Evidence that M₁ muscarinic receptors enhance noradrenaline release in mouse atria by activating protein kinase C

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1 The M₁ selective muscarinic agonist, McNeil A 343, enhanced the electrically evoked release of noradrenaline from postganglionic sympathetic nerves in mouse atria. This has been found previously to be due to activation of muscarinic receptors of the M₁ subtype, probably located on sympathetic nerve terminals. The present study investigated the signal transduction mechanisms involved in the release-enhancing effects of McNeil A 343. The release of noradrenaline from mouse atria was assessed by measuring the electrically-induced (3 Hz, 60 s) outflow of radioactivity from atria which had been pre-incubated with [³H]-noradrenaline.

2 8-Bromo cyclic AMP in the presence of IBMX was used to enhance maximally S-I noradrenaline release through cyclic AMP-dependent mechanisms. However, the facilitatory effect of McNeil A 343 (10 µM) was not different from the effect in the absence of these drugs, suggesting that McNeil A 343 enhances noradrenaline release independently of the cyclic AMP system. Furthermore, the release-enhancing effect of McNeil A 343 (10 µM) on noradrenaline release was also not altered by the 5-lipoxygenase inhibitor, BW A4C.

3 The facilitatory effect of McNeil A 343 was not altered in the presence of drugs (trifluoperazine, W7, and calmidazolium) which inhibit calmodulin-dependent processes, suggesting that the mechanisms of action of McNeil A 343 does not depend on calmodulin.

4 It was considered likely that the facilitatory effect of McNeil A 343 on noradrenaline release may be due to activation of protein kinase C, since activators of protein kinase C enhance noradrenaline release. The facilitatory effect of McNeil A 343 was abolished by the non-selective protein kinase C inhibitor, K-252a. To investigate further the involvement of protein kinase C, mouse atria were chronically incubated (9–10 h) with the protein kinase C activator, 4β-phorbol dibutyrate (1.0 µM) in order to down-regulate protein kinase C activity. In protein kinase C-down-regulated atria, the facilitatory effect of McNeil A 343 (30 µM) was abolished. Incubation with 4α-phorbol dibutyrate which does not affect protein kinase C did not reduce the facilitatory effect of McNeil A 343. This provides evidence that activation of protein kinase C is involved in the signal transduction process of McNeil A 343.

Keywords: Noradrenaline release; M₁ muscarinic receptors; McNeil A 343; calmodulin antagonists; protein kinase C

Introduction

It is well established that postganglionic sympathetic nerve terminals are endowed with release-inhibiting muscarinic receptors (see Muscholl, 1980; Mitchelson, 1988). However, the muscarinic agonist, McNeil A 343, enhances action-potential evoked release of noradrenaline from sympathetically innervated tissues (Allen *et al.*, 1972; 1974; Fozard & Muscholl, 1974; Nedergaard, 1980; Arbilla *et al.*, 1986; Vizi *et al.*, 1989). In the past this effect was thought not to involve muscarinic receptors as it was resistant to blockade by atropine (Allen *et al.*, 1972; 1974; Fozard & Muscholl, 1974; Nedergaard, 1980; Arbilla *et al.*, 1986; Vizi *et al.*, 1989). However, in a recent study in mouse atria (Costa & Majewski, 1991) it was found that the facilitatory effect of low concentrations of McNeil A 343 on stimulation-induced release of noradrenaline was blocked by the muscarinic receptor antagonists, atropine, pirenzepine, dicyclomine and methoctramine, with the rank order of antagonism (pirenzepine > methoctramine) suggesting that an M₁ receptor was involved. On the other hand, the inhibitory effect of carbachol on noradrenaline release was blocked with a rank order of antagonism methoctramine > pirenzepine, suggesting that this was mediated through M₂ muscarinic receptors. Thus it may be that noradrenaline release from sympathetic nerves is under dual M₁ and M₂ modulation. In field stimulated mouse atria, the facilitatory M₁ receptors on sympathetic nerves were not activated by endogenously released acetylcholine since pirenzepine did not inhibit

noradrenaline release (Costa & Majewski, 1991). However, in rabbit atria, pirenzepine did inhibit noradrenaline release during concomitant parasympathetic/sympathetic nerve stimulation (Muscholl *et al.*, 1989; Altes *et al.*, 1990; Harbermeier-Muth *et al.*, 1990), suggesting physiological activation of M₁-receptors by neuronally released acetylcholine.

The aim of the present study was to characterize further the M₁ facilitatory mechanism on sympathetic nerves by investigating the signal transduction pathway through which it operates. Candidate pathways include the adenosine 3':5'-cyclic monophosphate (cyclic AMP) system since activation of this system results in enhanced release of noradrenaline (see Majewski *et al.*, 1988); arachidonic acid metabolites since activation of muscarinic receptors releases arachidonic acid metabolites in many tissue and cell types (see Nathanson, 1987); and the phospholipase C pathway since in many systems M₁ receptors transduce their effect through this system (see Fisher & Agranoff, 1987). Two important second messengers are formed as a result of phospholipase C activity; inositol trisphosphate and diacylglycerol (see Berridge, 1987). The major action of inositol trisphosphate is to release calcium from intracellular stores (Berridge & Irvine, 1984), and this may influence neurotransmitter release since calcium binds to calmodulin which may then activate calcium-calmodulin protein kinases thought to be involved in the neurotransmitter release process (see Greengard, 1987). Diacylglycerol, on the other hand, activates protein kinase C, a process which enhances noradrenaline release from many tissues both in the central nervous system and periphery (see Majewski *et al.*, 1990). In this study all of these mechanisms

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were investigated to determine the signal transduction pathway utilized by release-enhancing M_1 receptors in sympathetic nerves.

Methods

[3H]-noradrenaline release

Mice of either sex (15–25 g) were decapitated and the heart was rapidly removed and placed in Krebs-Henseleit solution which was bubbled with a mixture of 5% CO_2 , 95% O_2 . The atria were dissected free of the surrounding tissue and placed in an organ bath with Krebs-Henseleit solution which was maintained at 37°C and constantly gassed with 5% CO_2 , 95% O_2 . The atria were incubated with [3H]-noradrenaline ($15 \mu Ci ml^{-1}$; $0.37 \mu M$) for 20 min to label transmitter noradrenaline stores. Following incubation the atria were tied loosely to a disc and transferred to individual flow cells. The tissues were washed by superfusing them at a rate of $2 ml min^{-1}$ for 60 min with Krebs-Henseleit solution which was maintained at 37°C and constantly gassed with 5% CO_2 , 95% O_2 . A priming stimulation was delivered to the atria 45 min after the beginning of the washing period. The priming stimulation consisted of square wave pulses, 1 ms duration, $15 V cm^{-1}$ field gradient, and was delivered at 3 Hz for 60 s through two platinum electrodes which were fixed into the flow cell and situated either side of the tissue. The washing procedure and the priming stimulation were designed to remove unbound and loosely bound radioactive compounds from the atria. After the washing period, 3 min fractions of the solution superfusing the atria were collected for the next 54 min. There were two test stimulation periods (S1 and S2), delivered at 6 and 42 min from the onset of sample collection. The stimulation parameters for the test stimulations were the same as those for the priming stimulations ($15 V cm^{-1}$, 3 Hz, 60 s). The effects of drugs were assessed by superfusing them only during the second stimulation period. In most experiments drugs were present from either 24 min or 15 min prior to the second stimulation period.

At the end of all experiments the atria were dissolved overnight in 2 ml Soluene 350 (Packard Instruments, Wilmington, U.S.A.). The samples of the superfusion solution and the dissolved atria were added to 6–12 ml Picofluor 40 (Packard), and the radioactive content of the samples and the atria was estimated with a liquid scintillation counter. Corrections for counting efficiency were determined by automatic external standardization, and all measurements were expressed as disintegrations per min (d.p.m.).

Chronic experiments with phorbol dibutyrate

Mouse isolated atria were dissected free and placed in 25–50 ml of tissue culture medium to which either vehicle (dimethylsulphoxide, DMSO, 0.05% v/v) or phorbol ester ($1.0 \mu M$) had been added. The solution containing the atria was placed in an incubator which had a 95% O_2 , 5% CO_2 environment and was maintained at 37°C, for 9–10 h. The culture medium had been equilibrated in the incubator for 20 min before the atria were added. Following the preincubation the atria were removed, rinsed in Krebs-Henseleit solution and then a [3H]-noradrenaline release experiment was conducted as described by Foucart *et al.* (1991), where instead of flow cells, the atria was placed in an organ bath and the outflow of radioactivity measured in 3 min samples of the bathing fluid. Some experiments on non-preincubated atria were also performed in organ baths as indicated in the results.

Calculation of radioactive outflow

The fractional radioactive outflow of a sample was calculated as the radioactive content of the sample expressed as a

percentage of the total tissue radioactivity at the time the sample was collected. The spontaneous (resting) outflow of radioactivity was calculated as the mean value of the fractional radioactive content of the sample taken immediately before the stimulation and of the sample taken 9 min following the onset of stimulation. The fractional stimulation-induced outflow of radioactivity was calculated by adding the fractional radioactive content of the sample during which stimulation occurred and the sample immediately following, and then subtracting twice the mean fractional resting outflow of radioactivity.

Statistical analysis of results

All data are expressed as mean and standard error of the mean (s.e.mean). The data were analysed by Student's *t* tests. Where multiple comparisons against a single control were made, one way analysis of variance (ANOVA) followed by Dunnett's tests were done. Where appropriate, two-way ANOVA were also carried out to determine whether effects of McNeil A 343 in relation to drug-free control experiments (A) were affected by a particular treatment (B). In this case the significance was determined from the interaction term A*B in the analysis of variance table, where variable A comprised McNeil A 343 and control experiments and variable B comprised McNeil A 343 and control experiments after the treatment. Probability levels (*P*) of less than 0.05 were taken to indicate statistical significance in all cases.

Materials

The following drugs were used: (–)-2,5,6-[3H]-noradrenaline (specific activity $40.8 Ci mmol^{-1}$; NEN, Boston, U.S.A.), 3-isobutyl-1-methylxanthine (IBMX), W-7 (N-(6-aminohexyl-5-chloro-1-naphthalenesulphonamide), 4 β -phorbol dibutyrate, calmidazolium (R 24 571) and trifluoperazine (Sigma, St Louis, U.S.A.); 8-bromoadenosine 3',5'-cyclic monophosphate (Boehringer-Mannheim, Mannheim, FRG); 4 α -phorbol dibutyrate (LC Services, Woburn, U.S.A.); McNeil A 343 (4-(*m*-chlorophenylcarbamoyloxy)-2-butylnyltrimethylammonium chloride; McNeil Laboratories, Australia). BW A4C (N-(3-phenoxycinnamyl) acetohydroxamic acid) was a gift from Wellcome, UK. K-252a ((8R*,9S*,11S*)-(–)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8-11-epoxy-1H,8H,11H-2,7b,11a-triazadi benz[a,g]cycloocta[c,d,e]trinden-1-one) was a generous gift from Dr H. Kase, Kyowa Hakko Kogyo, Japan.

Trifluoperazine, W-7, calmidazolium, BW A4C, phorbol dibutyrate and K-252a were dissolved in DMSO before being further diluted in Krebs-Henseleit. All other drugs were dissolved directly in Krebs-Henseleit solution.

The modified Krebs-Henseleit solution had the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, KH_2PO_4 1.03, $MgSO_4$ 0.45, D-(+)-glucose 11.1, disodium edetate 0.067, and ascorbic acid 0.14.

The tissue culture medium used was Medium 199 with Hank's salt without glutamine (Commonwealth Serum Laboratories, Parkville, Australia) to which bovine serum albumin ($5 g l^{-1}$) and sodium pyruvate (0.5 mM) were added.

Results

Cyclic AMP and McNeil A 343

Mouse atria were incubated with [3H]-noradrenaline and two stimulations were delivered (S1 and S2). The absolute fractional stimulation-induced (S-I) outflow of radioactivity for all experimental groups in the first stimulation period (FR1) is given in Table 1.

The M_1 selective agonist, McNeil A 343 ($10 \mu M$), significantly enhanced the fractional S-I outflow of radioactivity from mouse atria when it was present during the second

Table 1 The baseline parameters for the outflow of radioactivity corresponding to the first stimulation period (S1) from mouse atria pre-incubated with [³H]-noradrenaline

Treatment	n	S1 × 100	R1 × 100
<i>Flow cell experiments</i>			
No pre-incubation	80	0.43 ± 0.02	0.43 ± 0.01
<i>Organ bath experiments</i>			
<i>Series 1</i>			
No pre-incubation	10	1.37 ± 0.28	1.19 ± 0.12
DMSO incubation	8	1.22 ± 0.09	1.92 ± 0.11
4α-PDB incubation	8	1.43 ± 0.24	1.86 ± 0.15
<i>Series 2</i>			
DMSO incubation	13	1.14 ± 0.13	2.00 ± 0.23
4β-PDB incubation	12	1.60 ± 0.13	2.52 ± 0.21

Mouse atria were incubated with [³H]-noradrenaline, washed for 60 min and the outflow of radioactivity was measured. There were two test stimulation periods and the fractional stimulation-induced outflow of radioactivity in the first stimulation period (S1) multiplied by 100 is shown as is the corresponding fractional resting (spontaneous) outflow (R1). Mean ± s.e.mean values are given, and *n* is the number of experiments. Some atria were chronically incubated for 9–10 h with either DMSO or 4α- or 4β-phorbol dibutyrate (PDB) prior to the experiment. There were two series of these experiments. Both the fractional S-I outflow in S1 and the corresponding resting outflow of radioactivity were higher in organ bath experiments compared to the flow cells ($P < 0.05$, two way analysis of variance). However there were no significant differences in the fractional S-I outflow within the organ bath groups ($P > 0.05$, two way analysis of variance) but the resting outflow of radioactivity was significantly higher in all the pre-incubated atria compared to non-pre-incubated atria within the organ bath groups ($P < 0.05$, two way analysis of variance, followed by Dunnett's test).

Figure 1 The influence of 8-bromo cyclic AMP (8BR; 270 μ M) plus 3-isobutyl-1-methylxanthine (IBMX, 100 μ M) and BWA4C (BW; 1.0 μ M) on the facilitatory effect of McNeil A 343 (McN; 10 μ M) on the fractional stimulation-induced (S-I) outflow of radioactivity from mouse atria pre-incubated with [³H]-noradrenaline. There were two stimulation periods (3 Hz, 60 s) 36 min apart. All drugs were present from 15 min before the second stimulation period. The mean fractional S-I outflow in the second stimulation period (S2) expressed as a percentage of that in the first (S1) is shown. The columns represent the mean ± s.e.mean. The number of experiments is at the base of each column. *Represents a significant difference from control (Con; $P < 0.05$, Dunnett's test after one-way ANOVA), and †represents a significant difference from 8-bromo cyclic AMP plus IBMX or BW A4C as appropriate ($P < 0.05$, Student's *t* test). The enhancing effect of McNeil A 343 was not altered by 8-bromo cyclic AMP and IBMX, or BW A4C ($P < 0.05$, two-way ANOVA).

stimulation period (Figure 1). The combination of 3-isobutyl-1-methyl-xanthine (IBMX; 100 μ M) and 8-bromo cyclic AMP (270 μ M) enhanced the fractional S-I outflow of radioactivity (Figure 1), and it has been shown previously that this concentration of 8-bromo cyclic AMP in the presence of IBMX is maximally effective for enhancing noradrenaline release from mouse atria (Costa & Majewski, 1990). In the presence of 8-bromo cyclic AMP (270 μ M) and IBMX (100 μ M), the facilitatory effect of McNeil A 343 (10 μ M) on the fractional S-I outflow of radioactivity was not significantly different from the effect of McNeil A 343 in the absence of these drugs (Figure 1).

5-Lipoxygenase and McNeil A 343

The 5-lipoxygenase inhibitor BW A4C (1.0 μ M), present in the second stimulation period, did not alter the fractional S-I outflow of radioactivity ($P > 0.05$, Student's *t* test; Figure 1). When McNeil A 343 was present with BW A4C, the increase in the fractional S-I outflow of radioactivity was not different from that produced by McNeil A 343 alone (Figure 1).

Calmodulin antagonists and McNeil A 343

By itself, the calmodulin antagonist calmidazolium (1.0 μ M) did not alter the fractional S-I outflow of radioactivity when it was present only during the second stimulation period ($P > 0.05$, Dunnett's test after one-way ANOVA; Figure 2). However, two other compounds which inhibit calmodulin effects, trifluoperazine (1.0 μ M) and W-7 (10 μ M), significantly increased the fractional S-I outflow of radioactivity from mouse atria (Figure 2). The enhancing effect of McNeil A 343 (10 μ M) in the presence of calmidazolium (1.0 μ M), trifluoperazine (1.0 μ M) or W-7 (10 μ M) was not significantly different from the effect of McNeil A 343 in the absence of these calmodulin antagonists (Figure 2).

DMSO at a final concentration of 0.1% (solvent for BW A4C and calmodulin inhibitors) did not alter either the

Figure 2 The influence of calmidazolium (CMZ; 1.0 μ M), W-7 (10 μ M) and trifluoperazine (TFP; 1.0 μ M) on the facilitatory effect of McNeil A 343 (McN; 10 μ M) on the fractional stimulation-induced (S-I) outflow of radioactivity from mouse atria pre-incubated with [³H]-noradrenaline. There were two stimulation periods (3 Hz, 60 s) 36 min apart. McNeil A 343 was present from 15 min before the second stimulation period. All other drugs were present from 24 min before the second stimulation period. The mean fractional S-I outflow in the second stimulation period (S2) expressed as a percentage of that in the first (S1) is shown. The columns represent the mean ± s.e.mean. The number of experiments in each series was 4–6. *Represents a significant difference from the drug free control (Con; $P < 0.05$, Dunnett's test after one-way ANOVA), and †represents a significant difference from the respective drug control experiment ($P < 0.05$, Student's *t* test). The enhancing effect of McNeil A 343 was not altered by either calmidazolium, W-7 or trifluoperazine ($P > 0.05$, two-way ANOVA).

radioactive outflow from the tissue, or the effects of the drugs on the fractional S-I outflow of radioactivity (not shown).

Protein kinase C and McNeil A 343

The protein kinase C inhibitor K-252a (1.0 μM), present by itself in the second stimulation period did not alter the fractional S-I outflow of radioactivity ($P > 0.05$, Student's *t* test; Figure 3). In the presence of K-252a, McNeil A 343 (10 μM) did not facilitate the fractional S-I outflow of radioactivity from mouse atria (Figure 3).

Further experiments were conducted in organ baths instead of flow cells. In flow cells the atria are superfused with Krebs-Henseleit solution at 1 ml min⁻¹ whereas in organ bath experiments the atria were bathed in solution which was replaced every 3 min. The spontaneous and stimulation-induced outflow of radioactivity was less in flow cell experiments compared to organ bath experiments (Table 1). It is unclear why this was observed but it may be related to the more extensive washing by the superfusion in flow cells after incubation with [³H]-noradrenaline and/or different electrode geometry between the two systems. In atria which had been pre-incubated with either vehicle (0.05% DMSO) for 9–10 h or the phorbol ester which does not activate protein kinase C, 4 α -phorbol dibutyrate (1.0 μM), McNeil A 343 (30 μM) enhanced the S-I outflow of radioactivity to a similar extent (Figure 4). In a separate series of experiments (series 2), both 4 β -phorbol dibutyrate (1.0 μM) and McNeil A 343 (30 μM) added before the second stimulation significantly enhanced the S-I outflow of radioactivity from mouse atria which had been chronically (9–10 h) incubated with vehicle (0.05% DMSO; Figure 4). However, after pre-incubation with 4 β -phorbol dibutyrate (1.0 μM) for 9–10 h to down-regulate protein kinase C, subsequent addition of 4 β -phorbol dibutyrate (1.0 μM) did not alter the fractional S-I outflow of radioactivity indicating down-regulation of protein kinase C (Figure 4). In 4 β -phorbol dibutyrate pre-incubated atria, McNeil A 343 (30 μM) also did not alter the S-I outflow of radioactivity (Figure 4). In the pre-incubated atria, the fractional S-I outflow of radioactivity in the first stimulation period was not significantly different from non-pre-incubated atria in organ baths for any of the pre-incubated groups (DMSO, 4 α -phorbol dibutyrate, 4 β -phorbol dibutyrate, series

Figure 3 The influence of K-252a (1.0 μM) on the facilitatory effect of McNeil A 343 (McN; 10 μM) on the fractional stimulation-induced (S-I) outflow of radioactivity from mouse atria pre-incubated with [³H]-noradrenaline. There were two stimulation periods (3 Hz, 60 s) 36 min apart. McNeil A 343 was present from 15 min before the second stimulation period, and K-252a was present from 24 min before the second stimulation period. The mean fractional S-I outflow in the second stimulation period (S2) expressed as a percentage of that in the first (S1) is shown. The columns represent the mean $\pm$ s.e.mean. The number of experiments is at the base of each column. *Represents a significant difference from control (Con; $P < 0.05$, Dunnett's test after one way ANOVA); †indicates that the enhancing effect of McNeil A 343 was significantly inhibited by K-252a ($P < 0.05$, two-way ANOVA).

1 and series 2) (Table 1). The spontaneous (resting) outflow of radioactivity was significantly increased in pre-incubated atria over non-pre-incubated atria (Table 1).

Spontaneous outflow of radioactivity

McNeil A 343 (10 μM), either by itself or in the presence of other drugs, inhibited the spontaneous outflow of radioactivity by about 10% from mouse isolated atria which had been incubated with [³H]-noradrenaline and this was not affected by any of the other treatments used in combination with McNeil A 343 (not shown). Other drugs used in the present study had minor or no effect on the spontaneous outflow (not shown).

Figure 4 The effect of prolonged incubation (9–10 h) with 4 α - or 4 β -phorbol dibutyrate (α PDB, β PDB, 1.0 μM) on the facilitatory effect of subsequently applied 4 β -phorbol dibutyrate (1.0 μM) and McNeil A 343 (McN; 30 μM) on the fractional stimulation-induced (S-I) outflow of radioactivity from mouse atria pre-incubated with [³H]-noradrenaline. Mouse isolated atria were incubated with tissue culture medium containing 4 α - and 4 β -phorbol dibutyrate (1.0 μM) or DMSO (vehicle) for 9–10 h prior to being incubated with [³H]-noradrenaline. There were two series of experiments: series 1 and series 2 done at different times. In some experiments the atria were used without prior incubation. In all experiments there were two stimulation periods (3 Hz, 60 s) 36 min apart. McNeil A 343 was present from 15 min before the second stimulation period, and 4 β -phorbol dibutyrate was present from 24 min prior to the second stimulation period. The mean fractional S-I outflow in the second stimulation period (S2) expressed as a percentage of that in the first (S1) is shown. The columns represent the mean $\pm$ s.e.mean. The number of experiments is at the base of each column. *Represents a significant difference from the vehicle control (Con) ($P < 0.05$, Student's *t* test); †indicates that the enhancing effects of 4 β -phorbol dibutyrate or McNeil A 343 were significantly reduced in 4 β -phorbol dibutyrate pre-incubated atria ($P < 0.05$, two-way ANOVA).

Discussion

The present study was designed to elucidate possible transduction pathways associated with enhancement of noradrenaline release from sympathetic nerves through muscarinic receptors. In the present study, as previously observed (Costa & Majewski, 1991), the muscarinic agonist McNeil A 343 enhanced the stimulation-induced (S-I) release of noradrenaline from mouse atria. This effect was found most likely to be due to activation of M_1 muscarinic receptors at postganglionic sympathetic nerve varicosities (Costa & Majewski, 1991).

One intraneuronal pathway involved in the enhancement of noradrenaline release from nerve terminals is the adenylate cyclase-cyclic AMP system (see Majewski *et al.*, 1988). There have been reports that cyclic AMP levels in some neurones are raised following activation of muscarinic receptors (Hanley & Iversen, 1978; Lenox *et al.*, 1980; Briggs *et al.*, 1982), and a recent report indicates that muscarinic receptors in the rat olfactory bulb are linked directly to stimulation of adenylate cyclase activity (Onali & Olanas, 1990). In the present study the combination of 8-bromo cyclic AMP and the phosphodiesterase inhibitor, IBMX, enhanced the S-I release of noradrenaline. This has been shown previously in mouse atria (Costa & Majewski, 1988) where the cyclic AMP-dependent pathway for enhancing noradrenaline release was saturated using the same concentrations of these agents. Under these conditions the release-enhancing effects of prejunctional β -adrenoceptors and prejunctional ACTH receptors were abolished (Costa & Majewski, 1988), which is consistent with the linking of these receptors to adenylate cyclase (Göthert & Hentrich, 1984; Johnston *et al.*, 1987; Costa & Majewski, 1988; 1990). However, in the present study the release-enhancing effect of McNeil A 343 was maintained in the presence of 8-bromo cyclic AMP and IBMX, ruling out the possibility of a cyclic AMP-mediated mechanism in this case.

Activation of muscarinic receptors releases arachidonic acid from many tissues including the heart (Junstad & Wennmalm, 1974), and metabolites of arachidonic acid, particularly the prostaglandins, have been found to modulate noradrenaline release (see Hedqvist, 1977; Malik & Sehic, 1990). In a previous study in rabbit pulmonary artery it was found that cyclo-oxygenase metabolites of arachidonic acid did not mediate the facilitatory effect of McNeil A 343 on noradrenaline release (Nedergaard, 1980) although this was not directly tested in the present study. In the present study we have extended these findings to suggest that certain lipoxygenase metabolites of arachidonic acid are also not involved since BW A4C, which is a relatively selective 5-lipoxygenase inhibitor (Tateson *et al.*, 1988), did not alter the facilitatory effect of McNeil A 343 on noradrenaline release.

Muscarinic receptors of the M_1 subtype have been closely associated with the phospholipase C-inositol trisphosphate/diacylglycerol signal transduction mechanism in many systems (see Eglén & Whiting, 1986; Nathanson, 1987; Mitchellson, 1988). The second messenger inositol trisphosphate releases Ca^{2+} from intracellular stores, which by binding to calmodulin may activate calmodulin-dependent enzymes thought to have a role in neurotransmitter release (see Greengard, 1987). However, in the present study the facilitatory effect of McNeil A 343 on noradrenaline release was not altered in the presence of the calmodulin antagonists, calmidazolium (Van Belle, 1981), W-7 (Hidaka *et al.*, 1981) or trifluoperazine (Levin & Weiss, 1979), suggesting that this pathway was not involved.

None of the calmodulin antagonists by themselves decreased the S-I release of noradrenaline. This is in marked contrast to the release-inhibiting effects of these agents (W7 and trifluoperazine) in brain synaptosomes (DeLorenzo, 1985), which has been interpreted as evidence for a role for calmodulin in the process of neurotransmitter release (DeLorenzo, 1981; 1985; see also Llinás *et al.*, 1985). How-

ever, other studies have also failed to observe inhibition of neurotransmitter release in various systems (Publicover, 1985; Jinnai *et al.*, 1986; Reimann *et al.*, 1988) using W7, trifluoperazine and calmidazolium. Thus, there may not be a universal role for calcium-calmodulin kinases in neurotransmitter release processes.

The other metabolite formed by the action of phospholipase C is diacylglycerol, which can activate protein kinase C to phosphorylate proteins associated with the process of neurotransmitter release (Nishizuka, 1984; 1986). Indeed, protein kinase C activators such as phorbol esters enhance the release of noradrenaline from many tissues including mouse atria (see Musgrave & Majewski, 1989). In the present study the protein kinase C inhibitor K-252a (Kase *et al.*, 1987) abolished the facilitatory effect of McNeil A 343 on noradrenaline release. Similar results have been observed with other protein kinase C inhibitors on muscarinic enhancement of neurotransmitter release in the central nervous system (Diamant *et al.*, 1988; Xu *et al.*, 1990), where a role for protein kinase C was assumed. However, some caution must be used since protein kinase C inhibitors are in general relatively non-selective. For example, K-252a has inhibitory effects on a wide range of other intracellular systems including cyclic AMP-dependent protein kinase, cyclic GMP-dependent protein kinase, myosin light chain kinase, as well as calmodulin and some calmodulin-dependent enzymes (Kase *et al.*, 1987; Nakanishi *et al.*, 1988; see Ruegg & Burgess, 1989). In the present study it is unlikely that K-252a inhibited the effect of McNeil A 343 by altering cyclic AMP- or calmodulin-dependent transduction systems since the experiments described above rule out their involvement.

Another approach taken to test whether protein kinase C was involved was to down-regulate protein kinase C by long-term exposure (9–10 h) to protein kinase C activating phorbol esters such as 4 β -phorbol dibutyrate (Mathies *et al.*, 1987; Young *et al.*, 1987). In the present study, after 4 β -phorbol dibutyrate pretreatment the facilitatory effect of subsequently applied phorbol esters on noradrenaline release was abolished, indicating functional protein kinase C down-regulation. Similar results were obtained previously in mouse atria by Foucart *et al.* (1991) who also showed that 4 α -phorbol dibutyrate, which does not activate protein kinase C but has the other chemical characteristics of 4 β -phorbol dibutyrate (Niedel & Blackshear, 1986), did not produce down-regulation of protein kinase C. Using 4 β -phorbol dibutyrate treatment in the present study, the facilitatory effect of McNeil A 343 on noradrenaline release was abolished, strongly suggesting that a functional protein kinase C mechanism was necessary for enhancement of noradrenaline release through M_1 receptors. After 4 α -phorbol dibutyrate treatment the facilitatory effect of McNeil A 343 on noradrenaline release was still observed indicating that the inhibition of this effect of McNeil A 343 produced by 4 β -phorbol dibutyrate was probably a specific protein kinase C effect.

There are two possible roles for protein kinase C. The first is that it is part of a final common pathway in the release process, necessary for the full manifestation of enhanced transmitter release. For example, the ability of many drugs (such as 8-bromo cyclic AMP, isoprenaline, idazoxan, tetraethylammonium and angiotensin II, as well as high frequency electrical stimulation) to enhance noradrenaline release in mouse atria is reduced but not abolished by protein kinase C inhibitors and protein kinase C down-regulation (Foucart *et al.*, 1991; Musgrave *et al.*, 1991). Thus, a partially reduced effect of McNeil A 343 by anti-protein kinase C measures may be expected. However, given the direct linking of M_1 receptors to protein kinase C in other systems (see above), a second alternative is that protein kinase C may directly transduce M_1 -facilitation on noradrenaline release especially since the PKC inhibitor and PKC down regulation completely inhibited the facilitatory effect of McNeil A 343. A similar

observation was made with angiotensin II (Musgrave *et al.*, 1991; Foucart *et al.*, 1991) where it was also suggested that protein kinase C directly transduces the facilitatory effect on noradrenaline release.

It is not clear whether these facilitatory M_1 receptors are of physiological significance, particularly for the tonic modulation of noradrenaline release *in vivo*, since in a previous study the M_1 antagonist, pirenzepine, by itself did not inhibit noradrenaline receptors from field stimulated mouse atria (Costa & Majewski, 1991), suggesting that they were not activated by endogenously released acetylcholine. However,

another possibility is that modulation of neurotransmitter release is not the only function of M_1 receptors especially since protein kinase C is involved in a number of cell functions such as neurotransmitter synthesis, as well as growth and development (Nishizuka, 1986). A physiological role for M_1 receptors in this arena remains to be investigated.

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