before S_2 which was commenced at t=0. (○) Drug free experiments (vehicle added) and (●) PKC inhibitor present. Means and s.e.mean (vertical lines) are represented; $n=6-11$ for each experiment. *Significant difference between the two curves from t=0–t=10 min, $P<0.05$, two way analysis of variance with repeated measures. Ro 318220 had a significantly greater effect at (c) 10 Hz compared to (d) 5 Hz ($P<0.05$, three way analysis of variance, interaction term).

of radioactivity which was apparent after 30 s of stimulation and was maintained for 10 min (Figure 1). In contrast, in rat atria stimulated at 5 Hz for 10 min, polymyxin B did not alter the fractional S-I outflow of radioactivity and the inhibitory effect of Ro 318220 was greatly reduced compared with that observed in atria stimulated at 10 Hz (Figure 1).

Stimulation for 5, 10 or 15 seconds

In this series of experiments shorter duration stimulations (5–15 s) were employed. In discrete experiments all at 10 Hz, the stimulation train length was altered from 5 through to 15 s which resulted in a graded increase in fractional S-I outflow. Polymyxin B (21 μM) did not significantly reduce the fractional S-I outflow of radioactivity after 5 s or 10 s. However, a significant inhibitory effect was observed at 15 s (Figure 2). Indomethacin (10 μM) did not change the inhibitory effect of polymyxin B (21 μM; Figure 2). Polymyxin B slightly elevated the resting outflow of radioactivity (Table 1).

The diacylglycerol kinase inhibitor R59949 (1 μM) enhanced the fractional S-I outflow of radioactivity from atria stimulated at 10 Hz for either 10 or 15 s (Figure 3). This effect was abolished by the protein kinase C inhibitor polymyxin B (21 μM). Polymyxin B alone inhibited the fractional S-I outflow of radioactivity from rat atria at both train lengths.

The phospholipase C inhibitor U73122 (1 and 3 μM) caused a concentration-dependent inhibition of the fractional S-I outflow of radioactivity from rat atria stimulated at 10 Hz for 10 s (Figure 4). The structurally related compound U73343, often used as a negative control (see Hawes *et al*., 1992), enhanced the fractional S-I outflow of radioactivity (Figure 4). In the presence of polymyxin B (21 μM) which by itself inhibited the fractional S-I outflow, U73122 (3 μM) no longer inhibited

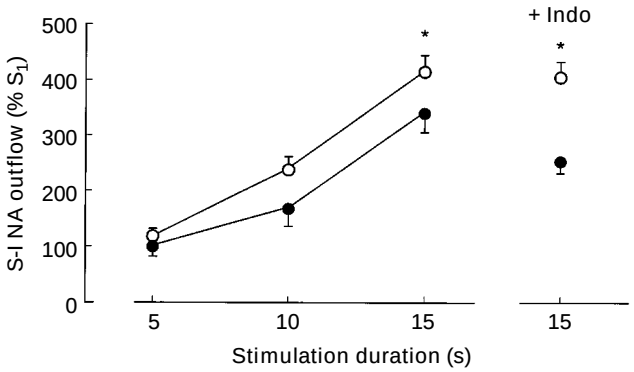

Figure 2 Effect of polymyxin B (21 μM) on the fractional S-I outflow of radioactivity (S-I noradrenaline (NA) outflow) from rat atrial strips incubated with [^{3}H]-noradrenaline. There were two periods of stimulation S_1 (5 Hz for 10 s) and S_2 (10 Hz at either 5, 10 or 15 s duration). The fractional S-I outflow in S_2 was expressed as a percentage of that in S_1. Polymyxin B and indomethacin (Indo) (10 μM) were present for S_2. Means and s.e.means (vertical lines) are given; $n=10-18$ for each group. (○) Refer to control experiments and (●) to experiments where polymyxin B was present. *Represents significant difference from respective polymyxin B-free experiments; $P<0.05$, Mann-Whitney test.

Table 1 Effect of drugs on the resting outflow of radioactivity from rat atria incubated with [^{3}H]-noradrenaline

Experiment	*R_2 as % of R_1*	*s.e.mean*	n
Control	82	1	37
PXB	85*	2	40
DMSO 0.05% in R_2			
Control	88	2	17
PXB	93*	1	17
R59949	89	1	13
PXB + R59949	96*	2	12
Wort	95*	2	4
PXB + Wort	99*	2	5
DMSO 0.2% in R_2			
Control	83	3	12
U73122 1μM	172*	26	8
U73122 3μM	261*	34	6
U73343 3μM	226*	6	6
PXB	88	1	5
PXB + U73122 3μM	180*	27	6
PXB + U73343 3μM	279*	8	4
H_2O infusion in R_2			
Control	82	1	4
Ethanol	86*	2	4
Na_2CO_3 0.1M in R_2			
Indo	76	3	7
Indo + PXB	82	4	7

There were two periods of stimulation S_1 and S_2. For all experiments combined, the mean value of S_1 as a % of tiissue radioactivity was = 0.080 ± 0.002%, $n = 253$; resting outflow immediately before S_1 was 0.063 ± 0.002% min^{-1}, and the tissue radioactivity was 2253685 ± 55240 d min^{-1}. The resting outflow of radioactivity for the second stimulation (R_2) was expressed as a percentage of that in the first (R_1). See Methods for calculation details. Drugs were present for R_2. *Significant difference from appropriate control ($P < 0.05$, Mann Whitney test). Abbreviations: PXB, polymyxin B; Wort, wortmannin; Indo, indomethacin. These data are composites from Figures 2 to 5.

the fractional S-I outflow of radioactivity but U73343 still enhanced the fractional S-I outflow (Figure 4). U73122 and U73343 both markedly enhanced the resting outflow of radioactivity (Table 1).

The phospholipase D inhibitor wortmannin (1 μM) significantly reduced the fractional S-I outflow of radioactivity from rat atria stimulated at 10 Hz for 10 s (Figure 5). In the presence of polymyxin B (21 μM), wortmannin also reduced the fractional S-I outflow of radioactivity (Figure 5). Ethanol (100 mM) present from 7 min before the electrical stimulation, did not alter the fractional S-I outflow of radioactivity from atria (Figure 5). Wortmannin and ethanol had small effects on the resting outflow of radioactivity (Table 1).

Discussion

Previous studies with cardiac sympathetic nerves suggest that during high frequency stimulation there is an endogenous activation of protein kinase C and this is fundamental to maintain transmitter output (Musgrave & Majewski, 1989; Foucart *et al*., 1991). Evidence for this hypothesis was that PKC inhibitors decreased noradrenaline release induced by high frequency stimulation (10 Hz) but not the release induced by low frequency stimulation (Musgrave & Majewski, 1989) and PKC down-regulation had the same effect (Foucart *et al*., 1991). However, in mouse cortex noradrenaline release was not differentially altered at high and low frequencies by PKC inhibitors or down regulation; PKC inhibitors decreasing transmitter release at all frequencies tested (Schroeder *et al*, 1995). The present study investigated this phenomenon in

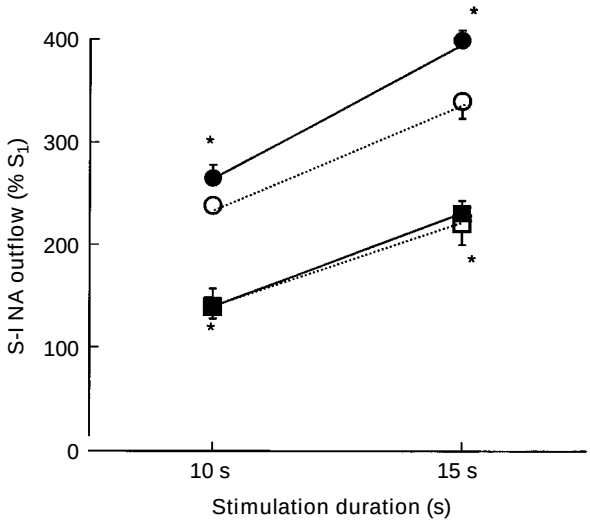

Figure 3 Effect of the diacylglycerol kinase inhibitor R59949 (1 μM) on the fractional S-I outflow of radioactivity (S-I noradrenaline (NA) outflow) from rat atrial strips incubated with [^{3}H]-noradrenaline. There were two periods of stimulation S_1 (5 Hz for 10 s) and S_2 (10 Hz at either 10 or 15 s duration). The fractional S-I outflow in S_2 was expressed as a percentage of that in S_1. R59949 (1 μM) and/or polymyxin B (21 μM) was present for S_2. Means and s.e.means (vertical lines) are given; $n = 5-12$ for each group. (○) Refer to control experiments and (●) experiments where R59949 was present; (□) refer to experiments where polymyxin B was present and (■) experiments where both R59949 and polymyxin B were present. *Represents significant difference from respective control; $P < 0.05$, Mann-Whitney test. In the presence of polymyxin B, R59949 had no effect on fractional S-I outflow ($P > 0.05$, Mann-Whitney test).

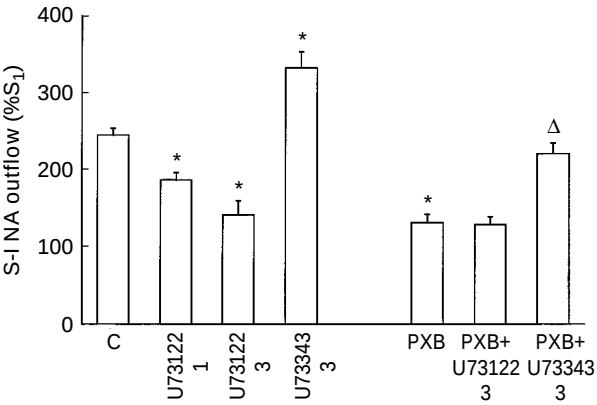

Figure 4 Effect of phospholipase C inhibitor U73122 n the fractional S-I outflow of radioactivity (S-I noradrenaline (NA) outflow) from rat atrial strips incubated with [^{3}H]-noradrenaline. There were two periods of stimulation S_1 (5 Hz for 10 s) and S_2 (10 Hz for 10 s). The fractional S-I outflow in S_2 was expressed as a percentage of that in S_1. Drugs (U73122 (1 or 3 μM) its inactive analogue U73343 (3 μM) and/or polymyxin B (PXB, 21 μM) were present for S_2. Means and s.e.means (vertical lines) are given; $n = 5-12$ for each group. *Represents significant difference from respective control (c); $P < 0.05$, Mann-Whitney test. ΔRepresents significant difference from polymyxin B; $P < 0.05$, Mann-Whitney test. In the presence of polymyxin B, U73122 had no effect on the fractional S-I outflow ($P > 0.05$, Mann-Whitney test).

sympathetic nerves further. Firstly, rat atria were chosen to determine whether the frequency-dependent action of PKC inhibitors was species-dependent. The atria were incubated with [^{3}H]-noradrenaline and the electrical field stimulation-induced outflow of radioactivity from the tissue was used as an index of the exocytotic release of endogenous noradrenaline. During sustained trains of stimulation (for 10 min), the PKC

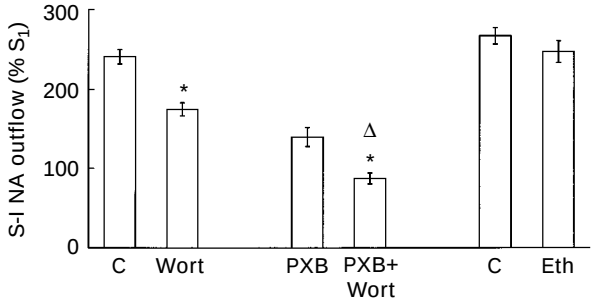

Figure 5 Effect of phospholipase D inhibitor wortmannin (Wort) and ethanol (Eth) on the fractional S-I outflow of radioactivity (S-I noradrenaline (NA) outflow) from rat atrial strips incubated with [^{3}H]-noradrenaline. There were two periods of stimulation S_1 (5 Hz for 10 s) and S_2 (10 Hz for 10 s). The fractional S-I outflow in S_2 was expressed as a percentage of that in S_1. Drugs (wortmannin (1 μM) or ethanol (100 mM) and/or polymyxin B (PXB, 21 μM) were present for S_2. Means and s.e.means are given; $n=5-12$ for each group. *Represents significant difference from respective control (C); $P<0.05$, Mann-Whitney test. $^{\Delta}$Represents significant difference from polymyxin B; $P<0.05$, Mann-Whitney test.

inhibitors polymyxin B and Ro 318220 both inhibited electrical field-stimulated noradrenaline release at a frequency of 10 Hz, but this effect was either greatly diminished or absent in atria stimulated at 5 Hz. This is consistent with previous work in mouse atria (Musgrave & Majewski, 1989).

One of the problems in relying on the actions of PKC inhibitors is that the drugs are relatively non-selective (Schächtele *et al*., 1989; Rüegg & Burgess, 1989). However, the inhibitory action of polymyxin B on noradrenaline release in mouse cortex at the concentration used in the present study (21 μM) disappeared after PKC down-regulation (Schroeder *et al*., 1995) and Ro 318220 is a PKC inhibitor with marked selectivity for PKC (Davis *et al*., 1992). Furthermore the actions of both compounds were only apparent at the 10 Hz stimulation ruling out non-specific depression. Interactions with the phospholipase C inhibitor described below also indicate selectivity.

If PKC is active during a train of nerve impulses then there are two possible ways in which this could occur: (1) that there is a high basal tone of PKC or (2) that during the stimulation train events are set in motion to activate PKC. Our data support the latter hypothesis since the effects of the PKC inhibitors were only apparent at the high stimulation frequency (10 Hz) and when we conducted experiments with shorter trains of stimulation (5, 10 and 15 s, at 10 Hz), the inhibitory effect of polymyxin B on noradrenaline release became progressively greater over the time period 5 to 15 s.

We assume that the site of activation of PKC which is responsible for enhanced transmitter release is within the neurone and not in the postjunctional tissue, although we have no evidence for this. This assumption comes from the observation that activation of PKC in pure neuronal systems such as synaptosomes results in enhanced transmitter release (see Nichols *et al*., 1987) and we do not know of any PKC activated process in muscle which could affect transmitter release.

The activation of protein kinase C during a high frequency train (10 Hz) could be a consequence of Ca^{2+} entry during depolarization, due to depolarization-mediated events or due to some event activated by released neurotransmitters. A major activation pathway for PKC involves phospholipase C (PLC) and the formation of diacylglycerol (see Nishizuka, 1992). Thus PLC may be the first link in the signal transduction cascade and we present evidence for this below. Importantly, in neuroblastoma cells PLC activation has been shown to be due to Ca^{2+} entry induced by either K^+ or the ionophore ionomycin (Smart *et al*, 1995), suggesting that Ca^{2+} may indeed be the initiator of PKC activation.

Alternatively there are several presynaptic receptors which are possibly coupled to PLC, among these are prostaglandin receptors, α_1-adrenoceptors, neuropeptide Y (NPY) and M_1/M_3 muscarinic receptors (see Watson & Girdlestone, 1996), and it is possible that these receptors may participate in the activation of PLC in field stimulated cardiac tissue as they have been described to modulate transmitter release in cardiac preparations (prostaglandins, Khan & Malik, 1982; α_1-adrenoceptors, Story *et al*., 1985; NPY receptors, Foucart *et al*., 1990; M_1 receptors, Costa *et al*, 1993). Prostaglandins appear not to be involved since the cyclo-oxygenase inhibitor indomethacin did not affect the inhibitory actions of polymyxin B on noradrenaline release. Furthermore, from previous work it is unlikely that α_1-adrenoceptors are involved, since polymyxin B inhibited noradrenaline release in mouse atria in the presence of the α-adrenoceptor blocker phentolamine (Musgrave & Majewski, 1989), and NPY modulation of noradrenaline release does not involve PKC (Foucart *et al*, 1990). Although activation of M_1 muscarinic receptors has been suggested to facilitate noradrenaline release in mouse atria through activation of PKC (Costa *et al*., 1993), no endogenous activation of the M_1 receptors by acetylcholine was observed (Costa & Majewski, 1991). Despite this it is clear that further work needs to be done to exclude the possibility that presynaptic receptors are involved in the endogenous activation of PKC seen at high frequency stimulation.

We tested the involvement of diacylglycerol in the maintenance of noradrenaline release at high frequency by use of a diacylglycerol kinase inhibitor, since diacylglycerol kinase is a major site of loss of diacylglycerol through the phosphorylation of diacylglycerol to phosphatidic acid (de Chaffoy de Courcelles, 1990; Kanoh *et al*., 1990). We used R59949 which has been shown to be a relatively selective diacylglycerol kinase inhibitor (de Chaffoy de Courcelles, 1990). Consistent with a role for diacylglycerol, R59949 elevated noradrenaline release during 10 and 15 s trains of stimulation at 10 Hz and, importantly, this effect was completely blocked by polymyxin B. This result is entirely consistent with the hypothesis that diacylglycerol activation of PKC is involved in maintaining transmitter output.

The suggestion that diacylglycerol was involved in the activation of protein kinase C implicates a role for phospholipases in the modulation of noradrenaline release. Diacylglycerol may be released from membrane-bound phospholipids such as phosphatidylinositol 4,5-bisphosphate or phosphatidylcholine by the action of phospholipase C (PLC) or phospholipase D (PLD) respectively (Haeffner, 1993). In the present study the phospholipase C inhibitor U73122 (Bleasdale *et al*., 1990) caused a concentration-dependent inhibition of noradrenaline release during a 10 s train. We suggest that this was due to inhibition of diacylglycerol formation and thus PKC activation. U73122 may have non-selective actions. However, the inhibitory effect of U73122 was not additive with that of polymyxin B which is consistent with both drugs acting on the same PLC/PKC signalling pathway. An isomer of U73122, U73343 is often used as a negative control (Bleasdale *et al*., 1990) and it enhanced noradrenaline release, an effect which was not altered by polymyxin B. It should be noted that U73122 and U73443 both substantially increased the resting outflow of radioactivity, which suggests that some caution should be used in interpreting the actions of these compounds on the fractional S-I outflow.

Although the above results with U73122 suggest that PLC is involved in the activation of PKC during a high frequency train, we also examined the role of phospholipase D, since it has been proposed that phospholipase D is involved in the formation of diacylglycerol in neural tissue (Wakade *et al*., 1991). Phospholipase D hydrolyzes phosphatidylcholine to form free choline and phosphatidic acid, which can then be dephosphorylated to form diacylglycerol. The role of phospholipase D was investigated by use of wortmannin, a compound with diverse pharmacological effects including inhibition of receptor-coupled phospholipase D activation (Bonser *et al*., 1991).

Wortmannin inhibited noradrenaline release from rat atria. However, the effect persisted in the presence of polymyxin B, suggesting that the effect of wortmannin did not involve the PLC/PKC pathway and may indicate the action of other enzymes in the modulation of noradrenaline release, such as phosphatidylinositol 3-kinase which wortmannin is known to inhibit (Arcaro & Wymann, 1993). Primary alcohols such as ethanol substitute for water in the phospholipase D reaction and phosphatidylethanol is preferentially formed instead of phosphatidic acid, thus preventing diacylglycerol formation (Thompson *et al*., 1991). In the present study noradrenaline release was not reduced in the presence of ethanol (100 mM), a concentration known to reduce phosphatidic acid formation through phospholipase D (Chalifa *et al*., 1990). This is consistent with the wortmannin data and suggests that diacylglycerol formation through the phospholipase D pathway is not important in high-level noradrenaline release. Other studies have also found that ethanol does not appreciably affect noradrenaline release (e.g. Bültmann *et al*., 1993).

Throughout the study we used drugs which had to be dissolved in DMSO and indeed U73122/U73343 required 0.2% DMSO in the physiological salt solution to remain in solution. A repeated observation was that the inhibitory effect of polymyxin B on noradrenaline release was significantly greater in the presence of DMSO (compare Figure 2 with Figures 3 and 4). The reason for this is unclear. It may be that DMSO *per se* can initiate PKC activation or that effects of polymyxin B are increased because of some physical effect of DMSO. The actions of polymyxin B involve interactions with charged phospholipid sites in the cell membrane (Mazzei *et al*., 1982) and these may be affected by DMSO.

Taken together, the results of the present study, in which a variety of pharmacological probes were used, suggest that a series of events which involve PLC, diacylglycerol and PKC activation is important in maintaining noradrenaline release from sympathetic nerves during a high frequency train of nerve impulses. The trigger for this may be either Ca^{2+} inflow or the activation of presynaptic receptors by neurotransmitters, co-transmitters or substances released from the myocardium. This remains to be investigated.

The work was supported by a grant from the National Health and Medical Research Council of Australia.

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(Received September 20, 1996
Revised March 26, 1997
Accepted April 9, 1997)