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The influence of activation or inhibition of protein kinase C on the release of radioactivity from rat isolated atria labelled with [^{3}H]-noradrenaline

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1 The release of radioactivity from rat isolated atria preloaded with [^{3}H]-noradrenaline ([^{3}H]-NA) evoked by electrical field stimulation (2 Hz, 1 ms, 60 s) of intraneuronal sympathetic nerves, high potassium (64.7 mM) or tyramine (0.3 μM) was used as an index of noradrenaline release.

2 Activation of protein kinase C by phorbol 12-myristate 13-acetate (PMA) produced a concentration-dependent enhancement of field stimulation-induced outflow of radioactivity, whereas polymyxin B, an inhibitor of protein kinase C, reduced [^{3}H]-NA release evoked by field stimulation. The enhancement observed in the presence of PMA was attenuated by polymyxin B (10 and 70 μM).

3 Release of noradrenaline evoked by membrane depolarization in a high potassium medium was similarly affected by PMA and polymyxin B.

4 In contrast, the release of noradrenaline evoked by the indirectly acting sympathomimetic amine, tyramine, was not altered by PMA. Polymyxin B in a concentration of 70 μM, but not 10 μM caused a slight reduction in tyramine-induced outflow of radioactivity.

5 The spontaneous outflow of radioactive compounds was not affected by either PMA or polymyxin B in the bathing medium.

6 The findings suggest that protein kinase C may play a role in the exocytotic release of noradrenaline but not in the displacement of noradrenaline by indirectly acting sympathomimetic amines.

Introduction

It is well established that an increase in the intraneuronal concentration of calcium is an essential step in the exocytotic release of neurotransmitters from nerve terminals (Augustine *et al.*, 1987). However, the biochemical events that occur between depolarization of the nerve terminal and the release of the transmitter are unknown. Recently the calcium/phospholipid-dependent protein kinase, protein kinase C, has been reported to be highly localized in neuronal tissue and in particular presynaptic nerve terminals (Wood *et al.*, 1986). Receptors coupled to phospholipase C lead to the hydrolysis of phosphatidylinositol 4,5 bisphosphate with the generation of two second messengers, inositol 1,4,5 trisphosphate (IP_3) and 1,2-diacylglycerol (DG). IP_3 results in the mobilization of calcium from the endoplasmic reticulum; and DG in the translocation and activation of protein kinase C (Williamson, 1986). DG decreases the calcium requirement of protein kinase C to the physiological range with subsequent phosphorylation of a number of substrate proteins (Nishizuka, 1986; Rodnight & Perrett, 1986). Protein kinase C can phosphorylate a number of cellular proteins including those which are neurone-specific and postulated to be involved in neurotransmission (Nishizuka, 1986; Gispen, 1987). Activation of protein kinase C has been reported to enhance the release of dopamine from cultured foetal brain neurones (Zurgil & Zisapel, 1985), of noradrenaline from guinea-pig sinus node (Shuntoh & Tanaka, 1986) and of acetylcholine from brain slices (Tanaka *et al.*, 1986). The present study was designed to examine

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the effect of activation and inhibition of protein kinase C on the exocytotic and non-exocytotic release of noradrenaline from sympathetic nerve terminals. We have examined the effect of the tumour promoting agent, phorbol 12-myristate 13-acetate, an activator of protein kinase C on the release of the sympathetic transmitter from rat isolated atria. Release was evoked in an exocytotic, calcium-dependent manner by electrical field stimulation and by membrane depolarization by increasing the potassium concentration in the atrial bathing solution, and also by a non-exocytotic, calcium-independent process by the indirectly acting sympathomimetic amine, tyramine (Smith & Winkler, 1972, Brandão *et al.*, 1978). Sympathetic noradrenergic transmission was chosen for this study on the role of protein kinase C on neurotransmitter release, as it is the best characterized of the transmitter systems. We demonstrate that phorbol ester enhances only the calcium-dependent release of the sympathetic neurotransmitter, noradrenaline.

Methods

Male Wistar rats (200–250 g) were decapitated and the heart removed. The atria were dissected free of surrounding tissue and suspended between two platinum electrodes in an organ bath containing 4 ml of Krebs-Henseleit solution (composition in mM: NaCl 118, KCl 4.7, $NaHCO_3$ 25.0, KH_2PO_3 1.03, $MgSO_4$ 0.45, $CaCl_2$ 2.5, glucose 11.1, Na_2EDTA 0.07 and ascorbic acid 0.14) gassed with 5% CO_2 in O_2 and maintained at 37°C. Atria were incubated with (−)-7,8-[^{3}H]-noradrenaline (2.5 μCiml^{-1}; 0.2 μM) for 20 min and then repeatedly washed with Krebs-Henseleit solution for 1 h. A priming stimulation (2 Hz, 1 ms, 30 s) was delivered 40 min after the start of the washing procedure to remove loosely bound radioactive compounds from the tissue. Following the washing procedure Krebs-Henseleit solution was continuously collected after 3 min periods of contact with the tissue. Unless otherwise stated, two test stimulations were applied 69 min (S_1) and 99 min (S_2) after the start of the washing procedure. Electrical field stimulation-induced outflow of radioactivity was evoked by delivering 60 s trains of square wave pulses at a frequency of 2 Hz (1 ms, 15 Vcm^{-1}). For release evoked by high potassium, atria were exposed for 3 min periods (S_1 and S_2) to a modified Krebs-Henseleit solution containing 64.7 mM K^+ ions. For tyramine-evoked outflow of radioactivity, atria were exposed to tyramine (0.3 μM) for 3 min periods (S_1 and S_2). The spontaneous outflow of radioactivity was taken as the mean radioactive content of the bathing solution during the 3 min period before stimulation by field pulses, potassium-enriched solution or tyramine, and the 3 min period starting 6 min after the onset of stimulation. The stimulation-induced (S-I) component of the radioactive outflow (S_1 and S_2) was calculated in each case by subtracting the resting outflow from the mean radioactivity in the two 3 min samples collected during and immediately after stimulation. S-I outflow was expressed as a ratio of S_2/S_1. The radioactivity present in the atria was determined after dissolving the tissue in 2 ml of Soluene 350 (Packard Instruments). Radioactivity present in samples of the bathing solution and tissue was determined by liquid scintillation counting in Picofluor (Packard Instruments). Measurements were expressed as disintegrations per min (d.p.m.) after corrections were made for counting efficiency (approximately 25%) by automatic external standardization.

Tension developed by isometric contractions of atria was measured with a Grass FT03 transducer connected to a Grass Polygraph (Model 79C). The rate of beating of atria was recorded with a Grass tachygraph which was triggered by the contractions of the spontaneously beating atria.

Drugs were present only during the second stimulation period: the protein kinase C activator, phorbol 12-myristate 13-acetate (PMA), protein kinase C inhibitor, polymyxin B sulphate (PXB) and tetrodotoxin (TTX) were added 15, 24 and 24 min before the second stimulation period (S_2), respectively.

Statistical analysis

Data are expressed as mean ± s.e. mean. Differences between groups of data were analysed by Student's *t* test (unpaired, two-tailed) subsequent to one-way analysis of variance. Probability levels of less than 0.05 were considered to be statistically significant.

Drugs and chemicals

The following drugs were used: (−)-7,8-[^{3}H]-noradrenaline (specific activity 10–15 Ci mmol^{-1}, Radiochemical Centre, Amersham). Phorbol 12-myristate 13-acetate (PMA), polymyxin B sulphate (PXB), tetrodotoxin (TTX) and tyramine HCl were obtained from Sigma Chemical, USA. All other chemicals were of reagent grade.

Results

Electrical field stimulation-induced release

Rat isolated atria were incubated with [^{3}H]-noradrenaline ([^{3}H]-NA) and the outflow of radio-

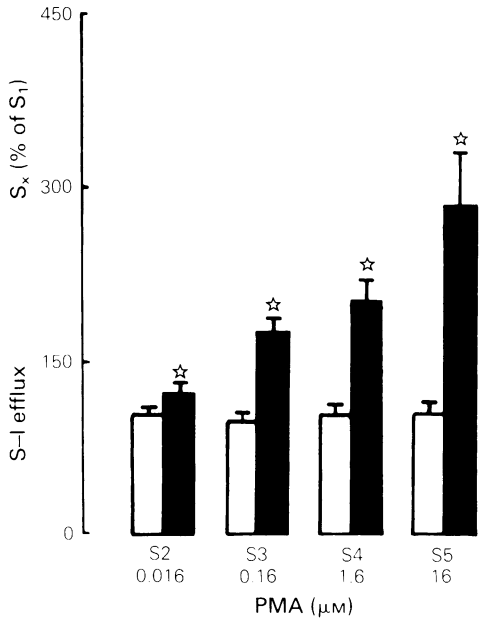

Figure 1 The effect of phorbol 12-myristate 13-acetate (PMA) on the electrical field stimulation-induced (S-I) release of [^{3}H]-noradrenaline ([^{3}H]-NA) from rat isolated atria preloaded with [^{3}H]-NA. Electrical field stimulation (2 Hz, 1 ms, 60 s) was applied at 69, 99, 129, 159 and 189 min (S_1, S_2, S_3, S_4 and S_5 respectively) after the start of the washing procedure to remove loosely bound radiolabel. Results are expressed as evoked release of [^{3}H]-NA, S_x as a percentage of S_1. Each column represents the mean of 5 experiments with s.e. mean shown by vertical lines. PMA was added 15 min prior and during stimulation at the concentrations as shown. Basal efflux of [^{3}H]-NA was 10277 ± 436 d.p.m. (n = 10). Electrical field stimulation-evoked release (S_1) for control and PMA-treated groups were 35887 ± 2540 d.p.m. (n = 5) and 38439 ± 3415 d.p.m. (n = 5) respectively. Open columns represent vehicle control, 0.01% dimethyl sulphoxide (DMSO). This concentration of DMSO did not affect the release of radioactivity. Solid columns represent PMA at the concentrations depicted.
☆ Significant difference from corresponding control ($P < 0.05$).

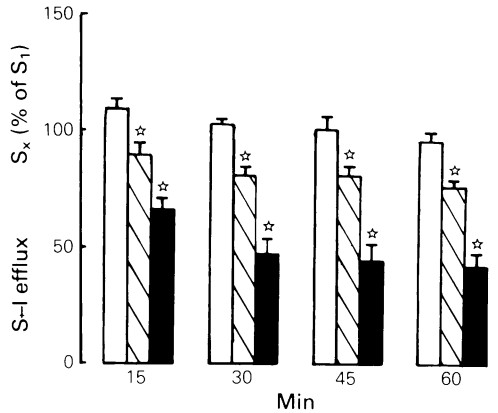

Figure 2 The effect of various periods of exposure to polymyxin B (PXB; 10 and 70 μM) on the electrical field stimulation-induced (SI) release of [^{3}H]-noradrenaline ([^{3}H]-NA]) from rat isolated atria. Electrical field stimulation (2 Hz, 1 ms, 60 s) was applied at 69, 84, 99, 114 and 129 min (S_1, S_2, S_3, S_4 and S_5 respectively) after the start of washing procedure. Results are expressed as evoked release of [^{3}H]-NA, S_x as a percentage of S_1. Each column represents the mean of 3 to 4 experiments; s.e. mean shown by vertical lines. Open columns represent no drug, hatched columns represent in the presence of PXB (10 μM) and solid columns PXB (70 μM) at the times indicated. Basal efflux of [^{3}H]-NA was 10786 ± 398 d.p.m. (n = 11). Electrical field stimulation-evoked release (S_1) for control, PXB (10 μM) and PXB (70 μM) were 38845 ± 2774 d.p.m. (n = 4), 36713 ± 1012 d.p.m. (n = 3) and 36089 ± 1208 d.p.m. (n = 4) respectively.
☆ Significant difference from corresponding control ($P < 0.05$).

activity was collected. Electrical field stimulation increased the outflow of radioactivity from a resting level of 10546 ± 272 d.p.m. to 38114 ± 1022 d.p.m. (S_1, n = 12). As shown in Figure 1, phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C, caused a concentration-dependent (0.016–16 μM) enhancement of electrical field stimulation-evoked release of radioactivity. Spontaneous outflow of radioactivity and resting atrial rate were not altered by the presence of PMA. Polymyxin B (10 and 70 μM), when present in the incubation medium, reduced the stimulation-induced (S-I) outflow of radioactivity in a concentration and time-dependent manner (Figure 2). Polymyxin B (10 μM) reduced S-I efflux of radioactivity by 18% at 15 min and 22% at 30 min, whereas at a concentration of 70 μM the reduction was 38% and 54% respectively. No further increase in the inhibition of S-I efflux was observed after 30 min with either concentration of polymyxin B. In the concentrations used, polymyxin B (10 and 70 μM) did not alter the basal outflow of radioactivity. However with the higher concentration, a significant decrease in resting atrial rate from 285 ± 8 b min^{-1} to 243 ± 8 b min^{-1} (n = 12) was observed. The PMA-induced (0.16 μM) enhancement in electrically-evoked radioactive outflow was markedly attenuated by polymyxin B (10 and 70 μM) compared to atria treated with PMA alone (Figure 3). The presence of tetrodotoxin (1 μM) in the incubation medium 24 min before S_2 reduced the electrical field

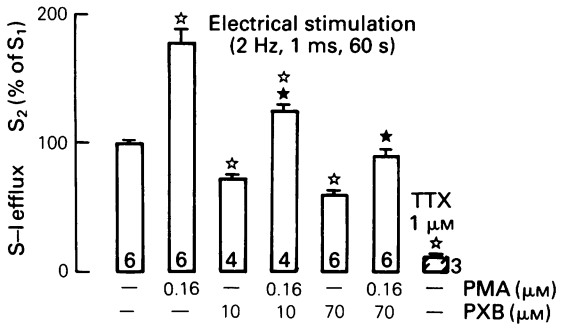

Figure 3 The effect of phorbol 12-myristate 13-acetate (PMA), polymyxin B (PXB) and tetrodotoxin (TTX) on the outflow of radioactivity from rat isolated atria evoked by electrical field stimulation (S-I, 2 Hz, 1 ms, 60 s). Results are expressed as the evoked release of radioactivity in the second stimulation period (S_2) as a percentage of that evoked during the first stimulation period (S_1). PMA (0.16 μM), PXB (10 and 70 μM) and TTX (1 μM) were present from 15, 24 and 24 min respectively before the second stimulation period (S_2).
☆ Significant difference from corresponding control ($P < 0.05$).
* Significant difference from PMA alone ($P < 0.05$)

stimulation-evoked release of [^{3}H]-NA by 88% (Figure 3).

High potassium-induced release

An increase in the potassium concentration of the atrial bathing solution to 64.7 mM resulted in an increase in radioactive outflow of 130498 ± 13090 d.p.m. ($n = 23$). As shown in Figure 4 the presence of PMA (0.16 μM) enhanced potassium-induced [^{3}H]-NA release compared to control, 116 ± 3% ($n = 5$) and 94 ± 5% respectively (S_2% of S_1). Whereas inhibition of protein kinase C by polymyxin B (10 and 70 μM) decreased outflow by 35% and 77% respectively (Figure 4). The PMA-induced enhancement of potassium-evoked outflow of radioactivity was decreased significantly by polymyxin B (10 and 70 μM). Tetrodotoxin (1 μM) when present in the incubation medium caused a slight reduction of the potassium-evoked release of [^{3}H]-NA (Figure 4).

Tyramine-induced release

Tyramine in a concentration of 0.3 μM caused an increase in radioactive outflow of 40224 ± 2108 d.p.m. ($n = 10$). PMA did not significantly affect the tyramine-evoked release of radioactivity (Figure 5). In contrast, polymyxin B at 70 μM, but not at 10 μM reduced the tyramine outflow by 23%. The presence of tetrodotoxin (1 μM) in the incubation medium did not alter the tyramine-evoked release of radioactivity (Figure 5).

In a series of experiments the effect of polymyxin B upon the tissue uptake of [^{3}H]-NA during the loading period was investigated. Isolated atria were preincubated with polymyxin B (70 μM) 20 min before the addition of [^{3}H]-NA (0.02 μM, 0.25 μCi ml^{-1}) and tissue content of radioactivity was determined. The presence of polymyxin B did not alter the tissue uptake of [^{3}H]-NA (69661 ± 9023 d.p.m., $n = 5$) compared to corre-

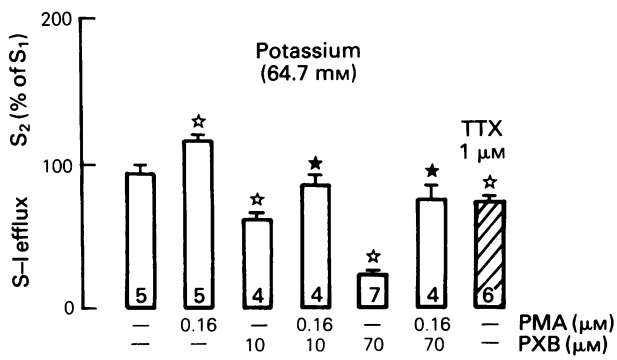

Figure 4 The effect of phorbol 12-myristate 13-acetate (PMA), polymyxin B (PXB) and tetrodotoxin (TTX) on the outflow of radioactivity from rat isolated atria evoked by a high potassium concentration (64.7 mM) in the bathing medium. Results are expressed as the evoked release of radioactivity (S-I) in the second stimulation period (S_2) as a percentage of that evoked during the first stimulation period (S_1). PMA (0.16 μM), PXB (10 and 70 μM) and TTX (1 μM) were present from 15, 24 and 24 min respectively before the second stimulation period (S_2).
☆ Significant difference from corresponding control ($P < 0.05$).
* Significant difference from PMA alone ($P < 0.05$).

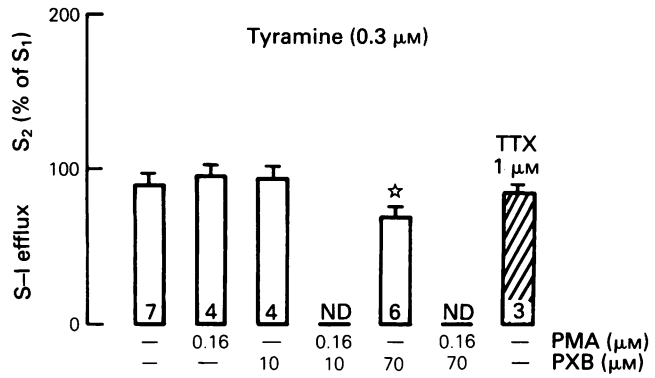

Figure 5 The effect of phorbol 12-myristate 13-acetate (PMA), polymyxin B (PXB) and tetrodotoxin (TTX) on the outflow of radioactivity (S-I) from rat isolated atria evoked by tyramine (0.3 μM). Results are expressed as the evoked release of radioactivity in the second stimulation period (S_2) as a percentage of that evoked during the first stimulation period (S_1). PMA (0.16 μM), PXB (10 and 70 μM) and TTX (1 μM) were present from 15, 24 and 24 min respectively before the second stimulation period (S_2). ND: not determined.
☆ Significant difference from corresponding control ($P < 0.05$).

sponding control tissues (61278 ± 5766 d.p.m., $n = 5$).

Discussion

The release of radioactivity from rat isolated atria preloaded with [^{3}H]-NA was used as an index of noradrenaline release from intraneuronal sympathetic nerves. In the present study we have provided evidence that exocytotic release of noradrenaline either by action-potential (electrical field stimulation) or by direct membrane depolarization with potassium is enhanced by the presence of phorbol ester. Tumour-promoting agents such as phorbol 12-myristate 13-acetate (PMA), activate protein kinase C (PKC) by mimicking the action of the endogenous activator 1,2-diacylglycerol (Kikkawa *et al.*, 1982). In rat isolated atria preloaded with [^{3}H]-NA, the efflux of radioactivity evoked by electrical field stimulation (2 Hz, 1 ms, 60 s) was progressively enhanced by PMA in a concentration-dependent manner (0.016–16 μM). Tetrodotoxin (TTX) suppresses neuronal depolarization by inhibiting the Na^+ channels to decrease propagated action-potentials with subsequent decrease in the release of neurotransmitter (Kao, 1966). The sensitivity of electrical field stimulation-induced release of noradrenaline to TTX (1 μM) suggests that the [^{3}H]-NA efflux was action-potential-induced and thus exocytotic in nature. Noradrenaline release has also been found to be enhanced by phorbol ester in rat and rabbit hippocampus slices (Allgaier & Hertting, 1986; Allgaier *et al.*, 1986; Versteeg & Florijn, 1987), guinea-pig sinus node (Shuntoh & Tanaka, 1986), rat salivary gland (Wakade *et al.*, 1985), rat brain synaptosomes (Nichols *et al.*, 1987), rabbit ear artery (Meehan & Ishac, 1987) and adrenal medullary chromaffin cells (Pocotte *et al.*, 1985). A possible role of PKC in the modulation of tyrosine hydroxylase, the rate-limiting enzyme in noradrenaline biosynthesis, has been suggested (Albert *et al.*, 1984; Vulliet *et al.*, 1985). Indeed, Albert and her colleagues report that phosphorylation of tyrosine hydroxylase by PKC leads to an increase in enzyme activity similar to that observed to cyclic AMP-dependent protein kinase. This would tend to decrease stimulation-induced release of radioactivity rather than the enhanced release observed in the present study and therefore excluded as a possible site of action of PMA.

Phorbol esters have also been demonstrated to enhance stimulation-induced release of acetylcholine (Murphy & Smith, 1987; Shapira *et al.*, 1987; Versteeg & Florijn, 1987), dopamine (Pozzan *et al.*, 1984; Zurgil & Zisapel, 1985; Zurgil *et al.*, 1986; Nichols *et al.*, 1987), 5-hydroxytryptamine (5-HT) (Wang & Friedman, 1987), histamine (Cantwell & Foreman, 1987) and also to enhance amylase secretion (De Pont & Fleuren-Jakobs, 1984). These results are consistent with the view that PKC plays a role in the mechanism associated with, not only neurotransmitter release, but secretion of hormones in general. This view is also supported by the finding in this study that polymyxin B, a putative inhibitor of PKC (Kuo *et al.*, 1983), reduced the electrical field stimulation-evoked release of [^{3}H]-NA and attenuated the PMA-induced enhancement of the evoked

release of noradrenaline. Pickard & Hawthorne (1978) have demonstrated that depolarization of synaptosomal plasma membranes with electrical stimulation leads to hydrolysis of phosphatidylinositol and the production of diacylglycerol. Thus the inhibition of release observed in the presence of polymyxin B is most probably due to blockade of diacylglycerol-induced increase in PKC activity. Polymyxin B is a polypeptide antibiotic that inhibits PKC by disrupting the membrane environment to which the enzyme binds (Kuo *et al.*, 1983; Wooten & Wrenn, 1984). Unfortunately as an inhibitor of PKC, polymyxin B is not as selective as PMA is an activator of PKC and thus may disrupt other membrane-dependent functions. We have used polymyxin B in two concentrations, 10 and 70 μM, which are 2.5 and 17 fold higher than the IC_{50} concentration for inhibition of PKC *in vitro* (Kuo *et al.*, 1983; Greenberg *et al.*, 1987). In the lower concentration, polymyxin B (10 μM) did not alter the spontaneous rate of beating of the rat isolated atria. Further, since polymyxin B does not inhibit cyclic AMP-dependent kinase, Ca^{2+}/calmodulin-dependent kinase or Ca^{2+} influx it can be considered relatively selective for PKC (Kuo *et al.*, 1983; Greenberg *et al.*, 1987), whereas H-7, another putative inhibitor of PKC inhibits these processes at approximately the same IC_{50} values (Hidaka *et al.*, 1984).

Exposure of rat atria to a high potassium concentration in the bathing solution leads to exocytotic, calcium-dependent outflow of neurotransmitter due to direct membrane depolarization. Kidokoro & Ritchie (1980) have shown that potassium-evoked release of adrenaline from chromaffin cells is partly due to direct depolarization and partly by the generation of action potentials. They demonstrate that at low potassium concentration (< 30 mM) transmitter release is action potential-dependent as indicated by it's sensitivity to TTX blockade, whereas at higher potassium concentrations (30–120 mM) transmitter release is due to direct depolarization and is consequently TTX-insensitive. This is consistent with the findings of the present study where potassium-evoked release (64.7 mM) is only partially sensitive to inhibition by TTX. In the present study the effect of PMA on potassium-evoked outflow of radioactivity was qualitatively similar to that observed with electrical stimulation. Phorbol esters have also been demonstrated to enhance potassium-evoked transmitter release in chromaffin cells (Brocklehurst & Pollard, 1986) and rat brain synaptosomes (Nichols *et al.*, 1987). Furthermore in this study, polymyxin B was observed to reduce the efflux of [^{3}H]-NA evoked by potassium. The observation that the effects of PMA are much smaller and polymyxin B much larger for potassium than electrical stimulation is consistent with a greater activation of PKC by potassium. Thus the effect of PMA would be expected to be smaller if the absolute magnitude of the evoked response is limited and conversely the effect of polymyxin B would be expected to be greater. An alternative explanation is that this may reflect a component that includes a non-specific action of polymyxin B on the membrane environment (Wooten & Wrenn, 1984).

In contrast to the effect of PMA on electrical field stimulation- and potassium-evoked release, tyramine-evoked efflux of radioactivity was unaltered by PMA. Tyramine induces non-exocytotic, calcium-independent release of noradrenaline due to displacement of noradrenaline from neuronal stores (Smith & Winkler, 1972). In dog saphenous vein, tyramine results in the efflux of a large proportion of the deaminated metabolites suggesting that noradrenaline is released from the storage vesicle into the axon cytoplasm and then metabolized by intraneuronal monoamine oxidase before release (Brandão *et al.*, 1978). Whereas noradrenaline release elicited by electrical stimulation consists of a large proportion of unchanged noradrenaline with only small amounts of metabolites (Brandão *et al.*, 1978). The overflow of radioactivity induced by tyramine was undiminished by polymyxin B in a concentration of 10 μM and only slightly reduced in a concentration of 70 μM. The reduction observed with the higher concentration is unlikely to be due to blockade of neuronal uptake since polymyxin B did not reduce the tissue accumulation of [^{3}H]-NA and is most likely due to a non-specific action of polymyxin B on the membrane environment.

Although the underlying mechanism(s) by which activation of PKC leads to enhanced transmitter release are unknown, it appears likely that an increase in PKC activity involves phosphorylation of neuronal protein(s) that are either coupled to or closely associated with exocytotic release of neurotransmitter. Inhibition or impairment of either high affinity neuronal uptake or inhibitory presynaptic α_2-adrenoceptors could account for the observed changes. However neither blockade of neuronal uptake with cocaine or desipramine nor blockade of prejunctional α_2-adrenoceptors with yohimbine abolish the response to phorbol ester and can therefore be excluded (Wakade *et al.*, 1985; Allgaier *et al.*, 1987). Alternatively PKC may modulate the release of the transmitter noradrenaline by the regulation of ion channel function (Kaczmarek, 1987). Briefly, activation of PKC with phorbol ester has been demonstrated to decrease the conductance of K^+-channels (Farley & Auerbach, 1986) and to increase calcium influx (De Riemer *et al.*, 1985; Wakade *et al.*, 1986), either of which could lead to enhanced transmitter efflux. In view of the high localization of PKC within synaptic nerve terminals and possibly in association

with the storage vesicles (Wood *et al.*, 1986), protein phosphorylation may be involved with the transport of storage vesicles to the plasma membrane or fusion of storage vesicles with the plasma membrane. Protein phosphorylation by PKC could increase the sensitivity to Ca^{2+} of Ca^{2+}-dependent processes (Shuntoh & Tanaka, 1986) which would lead to enhanced S-I transmitter efflux. Neurone-specific phosphoproteins that are substrates for PKC have been identified, however their possible role in neurotransmission remains in doubt (Nishizuka, 1986; Rodnight & Perrett, 1986; Gispen, 1987).

Finally, recent studies indicate that PKC consists of a family of at least four distinct but closely related proteins (Coussens *et al.*, 1986; Knopf *et al.*, 1986; Ohno *et al.*, 1987; Brandt *et al.*, 1987). Indeed, Brandt and colleagues report the presence of three PKC subtypes, their results suggest that PKC-I protein is located postsynaptically, whereas PKC-II and PKC-III are located presynaptically. Multiple classes of PKC may represent the requirement for specific activators or substrate proteins involved in the regulation of neurotransmitter release.

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