Evidence for facilitatory and inhibitory muscarinic receptors on postganglionic sympathetic nerves in mouse isolated atria

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1 McNeil A 343 (10 μM –30 μM) enhanced the fractional stimulation-induced (S-I) outflow of radioactivity from mouse isolated atria which had been incubated with [^3H]-noradrenaline. The enhancing effect of McNeil A 343 was not altered by hexamethonium (300 μM) suggesting that it was not due to an action at nicotinic receptors. It is also unlikely that McNeil A 343 enhanced the S-I outflow of radioactivity in mouse atria by blocking neuronal reuptake of noradrenaline since the effect persisted in the presence of cocaine (30 μM).

2 The facilitatory effect of McNeil A 343 on the S-I outflow of radioactivity was attenuated by atropine (0.3 μM), pirenzepine (0.2 μM or 1.0 μM), dicyclomine (1.0 μM) and methoctramine (1.0 μM) and was thus due to activation of muscarinic receptors.

3 In contrast to the effect of McNeil A 343, another muscarinic receptor agonist, carbachol (3.0 μM) significantly decreased the S-I outflow of radioactivity. The receptors through which McNeil A 343 acts to enhance the S-I outflow of radioactivity appear to be distinct from inhibitory prejunctional muscarinic receptors. The relatively M_1 -selective antagonist, pirenzepine (0.2 μM), attenuated the facilitatory effect of McNeil A 343 whereas a higher concentration (1.0 μM) was required to block the inhibitory effect of carbachol. Conversely, the relatively M_2 -selective antagonist, methoctramine (0.1 μM), blocked the inhibitory effect of carbachol but a higher concentration of methoctramine (1.0 μM) was required to block the facilitatory effects of McNeil A 343. These results tentatively ascribe facilitatory muscarinic receptors as belonging to the M_1 subtype and inhibitory muscarinic receptors as belonging to the M_2 subtype.

4 The non-selective muscarinic receptor antagonist, atropine, enhanced the S-I outflow of radioactivity, suggesting that there was tonic activation of inhibitory prejunctional muscarinic receptors by endogenous acetylcholine released from parasympathetic nerves. However, pirenzepine (0.03 μM –1.0 μM) did not decrease the S-I outflow of radioactivity, suggesting that under the conditions of the present study, facilitatory muscarinic receptors are not tonically activated by endogenous acetylcholine.

Keywords: McNeil A 343; muscarinic receptors; methoctramine; noradrenaline release; pirenzepine

Introduction

Activation of prejunctional muscarinic receptors on postganglionic sympathetic nerve terminals inhibits noradrenaline release from a range of tissues (see Starke, 1977; Muscholl, 1980). Muscarinic receptor antagonists enhance noradrenaline release in the heart, suggesting that there is tonic activation of inhibitory prejunctional muscarinic receptors by endogenous acetylcholine released from nearby parasympathetic nerves (Levy & Blattberg, 1976; Muscholl & Muth, 1982; Habermeier-Muth & Muscholl, 1988). In rat heart (Fuder, 1982; Fuder *et al.*, 1982) and rabbit heart (Fuder *et al.*, 1981; Fuder, 1982), inhibitory prejunctional muscarinic receptors are similar to postjunctional muscarinic receptors in these tissues, and thus resemble the M_2 subtype (Fuder, 1982; Fuder *et al.*, 1981; 1982; see also Mitchelson, 1988).

The muscarinic receptor agonist, McNeil A 343 inhibited noradrenergic transmission in rabbit ear artery (Rand & Varma, 1971; Allen *et al.*, 1974; Choo *et al.*, 1985) but enhanced noradrenaline release from guinea-pig atria (Allen *et al.*, 1972; 1974; Vizi *et al.*, 1989), rabbit heart (Fozard & Muscholl, 1974), rabbit pulmonary artery (Nedergaard, 1980; 1981) and rat atria (Arbilla *et al.*, 1986). In these tissues where McNeil A 343 enhanced noradrenaline release, other muscarinic receptor agonists such as acetylcholine, carbachol and methacholine consistently inhibited noradrenaline release (see Starke, 1977). McNeil A 343 is not a typical muscarinic receptor agonist and appears to be functionally selective for M_1 receptors (see Birdsall *et al.*, 1988; Mitchelson, 1988). Thus it is possible that the enhancing effect of McNeil A 343 on noradrenergic transmission may be due to activation of a specific prejunctional facilitatory M_1 muscarinic mechanism.

Indeed there is recent evidence for a prejunctional M_1 facilitatory muscarinic receptor site that is activated by endogenous acetylcholine in rabbit atria (Muscholl *et al.*, 1989; Altes *et al.*, 1990).

The aim of the present study was to determine if the enhancing effect of McNeil A 343 on noradrenaline release could be blocked by antagonists which are able to discriminate between various muscarinic receptor subtypes, and thus provide evidence for the existence of facilitatory muscarinic receptors on sympathetic nerve terminals. Our hypothesis was that sympathetic nerve terminals are endowed with inhibitory M_2 muscarinic receptors and facilitatory M_1 muscarinic receptors.

Methods

Preparation of mouse atria

Female mice (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 and 95% O_2 . The atria were dissected free of the surrounding tissue and then 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 $\mu\text{Ci ml}^{-1}$; 0.37 μM), 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

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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 (S_1 and S_2), 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 stimulation (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 antagonists or blocking drugs were present from 24 min prior to the second stimulation period, whilst agonists were present from 15 min prior to the second stimulation period. In some experiments antagonists were present in the superfusion solution from just after the priming stimulation until the end of the experiment (designated 'throughout'). In one series of experiments, S_1 and S_2 were 69 min apart and pirenzepine was present from 57 min before the second stimulation period. At the end of all the experiments the atria were dissolved in Soluene (Packard Instruments, U.S.A.).

The samples of the superfusion solution and the atria were added to Picofluor 40 (Packard Instruments, U.S.A.), and the radioactive content of both the samples and the dissolved atria was measured with a liquid scintillation counter. Corrections for counting efficiency were made by automatic external standardization and all measurements were expressed as disintegrations per min (d.p.m.).

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 used. Where appropriate, two-way ANOVA were also carried out. 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 $40.8 \text{ Ci mmol}^{-1}$, N.E.N., U.S.A.), McNeil A 343 (4-(*m*-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium chloride, McNeil Laboratories, Australia), atropine sulphate (Sigma, U.S.A.), carbachol (carbamylcholine chloride, Sigma, U.S.A.), hexamethonium bromide (May and Baker, Australia), cocaine HCl (Macfarlan-Smith, Australia), methoctramine HCl (Research Biochemicals Inc., U.S.A.). dicyclomine HCl was a generous gift from Dr Fred Mitchell, Victorian College of Pharmacy, Australia. Pirenzepine HCl was a gift from Boehringer Ingelheim, Australia. All of the drugs except for dicyclomine HCl and methoctramine HCl were dissolved in Krebs-Henseleit solution. Dicyclomine and methoctramine were initially dissolved in distilled H_2O before being diluted 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.

Results

The effect of McNeil A 343 on the stimulation-induced outflow of radioactivity and the influence of hexamethonium and cocaine

Mouse atria were incubated with [^3H]-noradrenaline, two stimulations were delivered (S_1 and S_2) and the absolute fractional stimulation-induced (S-I) outflow of radioactivity in the first stimulation period (FR_1) is given in Table 1. McNeil A 343, present only in the second stimulation period concentration-dependently enhanced the fractional S-I outflow of radioactivity (Figure 1). When they were present only in the second stimulation period, cocaine ($30 \mu\text{M}$) but not hexamethonium ($300 \mu\text{M}$), significantly enhanced the fractional S-I outflow of radioactivity (Figure 2). The enhancing effect of McNeil A 343 ($10 \mu\text{M}$) on the S-I outflow of radioactivity was not altered in the presence of either cocaine or hexamethonium (Figure 2).

Table 1 Fractional stimulation-induced outflow of radioactivity in the first stimulation period (S_1) from mouse isolated atria

Drug	S_1	n
Control	0.45 ± 0.01	157
Pirenzepine ($0.2 \mu\text{M}$)	0.44 ± 0.04	12
Atropine ($0.3 \mu\text{M}$)	0.43 ± 0.02	17
Dicyclomine ($0.1 \mu\text{M}$)	0.34 ± 0.04	12
Dicyclomine ($1.0 \mu\text{M}$)	0.41 ± 0.03	10
Methoctramine ($0.1 \mu\text{M}$)	0.51 ± 0.03	14
Methoctramine ($1.0 \mu\text{M}$)	$1.09 \pm 0.09^*$	8

Mouse atria were incubated with [^3H]-noradrenaline and then continually superfused. There were two test stimulation periods, and the fractional S-I outflow of radioactivity during the first stimulation period is shown. Where they were present, drugs were added from 20 min prior to the first stimulation period. Figures are a percentage of the total tissue radioactivity. Mean $\pm$ s.e.mean values are given. *n* is the number of experiments. * represents a significant difference from control ($P < 0.05$, Dunnett's test after one-way ANOVA).

Figure 1 The effect of McNeil A 343 (McN A 343, $0.3 \mu\text{M}$ – $30 \mu\text{M}$) on the fractional stimulation-induced (S-I) outflow of radioactivity from mouse atria which had been incubated with [^3H]-noradrenaline. There were two stimulation periods (3 Hz, 60 s), and McNeil A 343 was present only for the second stimulation period. The mean fractional S-I outflow of radioactivity in the second stimulation period (S_2), expressed as a percentage of that in the first (S_1) is shown. The vertical lines represent s.e.mean, and the number of experiments is at the base of each column. * represents a significant difference from control ($P < 0.05$, Dunnett's test, after one way ANOVA).

Figure 2 The enhancing effect of McNeil A 343 (McN, 10 μ M) on the fractional stimulation-induced (S-I) outflow of radioactivity from mouse atria, and the influence of hexamethonium (C6, 300 μ M) or cocaine (Coc, 30 μ M) on the enhancing effect of McN A 343. There were two stimulation periods, and each of the drugs was present only for the second stimulation period. The mean fractional S-I outflow in the second stimulation period (S_2) expressed as a percentage of that in the first (S_1) is shown. * represents a significant difference from control ($P < 0.05$, Dunnett's test after one way ANOVA); † represents a significant difference from the appropriate McNeil A 343-free experiment ($P < 0.05$, Student's t test). When hexamethonium or cocaine were present, the effect of McNeil A 343 on the S-I outflow of radioactivity was not different from that in the absence of the drugs ($P > 0.05$, two-way ANOVA).

The effect of muscarinic receptor antagonists on the enhancement of S-I outflow of radioactivity by McNeil A 343

Pirenzepine Pirenzepine (0.2 μ M or 1.0 μ M), present only in the second stimulation period, had no significant effect on the fractional S-I outflow of radioactivity (Figure 3). However, pirenzepine (0.2 μ M or 1.0 μ M) significantly reduced the enhancing effect of McNeil A 343 on the S-I outflow of radioactivity (Figure 3). In the experiments shown in Figure 3

Figure 3 The influence of pirenzepine (0.2 μ M or 1.0 μ M) on the enhancing effect of McNeil A 343 (McN A 343, 3.0 μ M, 10 μ M or 30 μ M) on the fractional stimulation-induced (S-I) outflow of radioactivity from mouse atria which had been pre-incubated with [3 H]-noradrenaline. There were two stimulation periods, and drugs were present only for the second stimulation period. The mean fractional S-I outflow in the second stimulation period (S_2) expressed as a percentage of that in the first (S_1) is shown. On some points, the s.e.mean is not seen since the symbol size is larger than the standard error line. (○) represents experiments in the absence of pirenzepine; (□) represents experiments in the presence of pirenzepine (0.2 μ M), and (Δ) represents experiments in the presence of pirenzepine (1.0 μ M) ($n = 4-10$ for each point). Experiments in the absence of McNeil A 343 are shown on the left. The concentration-response relationship of McNeil A 343 was analysed by least squares regression. The lines for McNeil A 343 in the absence of pirenzepine and in the presence of pirenzepine (0.2 μ M or 1.0 μ M) did not differ significantly from parallelism ($P > 0.05$, two-way ANOVA), but did differ significantly in elevation ($P < 0.05$, two-way ANOVA), indicating a significant reduction in the effect of McNeil A 343 at both concentrations of pirenzepine.

pirenzepine was present for only 9 min before McNeil A 343 was introduced. Experiments were also done with pirenzepine present for 41 min (throughout) before the addition of McNeil A 343 (10 μ M). Under these conditions the facilitatory effect of McNeil A 343 (10 μ M) on the S-I outflow of radioactivity was also significantly reduced (Figure 4a). Pirenzepine (0.2 μ M) had no effect on absolute fractional S-I outflow of radioactivity in the first stimulation period (Table 1).

Atropine When atropine (0.3 μ M) was present during both stimulation periods, the enhancing effect of McNeil A 343 (10 μ M) on the fractional S-I outflow of radioactivity was significantly reduced (Figure 4a). Atropine had no effect on the absolute fractional S-I outflow of radioactivity in the first stimulation period (Table 1).

Dicyclomine Dicyclomine (0.1 μ M), present throughout the experiment, did not alter the enhancing effect of McNeil A 343 (10 μ M) on the fractional S-I outflow of radioactivity (Figure 5). In the presence of a higher concentration of dicyclomine (1.0 μ M) however, the effect of McNeil A 343 (10 μ M) was almost totally abolished (Figure 5). Dicyclomine (0.1 μ M or 1.0 μ M) had no effect on the absolute fractional S-I outflow of radioactivity in the first stimulation period (Table 1).

Methoctramine When methoctramine (0.1 μ M) was present during both stimulation periods, the enhancing effect of McNeil A 343 (10 μ M) on the fractional S-I outflow of radioactivity was not attenuated (Figure 4a). In the presence of a higher concentration of methoctramine (1.0 μ M) however, the

Figure 4 The effect of pirenzepine (Pir, 0.2 μ M), atropine (Atr, 0.3 μ M) and methoctramine (Meth, 0.1 μ M) on the modulation of the fractional stimulation-induced (S-I) outflow of radioactivity by McNeil A 343 (McN, 10 μ M) (a), or by carbachol (CCh, 3.0 μ M) (b). Mouse atria were pre-incubated with [3 H]-noradrenaline and stimulated twice. McNeil A 343 or carbachol were present only during the second stimulation period. Other drugs were present during both stimulation periods. The mean fractional S-I outflow in the second stimulation period (S_2) expressed as a percentage of that in the first (S_1) is shown. * represents a significant difference from the respective control ($P < 0.05$, Student's t test); † represents a significantly reduced effect of McNeil A 343 or carbachol in the presence of the antagonist compared to the effect of McNeil A 343 or carbachol alone ($P < 0.05$, two-way ANOVA).

Figure 5 The effect of McNeil A 343 (McN, 10 µM) in the presence of dicyclomine (DCM, 0.1 µM or 1.0 µM), on the fractional stimulation-induced (S-I) outflow of radioactivity from mouse atria which had been preincubated with [³H]-noradrenaline. The atria were stimulated twice, and McN A 343 was present only for the second stimulation period. Dicyclomine was present during both stimulation periods. The mean fractional S-I outflow in the second stimulation period (S₂) expressed as a percentage of that in the first (S₁) is shown. * represents a significant difference from the appropriate control ($P < 0.05$, Student's *t* test). † represents a significantly reduced effect of McNeil A 343 in the presence of dicyclomine compared to the effect of McN A 343 alone ($P < 0.05$, two-way ANOVA).

enhancing effect of McNeil A 343 (10 µM) was totally abolished. In this case the fractional S-I outflow of radioactivity in the second stimulation period as a percentage of that in the first was $92.6 \pm 5.3\%$ ($n = 4$) for methoctramine (1.0 µM) throughout, and $103.9 \pm 3.4\%$ ($n = 4$) for McNeil A 343 (10 µM) in the presence of methoctramine (1.0 µM) (compare with Figure 1). The absolute fractional S-I outflow of radioactivity in the first stimulation period was significantly increased when methoctramine (1.0 µM, but not 0.1 µM) was present throughout the experiment (Table 1).

The effect of muscarinic receptor antagonists on the inhibition of S-I outflow of radioactivity by carbachol

Carbachol (3.0 µM), present only in the second stimulation period, significantly reduced the fractional S-I outflow of radioactivity (Figure 4b). When atropine (0.3 µM) was present throughout the experiment, the inhibitory effect of carbachol was abolished (Figure 4b). Pirenzepine (0.2 µM), present throughout the experiment, did not alter the inhibitory effect of carbachol on the S-I outflow of radioactivity (Figure 4b). At the higher concentration, pirenzepine (1.0 µM), blocked the inhibitory effect of carbachol. In this case the fractional S-I outflow of radioactivity in the second stimulation period as a percentage of that in the first was $92.6 \pm 3.0\%$ ($n = 4$) for pirenzepine (1.0 µM) alone, and $85.6 \pm 6.7\%$ ($n = 4$) for carbachol in the presence of pirenzepine (compare with Figure 4b). When methoctramine (0.1 µM) was present throughout the experiment, the inhibitory effect of carbachol (3.0 µM) on the S-I outflow of radioactivity was totally abolished (Figure 4b).

The effect of muscarinic receptor antagonists on the S-I outflow of radioactivity

Atropine (0.3 µM), present only in the second stimulation period, significantly increased the S-I outflow of radioactivity. The fractional S-I outflow of radioactivity in the second stimulation period as a percentage of that in the first was $89.6 \pm 3.5\%$ ($n = 8$) for control, and $133.5 \pm 12.6\%$ ($n = 8$) for atropine.

Pirenzepine (0.03 µM–1.0 µM) did not alter the S-I outflow of radioactivity when it was present only in the second stimulation period (Figure 6). At the higher concentration of pirenzepine (10 µM), there was a significant increase in the S-I outflow of radioactivity (Figure 6). When the interval between the two stimulation periods was increased to 69 min to allow a longer contact time with the antagonist, pirenzepine (0.2 µM) present from 57 min before the second stimulation period, did

Figure 6 The effect of pirenzepine (Pir, 0.03 µM–10 µM) on the fractional stimulation-induced (S-I) outflow of radioactivity from mouse atria which had been pre-incubated with [³H]-noradrenaline and stimulated twice. Pirenzepine was present only for the second stimulation period. The mean fractional S-I outflow in the second stimulation period (S₂) as a percentage of that in the first (S₁) is shown. * represents a significant difference from control ($P < 0.05$, Dunnett's test after one way ANOVA).

not alter the S-I outflow of radioactivity. The fractional S-I outflow of radioactivity in the second stimulation period as a percentage of that in the first was $79.0 \pm 2.1\%$ for control and $81.7 \pm 2.1\%$ for pirenzepine ($n = 5$ in both cases).

When methoctramine (0.1 µM) was present in the second stimulation period, there was a significant increase in the S-I outflow of radioactivity. The fractional S-I outflow of radioactivity in the second stimulation period as a percentage of that in the first was $89.6 \pm 3.5\%$ ($n = 8$) for control, and $143.7 \pm 7.1\%$ ($n = 5$) for methoctramine.

The effect of McNeil A 343 on the spontaneous outflow of radioactivity from mouse atria

McNeil A 343 (10 µM or 30 µM), either by itself or in the presence of other drugs, inhibited the spontaneous outflow of radioactive compounds by about 10% from mouse isolated atria which had been incubated with [³H]-noradrenaline (not shown). In the presence of cocaine (30 µM), which by itself increased the spontaneous outflow of radioactive compounds by about 15%, McNeil A 343 did not affect the spontaneous outflow of radioactive compounds (not shown). None of the other drugs used in the present study altered the spontaneous outflow of radioactivity from mouse atria (not shown).

Discussion

Stimulation-induced (S-I) outflow of radioactivity from tissues which had been previously incubated with [³H]-noradrenaline has been determined to be an appropriate assessment of transmitter noradrenaline release (see Langer, 1974). In the present study in mouse atria which had been preincubated with [³H]-noradrenaline, the M₁-selective muscarinic receptor agonist McNeil A 343 (see Mitchelson, 1988; Birdsall *et al.*, 1988), enhanced S-I release of noradrenaline in a concentration-dependent manner. Facilitation of noradrenaline release by McNeil A 343 has been known for some time (see Introduction), however an adequate explanation for this effect is lacking. In mouse atria the enhancing effect of McNeil A 343 on the S-I release of noradrenaline was not prevented by the antinicotinic drug hexamethonium, thus it is unlikely that the effect was due to activating facilitatory nicotinic receptors. This is in accord with previous reports that McNeil A 343 has little or no activity at nicotinic receptors (Roszkowski, 1961; Nedergaard, 1980).

Previous studies have suggested that McNeil A 343 enhances noradrenaline release to some extent by blocking

neuronal reuptake of noradrenaline (Fozard & Muscholl, 1974; Allen *et al.*, 1972). In mouse atria, this mechanism probably does not contribute to a substantial proportion of the enhancing effect of McNeil A 343, since previously blocking neuronal uptake with a high concentration of cocaine (30 μM) did not alter the ability of McNeil A 343 to enhance noradrenaline release. Furthermore, McNeil A 343 (10 μM) increased noradrenaline release by almost twice as much as cocaine (30 μM) itself. It has been reported (Nedergaard, 1980) that McNeil A 343 is a much weaker neuronal uptake blocker than cocaine, therefore it is possible that blockade of neuronal uptake is only apparent at higher concentrations of McNeil A 343 (100 μM used in the study of Allen *et al.*, 1972; 20 μM used in the study of Fozard & Muscholl, 1974).

The possibility that McNeil A 343 enhances S-I noradrenaline release by a receptor-mediated process has been dismissed previously because the effect was found to be resistant to blockade by atropine (Fozard & Muscholl, 1974; Allen *et al.*, 1974; Nedergaard, 1980; 1981; Arbilla *et al.*, 1986). However in the present study in mouse atria, atropine markedly reduced the enhancing effect of McNeil A 343 on noradrenaline release. A similar finding in guinea-pig atria has also been reported by Vizi *et al.* (1989). It is possible that the effect of McNeil A 343 was resistant to blockade by atropine in previous studies because very high concentrations of McNeil A 343 were used and non-receptor-mediated actions may have contributed to the effect of McNeil A 343 on noradrenaline release.

In the present study in addition to atropine, the relatively M_1 -selective antagonists pirenzepine (Hammer & Giachetti, 1982), and dicyclomine (Giachetti *et al.*, 1986; Giachetti & Micheletti, 1988), and a high concentration of the relatively M_2 -selective antagonist methoctramine (Melchiorre *et al.*, 1987; Wess *et al.*, 1988), were also able to reduce the enhancing effect of McNeil A 343 on noradrenaline release. This further supports the proposal that the facilitatory effect of McNeil A 343 is by a receptor-mediated process.

The question arises as to why McNeil A 343 enhances noradrenaline release yet other muscarinic receptor agonists such as carbachol inhibit release of the transmitter. A likely explanation is that muscarinic receptors through which McNeil A 343 acts to facilitate noradrenaline release are different from inhibitory prejunctional muscarinic receptors. In the present study we were able to differentiate the effect of McNeil A 343 and that of carbachol using selective muscarinic receptor antagonists. Firstly 0.2 μM pirenzepine, a relatively M_1 -selective muscarinic receptor antagonist (Hammer & Giachetti, 1982; see also Egle & Whiting, 1986), reduced the facilitatory effect of McNeil A 343 without affecting the inhibitory action of carbachol on the S-I noradrenaline release. A higher concentration of pirenzepine (1.0 μM) however, blocked the effects of both drugs. An opposite order of antagonism was found with methoctramine, a relatively M_2 -selective muscarinic receptor antagonist (Melchiorre *et al.*, 1987; Wess *et al.*, 1988). At a low concentration methoctramine (0.1 μM) blocked the inhibitory effect of carbachol but did not reduce the facilitatory effect of McNeil A 343. However, a higher concentration of methoctramine (1.0 μM), blocked the effect of carbachol as well as that of McNeil A 343. Together these results suggest that facilitatory muscarinic receptors resemble the M_1

subtype. It should be noted that the concentrations of pirenzepine used to block the effects of McNeil A 343 were higher than its reported pA_2 values at M_1 receptors (about 8–8.4, see Mitchelson, 1988). Therefore, some caution must be used when assigning a definite muscarinic receptor subtype in spite of the obvious difference between the order of antagonist effectiveness for inhibitory and facilitatory muscarinic mechanisms.

It is widely accepted that acetylcholine released from parasympathetic nerves can activate inhibitory prejunctional muscarinic receptors on sympathetic nerve endings (see Muscholl, 1980). In mouse atria, it is likely that endogenous acetylcholine modulates noradrenaline release through inhibitory prejunctional muscarinic receptors since atropine was found to enhance noradrenaline release. Methoctramine, as well as a high concentration of pirenzepine (10 μM), also enhanced noradrenaline release which further indicates that in this field-stimulated preparation, inhibitory prejunctional muscarinic receptors were tonically activated by endogenous acetylcholine. Facilitatory prejunctional muscarinic receptors were probably not tonically activated since, as would have been expected, lower concentrations of pirenzepine (0.03 μM –1.0 μM) did not inhibit noradrenaline release. This lack of effect was probably not due to insufficient equilibration with the antagonist, since an even longer contact time with pirenzepine also did not reduce noradrenaline release.

In a recent study on a nerve-stimulated rabbit atria preparation (Habermeier-Muth & Muscholl, 1988), it was found that pirenzepine inhibited noradrenaline release under certain conditions of dual parasympathetic-sympathetic nerve stimulation, suggesting that endogenous acetylcholine may activate facilitatory prejunctional M_1 receptors (Muscholl *et al.*, 1989; Altes *et al.*, 1990). However in these studies it was found that the timing of the vagal nerve stimulation in relation to the sympathetic nerve stimulation was crucial (Muscholl *et al.*, 1989; Altes *et al.*, 1990). Pirenzepine did not affect noradrenaline release if the vagal pulses preceded the sympathetic nerve pulses by a short interval (3 ms) or a long interval (233 ms). However if the pulses arrived at an interval of 100 ms pirenzepine inhibited noradrenaline release (Muscholl *et al.*, 1989; Altes *et al.*, 1990). The reasons for these temporal effects are unclear. However, the lack of effect of pirenzepine on S-I noradrenaline release in the present study may be because with field stimulation there is concomitant parasympathetic and sympathetic nerve activation.

In conclusion, postganglionic sympathetic nerve terminals are endowed with prejunctional facilitatory muscarinic receptors in addition to typically observed inhibitory muscarinic receptors. The selectivity of pirenzepine and methoctramine for each of these receptors suggests that facilitatory prejunctional muscarinic receptors resemble the M_1 subtype, and inhibitory prejunctional muscarinic receptors resemble the M_2 subtype. While inhibitory prejunctional muscarinic receptors are probably activated by endogenous acetylcholine, this does not appear to be the case for facilitatory prejunctional muscarinic receptors in field-stimulated mouse atria.

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