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Cholinesterase activity and exposure time to acetylcholine as factors influencing the muscarinic inhibition of [^{3}H]-noradrenaline overflow from guinea-pig isolated atria

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1 Guinea-pig isolated atria were incubated and loaded with [^{3}H]-noradrenaline. The release of ^{3}H and of [^{3}H]-noradrenaline was induced by field stimulation (6–9 trains of 150 pulses at 5 Hz). The stimulation-evoked overflows of ^{3}H and of [^{3}H]-noradrenaline were determined.

2 In the absence of an inhibitor of acetylcholinesterase, acetylcholine (12 min preincubation before nerve stimulation, up to 10 μM) failed to inhibit the evoked [^{3}H]-noradrenaline overflow. In the presence of atropine, an increase by acetylcholine of evoked release was observed in the same atria. In contrast, the selective muscarinic agonist methacholine significantly decreased the evoked overflow. The inhibition was antagonized by atropine. Methacholine did not enhance release in the presence of atropine.

3 When present for only 2 min, acetylcholine 10 μM inhibited the evoked overflow and no facilitation of release was observed in the presence of atropine.

4 In the presence of physostigmine, acetylcholine (12 min preincubation, 1 and 10 μM) inhibited evoked [^{3}H]-noradrenaline overflow, but the overflow was increased by acetylcholine 10 μM in the presence of atropine.

5 In the presence of cocaine, corticosterone, phentolamine, propranolol and hexamethonium together, acetylcholine 1 μM inhibited the evoked [^{3}H]-noradrenaline overflow. The inhibition was significantly enhanced in the presence of physostigmine. It decreased with preincubation time of the agonist, despite the presence of physostigmine and constant replacement by new drug. Neither inhibition nor facilitation of evoked release was observed in the presence of atropine.

6 It is concluded that a muscarinic inhibition by acetylcholine (upon prolonged exposure time) may be masked by a concomitant facilitation of release and/or desensitization of the muscarinic inhibitory mechanism. Furthermore, degradation by acetylcholinesterase contributes in part to the ineffectiveness of acetylcholine as a presynaptic inhibitor. When a distortion of the overflow/release ratio was excluded, adrenergic and nicotinic effects were prevented, and acetylcholinesterase was inhibited, the fading of muscarinic inhibition by acetylcholine may have been exclusively due to a slow and moderate desensitization of the presynaptic muscarinic mechanism.

Introduction

Postganglionic adrenergic nerves of the peripheral autonomic nervous system are endowed with muscarinic receptors (Lindmar *et al.*, 1968; for review see Muscholl, 1980) which when activated by selective muscarinic agonists decrease the exocytotic release of noradrenaline. The occurrence of presynaptic muscarinic receptors appears to be a general rule as noradrenaline release from virtually all peripheral tissues investigated could be inhibited by muscarinic agonists (Starke, 1977; Westfall, 1977). However, one finding might be interpreted in favour of an exception to this general rule: the evoked release of ^{3}H from isolated guinea-pig atria (previously incubated with [^{3}H]-noradrenaline) was not decreased by acetylcholine in concentrations up to 10 μM (Story *et al.*, 1975). One (among others) possible explanation for such finding could be a lack of presynaptic muscarinic receptors and such a model would then represent an interesting tool potentially useful in the investigation

of muscarinic mechanisms. Results obtained with the whole perfused guinea-pig heart in which a presynaptic muscarinic inhibition was observed (Westfall & Hunter, 1974; Langley & Gardier, 1977) rendered the lack of muscarinic receptors in atria an unlikely cause for the lack of inhibition by acetylcholine. Thus, the reason for the apparent discrepancy between findings with acetylcholine in atria vs. whole heart remained obscure.

The aim of the present study was to confirm the results of Story *et al.* (1975), and if we could do this, to explain why acetylcholine failed to depress the release of [^{3}H]-noradrenaline.

A preliminary account of some of this work was given at the Joint Meeting of the French and German Pharmacological and Toxicological Societies in Freiburg (F.R.G.) (Fuder & Wolf, 1983).

Methods

Preparation of guinea-pig atria

Guinea-pigs weighing 300–400 g were exsanguinated after stunning and the hearts rapidly removed. The atria were dissected free and suspended vertically between two parallel platinum wire electrodes (4 mm apart) in an organ bath containing 5 ml of Tyrode solution of the following composition (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.05, $NaHCO_3$ 11.9, NaH_2PO_4 0.42, D-glucose 5.6, and (+)-ascorbic acid 0.057. The solutions in the organ bath and in the reservoirs supplying the organ bath were gassed with 5% CO_2 in O_2 and maintained at a temperature of 37°C. The spontaneously beating atria received an initial preload of 1 g.

After an equilibration time of 15 min (incubation fluid changed every 3 min), the atria were incubated with Tyrode solution containing 5 μCi (−)-[7,8-^{3}H]-noradrenaline (3.0 or 14.8 Ci mmol^{-1}; 0.33 or 0.06 μM, respectively) for 30 min. The end of the incubation with radioactivity is referred to as 'time 0'. Subsequently, the incubation medium was replaced with fresh solution at 5 to 10 min intervals for 75 min before the solution samples were counted for radioactivity.

Determination of stimulation-evoked [^{3}H]-noradrenaline overflow and experimental design

The incubation medium (5 ml) was replaced at 5 min intervals and collected in 10 min samples for 170 or 220 min (starting 75 min after 'time 0'). The intramural nerves of the atria were stimulated by 6 or 9 trains of field pulses (S1–S6 or S0–S8). Each train (30 min intervals between the start of subsequent trains) consisted of 150 monophasic square wave pulses of 1 ms duration at a frequency of 5 Hz. Different experimental designs were chosen. In procedure I, 6 trains of field stimulation were carried out, the first train (S1) starting 87 min after 'time 0'. Drugs to be investigated under these conditions were introduced into the Tyrode solution at various time points preceding S2–S6 and were present during respective trains as specified below. In procedure II, the following drugs were present in the incubation medium from 55 min after 'time 0' until the end of an experiment (concentration in μM): cocaine 30, corticosterone 50, hexamethonium 10, phentolamine 1 and propranolol 1.Moreover, 9 stimulation trains were applied. A first train (S0; 66 min after 'time 0') served as a conditioning stimulation period and release evoked by S0 was not determined. S1 started 86 min after 'time 0'.

The samples containing overflow of radioactivity from atria were collected into vials containing 1 M HCl (to adjust pH to 2–3) and 1 mg ascorbic acid. After removing 1 ml of the fluid to determine the total tritium content, the [^{3}H]-noradrenaline content of the samples was determined (Graefe *et al.*, 1973; Fuder *et al.*, 1982a). Noradrenaline values were corrected for minor cross contaminations (Fuder *et al.*, 1982a). The sample content of ^{3}H and of [^{3}H]-noradrenaline was expressed as fmol per 10 min referring to the specific activity of [^{3}H]-noradrenaline used for loading. Stimulation-evoked ^{3}H or [^{3}H]-noradrenaline overflow was determined by subtracting the mean overflows from 10 min samples collected before and after field stimulation from the overflow observed in the 10 min sample during which nerve stimulation was carried out.

A third type of experiment was set up after experiments concerned with presynaptic muscarinic inhibition in guinea-pig atria had been completed. We were startled by a recent paper of Angus *et al.* (1984) in which a stimulation-evoked efflux of tritium from an organ bath in the absence of biological tissue after incubation with [^{3}H]-noradrenaline was observed. [^{3}H]-noradrenaline was therefore dissolved in Tyrode solution and incubated in the organ bath containing tissue holder and electrodes, but no atria. Field stimulations (150 pulses) were carried out at various times (Figure 1) and ^{3}H and [^{3}H]-noradrenaline were determined in the bath fluid in 5 min samples. In 4 experiments, field stimulation increased total tritium overflow by a factor of 2.1 ± 0.37 (at FS1) or 1.8 ± 0.37 (at FS3), respectively, but no increase in [^{3}H]-noradrenaline overflow was observed (Figure 1). The evoked ^{3}H overflow in the absence of atria was also observed in the presence of tetrodotoxin 3 μM ($n = 2$, not shown). These results emphasize that, under our conditions, [^{3}H]-noradrenaline overflow, but not total tritium overflow, may serve as a reliable parameter for presynaptic adrenergic mechanisms.

In each experiment, the [^{3}H]-noradrenaline overflow (fmol per 150 pulses) evoked by S2 to S8 was

expressed as a percentage of that evoked by S1. In a few experiments atrial [^{3}H]-noradrenaline content was determined (Fuder & Jung, 1985) and the fractional rate of [^{3}H]-noradrenaline overflow per pulse calculated. Since the percentages for absolute evoked overflow values and for fractional rate of release were almost identical, the simpler calculation procedure was chosen for the evaluation of drug effects.

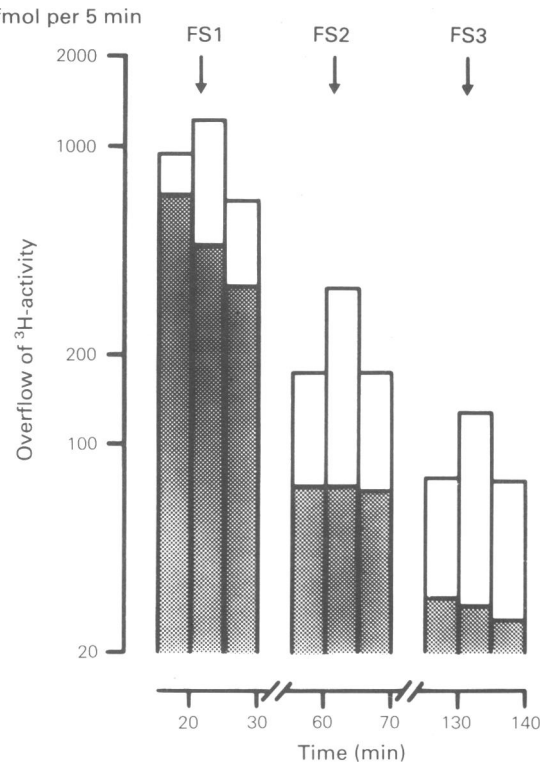

Figure 1 Representative examples of the effect of field stimulation on the efflux of total ^{3}H (stippled plus open column) and of [^{3}H]-noradrenaline (stippled column) from the organ bath previously filled with [^{3}H]-noradrenaline (5 μCi). The bath contained Tyrode solution (5 ml), tissue holder, and electrodes, but no biological tissue. Individual efflux values from one out of 4 similar experiments are expressed as fmol per 5 min (referring to the specific activity of [^{3}H]-noradrenaline used for preincubation, plotted on the logarithmic ordinate scale). Time (min) after the end of preincubation with radioactivity is shown on the abscissa scale. When indicated by arrows, 150 field pulses (5 Hz, 1 ms, 65 mA current strength; FS1–FS3) were delivered. Treatment of the bath surface with dichlorodimethyl silane failed to prevent the current-induced increase in tritium efflux. Surface-bound ^{3}H could be removed by acid washing.

Statistics and drugs

Results are given as the mean ± s.e.mean, and statistical significance was examined by use of Student's *t* test and, if more than one group of treatments was compared with one control group, by use of a modified *t* test according to Bonferroni (Wallenstein *et al.*, 1980).

The following drugs were used: acetylcholine chloride, (−)-cocaine hydrochloride, physostigmine salicylate (Merck, Darmstadt); atropine sulphate (Boehringer, Ingelheim); corticosterone, (±)-methacholine chloride, tetrodotoxin (Sigma, St. Louis); hexamethonium diiodide (Casstella, Frankfurt); phentolamine mesylate (Ciba Geigy, Wehr); propranolol hydrochloride (ICI, Plankstadt). Except for corticosterone, which was dissolved in propylene glycol, drugs were dissolved in distilled water. (−)-[7,8-^{3}H]-noradrenaline (3.0 or 14.8 Ci mmol^{-1}; NEN, Dreieich) was diluted in 0.01 M HCl.

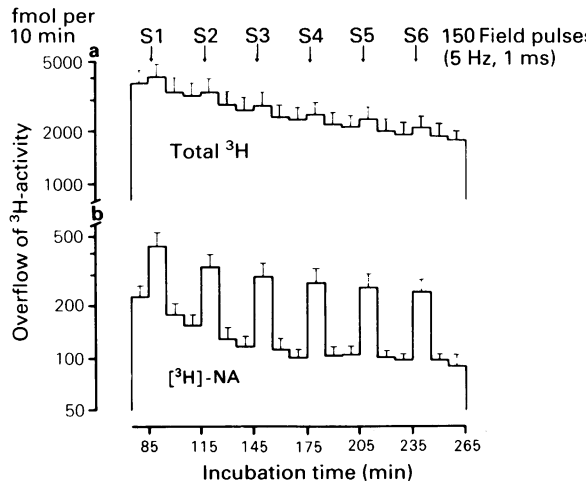

Figure 2 The effects of field stimulation on the overflow of total ^{3}H (a) and [^{3}H]-noradrenaline (NA; b) from 7 guinea-pig isolated atria incubated in Tyrode solution. Values are means with s.e.mean indicated by vertical line. Time (min) passed after the end of labelling is shown on the abscissa scale. When indicated by arrows, a series of 150 field pulses (5 Hz, 1 ms, 50 mA current strength; S1–S6) was delivered. Evoked [^{3}H]-noradrenaline overflow accounted for only 50–62% of the evoked total ^{3}H overflow. Under these conditions, the fractional rate of evoked [^{3}H]-noradrenaline overflow per pulse was $1-3 \times 10^{-5}$ ($n = 3$).

Results

Basal and evoked [^{3}H]-noradrenaline overflow

The basal [^{3}H]-noradrenaline overflow (Figure 2) was unaffected in the presence of muscarinic agonists, and/or physostigmine, and/or atropine (see legends of Figures 3–5). When the atria were incubated with Tyrode solution, the stimulation-evoked (50 mA, current strength) increase in [^{3}H]-noradrenaline overflow was decreased by tetrodotoxin 0.8 μM (reduction by 97 and 100%, $n = 2$) and 3 μM (reduction by 100 ± 0%, $n = 3$). In contrast, tetrodotoxin 3 μM reduced the evoked total tritium overflow by only 57.6 ± 7.5% ($n = 3$). Apparently field stimulation induced a comparatively large tetrodotoxin-insensitive increase in tritium compounds (other than [^{3}H]-noradrenaline) of probably non-neuronal origin that may in part be explained by current-induced efflux from bath surfaces, tissue holder, or electrodes (Figure 1).

Compared to experiments in which atria were immersed in Tyrode solution, the evoked [^{3}H]-noradrenaline overflow was 2–3.4 times higher in the presence of Tyrode solution containing cocaine, corticosterone, hexamethonium, phentolamine, and propranolol. The combination of drugs was chosen in order to block neuronal and extraneuronal uptake of released noradrenaline, to prevent a nicotinic component of acetylcholine action, and to eliminate the influence of well known presynaptic modulatory mechanisms that could vary with the biophase concentration of noradrenaline upon repeated stimulation.

Effects of muscarinic agonists

When added 12 min before nerve stimulation acetylcholine (0.1–10 μM) failed to affect the evoked [^{3}H]-noradrenaline overflow in the absence of atropine (Figure 3). This finding is in line with results from guinea-pig atria obtained previously with evoked tritium overflow (Story *et al.*, 1975). However, acetylcholine 10 μM enhanced the evoked [^{3}H]-noradrenaline overflow in the presence of atropine 5 μM (Figure 3). Hence, among other factors, a non-muscarinic

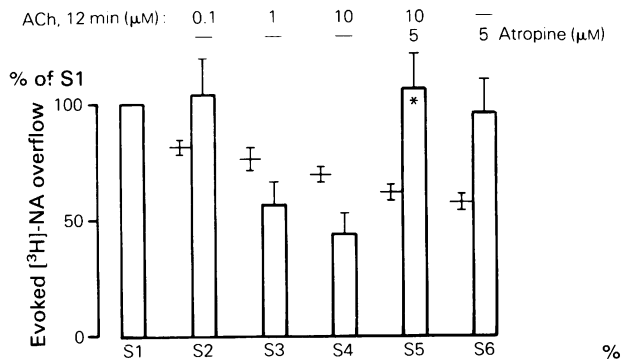

Figure 3 The effects of acetylcholine (ACh) in the absence and presence of atropine 5 μM on the stimulation-evoked [^{3}H]-noradrenaline (NA) overflow from 7 guinea-pig atria. Columns are means of evoked overflow expressed as a percentage of the overflow evoked by the first train of 150 pulses (S1; 5 Hz, 1 ms). ACh (0.1, 1, or 10 μM was introduced into the incubation medium 12 min before the respective stimulation period (S2, S3, S4, S5). ACh was present for 20 min and replaced 3 times by fresh solution at 5 min intervals. Before S5, atropine was added to the incubation medium and present during S5 (together with ACh 10 μM) and S6 (no ACh present). Before S1 or S5 the basal [^{3}H]-NA overflow was 129 ± 44 or 54 ± 11 fmol per 10 min, respectively. It was not changed by the presence of ACh and/or atropine. S1 increased the overflow of [^{3}H]-NA by 111 ± 19 fmol. The mean evoked control [^{3}H]-NA overflow observed in the absence of drugs (values of Figure 2, $n = 7$) is shown by a horizontal bar on the left hand side of the respective column. Note that ACh up to 10 μM failed to inhibit the evoked overflow significantly. In the presence of atropine and ACh 10 μM, the evoked [^{3}H]-NA overflow was significantly enhanced (*$P < 0.05$; modified t test according to Bonferroni for comparison of 3–4 different groups of treatment with one control group; Wallenstein *et al.*, 1980). Vertical lines represent s.e.mean.

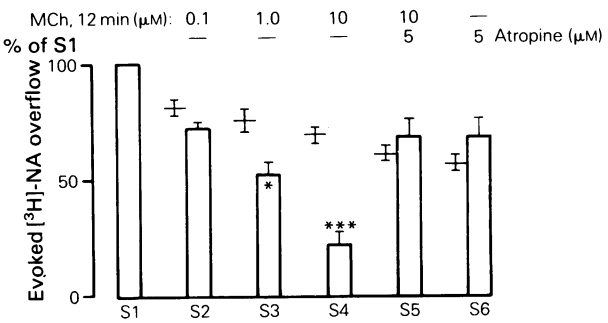

Figure 4 The effect of methacholine (MCh) in the absence and presence of atropine 5 μM on the stimulation-evoked [^{3}H]-noradrenaline (NA) overflow from guinea-pig atria. Columns are means of 5 observations with s.e.mean shown as vertical lines. For explanation of other symbols and of experimental design see Figure 3 (except that ACh was replaced by MCh). Before S1 or S5, the basal [^{3}H]-NA overflow was 120 ± 27 or 58 ± 9.3 fmol per 10 min, respectively. Neither MCh (0.1, 1, or 10 μM) nor atropine 5 μM or the combination of atropine plus MCh 10 μM affected the basal overflow. S1 increased the [^{3}H]-NA overflow by 170 ± 26 fmol. MCh 1 (*$P < 0.05$) and 10 μM (***$P < 0.001$, modified t test according to Bonferroni) significantly inhibited the evoked overflow. MCh 10 μM was ineffective in the presence of atropine.

facilitatory effect of acetylcholine may have functionally counteracted an inhibitory effect mediated by presynaptic muscarinic receptors.

In contrast, the selective muscarinic agonist methacholine (1 and 10 μM, 12 min preincubation time) inhibited the evoked [^{3}H]-noradrenaline overflow significantly, and the inhibition was not observed in the presence of atropine (Figure 4). Neither atropine (Figures 4 and 6) nor methacholine 10 μM plus atropine modulated the evoked overflow. The findings with methacholine suggest that functionally active presynaptic muscarinic receptors are present at adrenergic nerves of guinea-pig atria and that the lack of inhibition by acetylcholine is certainly not due to a lack of muscarinic receptors. Furthermore, the increase in evoked overflow observed in the presence of acetylcholine plus atropine cannot be due to effects of atropine unrelated to blockade of muscarinic receptors. Otherwise an increase by atropine should also have been visible in the presence or absence of methacholine.

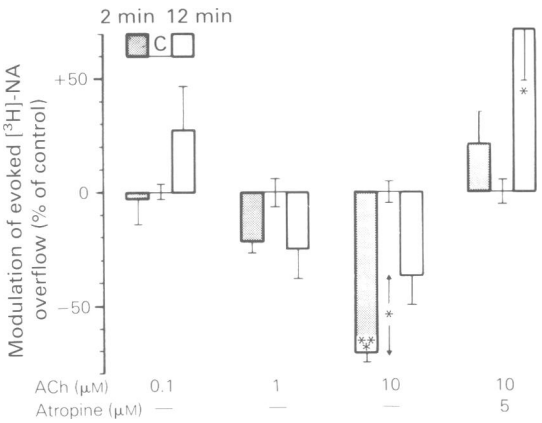

Figure 5 The effect of preincubation time with acetylcholine (ACh) before nerve stimulation on the modulation of evoked [^{3}H]-noradrenaline (NA) overflow from guinea-pig atria by ACh in the absence and presence of atropine 5 μM. Open columns represent values from Figure 3 transformed into % changes compared to controls. Atria were exposed to ACh starting 12 min before nerve stimulation. Normalized control values are shown by horizontal bars. Filled columns represent the modulation of evoked [^{3}H]-NA overflow observed in the presence of ACh introduced 2 min before nerve stimulation and maintained for 10 min. In this series of 7 experiments (6–7 observations) the basal [^{3}H]-NA overflow was 189 ± 62 or 50 ± 10 fmol per 10 min before S1 or S5, respectively. S1 increased the overflow by 135 ± 19 fmol. With 2 min preincubation time, ACh 10 μM inhibited the evoked [^{3}H]-NA overflow significantly (***$P < 0.001$, Bonferroni test), but not with 12 min (significant difference between the groups, *$P < 0.05$, unpaired two-tailed t test). In the presence of atropine (added from 22 min before S5 until after S6) ACh failed to modulate the evoked [^{3}H]-NA overflow after short, but significantly enhanced it after prolonged exposure time (see also Figure 3). All values are means and vertical lines represent s.e.mean.

Effects of acetylcholine preincubation time and of acetylcholinesterase inhibition

Surprisingly, acetylcholine (10 μM) inhibited the evoked [^{3}H]-noradrenaline overflow with a short (2 min) preincubation time, in contrast to results with 12 min preincubation (Figure 5). Moreover, acetylcholine 10 μM (2 min) failed to facilitate the evoked overflow in the presence of atropine (Figure 5), but atropine antagonized the inhibition. This result corroborates the view that upon prolonged (but not upon short) exposure time a facilitatory effect of acetylcholine opposes a muscarinic inhibition. It appears unlikely that the facilitation is nicotinic in origin, since nicotinic effects are prominent after short contact time and fade rapidly (Löffelholz, 1970a,b).

Since the inhibition was observed only in the presence of a high acetylcholine concentration, we investigated the influence of acetylcholinesterase inhibition on the acetylcholine effects. Physostigmine 1 μM either present alone or in combination with atropine failed to affect the evoked [^{3}H]-noradrenaline overflow. However, in the presence of physostigmine, a 12 min preincubation with acetylcholine 1 μM inhibited the evoked [^{3}H]-noradrenaline overflow to about the same extent as did acetylcholine 10 μM. Assuming that the concentration of physostigmine was sufficient to block acetylcholinesterase by more than 80% (Kilbinger & Wessler, 1980; Muscholl & Muth, 1982), it has to be concluded that the low maximum inhibition by acetylcholine compared to conditions described in Figure 7 cannot be due to degradation by acetylcholinesterase. Acetylcholine 10 μM increased the evoked [^{3}H]-noradrenaline overflow significantly in the presence of atropine plus physostigmine (Figure 6) thus confirming the results in the absence of physostigmine (Figure 5). It appears likely that the facilitation that occurred independently of the presence of physostigmine prevented the demonstration of a higher maximum of the muscarinic inhibition.

In order to find out whether effects other than breakdown of acetylcholine, functional antagonism by facilitation or distortion of the overflow/release ratio complicate the demonstration of muscarinic inhibition by acetylcholine, the experimental design was modified (Figure 7). In the presence of cocaine, corticosterone, hexamethonium, phentolamine, and propranolol, acetylcholine 1 μM inhibited the evoked overflow after 1 and 31 min preincubation time

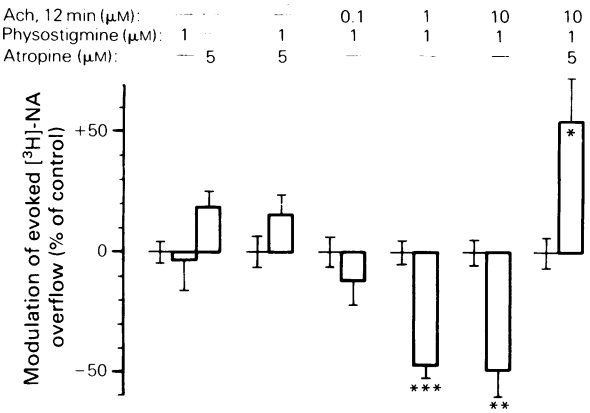

Figure 6 The modulation by acetylcholine (ACh) of the evoked [^{3}H]-noradrenaline (NA) overflow in the absence or presence of atropine and in the presence of physostigmine 1 μM. The preincubation time with ACh before nerve stimulation was 12 min. Columns are means of 5–7 observations. Normalized control values for the respective stimulation period in which drug effects were assessed are shown as horizontal bars on the left hand side of the columns. Neither physostigmine nor atropine, nor the combination of both affected the basal or evoked [^{3}H]-NA overflow significantly when added to the incubation medium 12 or 22 min, respectively, prior to S2. ACh 1 μM (***$P < 0.001$) and 10 μM (**$P < 0.01$; Bonferroni tests) decreased the evoked [^{3}H]-NA overflow significantly. In the presence of atropine the evoked overflow was significantly (*$P < 0.05$, Bonferroni test) enhanced, similar to results obtained in the absence of physostigmine after 12 min preincubation time for ACh. Vertical lines represent s.e.mean.

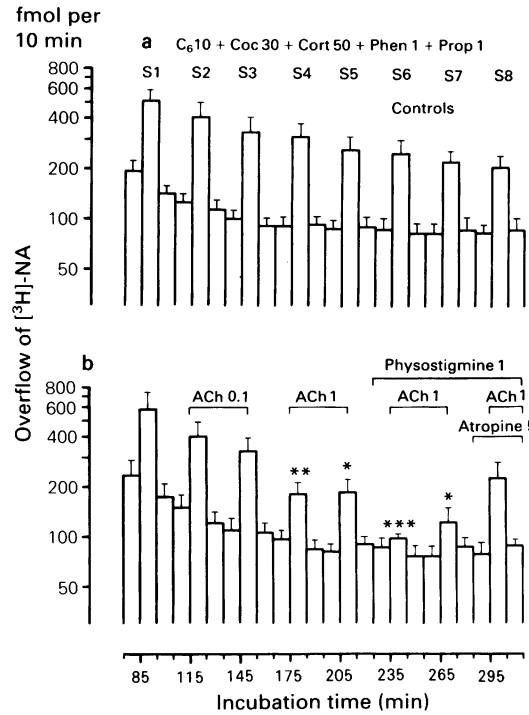

Figure 7 The effects of 8 trains of field stimulation (150 pulses, 5 Hz, 1 ms, 65 mA current strength; S1–S8) on the overflow of [^{3}H]-noradrenaline from guinea-pig atria in the presence of hexamethonium (C_6, 10 μM) + cocaine (Coc, 30 μM) + corticosterone (Cort, 50μM) + phentolamine (Phen, 1 μM) + propranolol (Prop, 1 μM). The drugs were introduced into the Tyrode solution 31 min prior to S1 and maintained until the end of an experiment. Numbers in connection with abbreviations of drug names give μM concentrations. Incubation time (abscissa scale, min) '0' corresponds to the end of the labelling procedure. Columns are means with s.e.mean as vertical lines from 5–6 observations. (a) Shows the pattern of [^{3}H]-NA overflow in the absence of other drugs (controls). In (b) the overflow is shown in the presence of acetylcholine ACh) and/or atropine 5 μM. ACh was introduced into the incubation medium 1 min prior to S2 (0.1 μM), S4 (1 μM), S6 (1 μM, physostigmine 1 μM present) and S8 (1 μM, physostigmine plus atropine 5 μM present; both drugs, 11 min preincubation time) and maintained for 35 min (i.e., 31 min, ACh preincubation time before S3, S5, S7). Asterisks within columns denote statistical differences (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; t test) of evoked [^{3}H]-NA overflow compared to controls (evoked overflow expressed as a percentage of that observed at S1). Note that the basal overflow was unaffected by ACh, physostigmine or atropine. After correction for recovery, the stimulation-evoked increase in [^{3}H]-NA overflow upon S1 represented 89 (a) or 88 (b) % of the stimulation-evoked increase in total ^{3}H overflow.

(Figure 8). A tendency towards a time-dependent loss of inhibition was observed. In the presence of physostigmine, the inhibition by acetylcholine after 1 min preincubation was significantly higher than in the absence of the acetylcholinesterase blocker (Figure 8). Despite the presence of physostigmine, the acetylcholine effect decreased significantly with time (Figure 8). The evoked [^{3}H]-noradrenaline overflow was unaffected (and clearly not enhanced) after 1 or 31 min preincubation time with acetylcholine plus atropine. Thus, under these conditions, a functionally counteracting facilitation cannot be the reason for loss of apparent muscarinic inhibition. The significant fading of the acetylcholine effect occurring only when the acetylcholine biophase concentration is kept high by acetylcholinesterase inhibition may result from desensitization of presynaptic muscarinic receptors or loss of coupling between receptor and effector mechanisms.

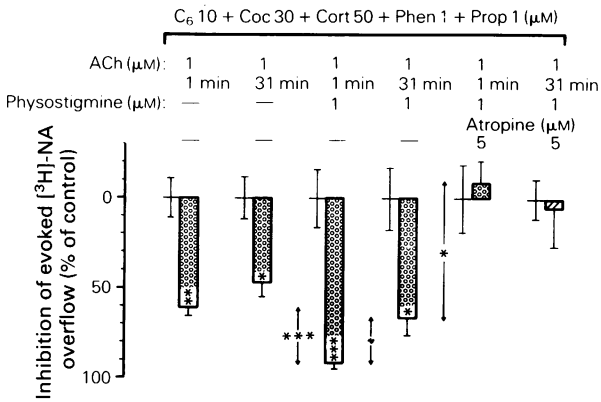

Figure 8 The inhibition of evoked [^{3}H]-noradrenaline (NA) overflow by acetylcholine (ACh) 1 μM after 1 or 31 min preincubation time and in the absence or presence of physostigmine 1 μM and/or atropine 5 μM. For abbreviations of other drugs present see Figure 7. Normalized mean control values for the respective stimulation period are shown by horizontal bars ± s.e.mean on the left hand side of columns ($n = 6$). The evoked overflow expressed as % of S1 was 82, 66, 63, 50, 48, 42, and 34 at S2–S8, respectively. Columns are means of 3–6 observations with s.e.mean derived from experiments shown on Figure 7 except values for ACh 31 min (atropine plus physostigmine present, hatched column) which stem from 3 independent experiments set up to match the scheme (for details see Figure 7). Significant inhibition of evoked [^{3}H]-NA overflow compared to controls is indicated by asterisks within the columns (as in Figure 7). Asterisks between columns denote significant differences between column heights indicated by arrows (paired t test). Note that the presence of physostigmine enhanced the degree of inhibition by ACh. Upon prolonged exposure time and in the presence of physostigmine, the inhibition was significantly less compared to short exposure time. Atropine abolished the inhibition. No facilitation upon prolonged ACh exposure time was observed in the presence of atropine plus physostigmine.

Discussion

The present paper was aimed at resolving an apparent discrepancy: why should acetylcholine fail to inhibit the evoked [^{3}H]-noradrenaline overflow from guinea-pig atria (Story *et al.*, 1975), when, in contrast, acetylcholine inhibits the evoked noradrenaline (Lindmar *et al.*, 1968) or [^{3}H]-noradrenaline overflow (Westfall & Hunter, 1974; Langley & Gardier, 1977) from guinea-pig perfused hearts? We adopted similar, though not identical methodology to that described by McCulloch *et al.* (1974) which was used in the paper reporting the lack of muscarinic inhibition by acetylcholine (Story *et al.*, 1975). The methodological modifications introduced by us (such as loading of the neuronal stores with lower concentrations of [^{3}H]-noradrenaline; 6–9 stimulation periods instead of only 2; physiological salt solution of a different composition) did not lead to important changes in presynaptic muscarinic mechanisms and, in agreement with the findings of Story *et al.* (1975), we failed to see a significant inhibition by acetylcholine of evoked [^{3}H]-noradrenaline overflow under certain experimental conditions. However, our conclusions are not based on the determination of ^{3}H overflow (as in most experiments of Story and coworkers), but on the overflow of [^{3}H]-noradrenaline. In our view, ^{3}H overflow that was increased by field stimulation in an organ bath containing electrodes and tissue holder, but no biological tissue (Angus *et al.*, 1984; Figure 1) does not represent a presynaptic parameter as reliable as the authentic labelled transmitter itself. In this context, it was important to show that the overflow of [^{3}H]-noradrenaline from the organ bath was not increased by field stimulation in the absence of atria (Figure 1). Moreover, the determination of [^{3}H]-noradrenaline becomes crucial when the stimulation-evoked ^{3}H overflow is not totally blocked by tetrodotoxin and omission of calcium. Thus, upon application of field pulses of high current strength (200 mA), the overflow of [^{3}H]-noradrenaline from mouse vas deferens was abolished in the presence of tetrodotoxin and absence of Ca^{2+} but an increase in ^{3}H overflow was still observed (Illes *et al.*, 1984). Such an increase could be due to current-induced tissue damage as suggested by Illes *et al.* (1984), but may in part be due to displacement of ^{3}H-compounds (other than noradrenaline) from plastic, glass, or metal surfaces (Angus *et al.*, 1984).

The results obtained in the presence of Tyrode solution (cocaine, corticosterone, etc. absent) will be discussed first. Under the present conditions, field stimulation does not selectively induce transmitter release from cardiac adrenergic nerves, but probably depolarizes all neuronal (and extraneuronal) membranes. However, the acetylcholine released cannot have reached adrenergic nerves to cause muscarinic inhibition. If this were true, atropine given alone should have increased, and physostigmine probably decreased, the evoked [^{3}H]-noradrenaline overflow, quite in contrast to our results (Figure 6). Thus, the lack of effect of exogenous acetylcholine cannot result from abundant amounts of endogenous acetylcholine causing a maximum degree of muscarinic inhibition. Moreover, methacholine reduced the evoked overflow (Figure 4), indicating that presynaptic muscarinic receptors are present at guinea-pig atria adrenergic nerves, and, again, that muscarinic mechanisms are certainly not maximally activated by endogenous acetylcholine. Nevertheless, it should be noted that the degree of inhibition observed after methacholine

10 μM was only 68%. This could be due to some degradation by acetylcholinesterase and/or desensitization of muscarinic inhibition. Perhaps a greater inhibition of release would have occurred if the agonist preincubation time had been shorter and acetylcholinesterase inhibited. The (+)-methacholine stereoisomer responsible for the presynaptic action (Fuder & Jung, 1985) can be hydrolyzed by acetylcholinesterase (Beckett *et al.*, 1963).

Clearly, more than one mechanism must be responsible for the apparent failure of acetylcholine to induce muscarinic inhibition after an agonist pre-equilibration time of 12 min. Firstly, it is evident that a lack of muscarinic inhibition in the absence, or a comparatively low maximum inhibition by acetylcholine in the presence, of an acetylcholinesterase inhibitor occurred concomitantly with a moderate degree of facilitation by acetylcholine in the presence of atropine (Figures 3, 5 and 6). Such a facilitation was not observed after a short (2 min) exposure time to acetylcholine of the atria (Figure 5). The facilitation by acetylcholine of evoked [^{3}H]-noradrenaline overflow in the presence of atropine is probably not nicotinic in nature, since presynaptic nicotinic facilitation fades within 4–7 min (Löffelholz, 1970a). Moreover, the basal [^{3}H]-noradrenaline overflow was not increased by acetylcholine in the presence of atropine, indicating a lack of nicotinic exocytosis of noradrenaline. At first sight, this finding appears to contradict observations on the perfused guinea-pig heart in which an explosive exocytotic release of transmitter was observed (Lindmar *et al.*, 1968; Westfall & Brasted, 1972). However, the nicotinic increase in basal overflow depends on the biophase concentration of nicotinic agonists, becoming significant only at >60 μM acetylcholine in the presence of atropine (Muscholl, 1980). Low concentrations of agonist desensitize the receptor without inducing release (Löffelholz, 1970b). Nicotinic drugs diffusing through an incubated tissue (and being degraded by esterases) may reach the receptor in a low concentration and desensitize the receptor so that no effect can be observed. Thus, in incubated rat atria, no nicotinic stimulation of noradrenaline release was observed (Lindmar, 1962), in contrast to results obtained in the rat perfused heart where a nicotinic stimulation of basal [^{3}H]-noradrenaline overflow was seen (Fuder *et al.*, 1982b).

From the present results, the origin of facilitation remains undefined. It must, however, be due to one of the mechanisms blocked by any of the drugs present throughout in the experiments shown in Figures 7 and 8. Even with 31 min exposure time, no facilitation of release was observed in the simultaneous presence of inhibitors of neuronal and extraneuronal uptake, adrenoceptors, nicotinic and muscarinic receptors and acetylcholinesterase. Under these conditions (atropine absent), the maximum muscarinic inhibition by acetylcholine (1 min, physostigmine present) was the largest observed under all conditions investigated. This is a further argument for functional interference between facilitation and concomitant muscarinic inhibition induced by acetylcholine.

At postsynaptic sites, an excitatory effect of acetylcholine has been observed in cooled or deteriorated cardiac tissue (for review see Trautwein, 1963). Such an excitatory effect (perhaps also relevant for the presynaptic facilitation shown here) is probably due to the increase of a previously lowered membrane potential by enhanced potassium permeability of membranes. Thus, a previously lost ability to conduct action potentials is restored. In addition, a small increase in the rate of rise of the action potential in guinea-pig atria was observed in the presence of acetylcholine, possibly indicating a direct excitatory acetylcholine action (for discussion see Johnson & Robertson, 1958).

The second mechanism responsible for the weak muscarinic effect of acetylcholine is acetylcholinesterase activity. Acetylcholinesterase is present in guinea-pig atria in concentrations higher than in ventricles (for reviews see Koelle, 1963; Silver, 1974), and decreases the postsynaptic muscarinic effects of acetylcholine on force of contraction and action potential of guinea-pig atria (Furchgott *et al.*, 1960). Our presynaptic results show that the muscarinic inhibition is clearly enhanced in the presence of physostigmine 1 μM (compare Figures 3 and 6; Figure 8), a concentration known to block acetylcholinesterase activity in guinea-pig ileum (Kilbinger & Wessler, 1980) and rabbit atria (Muscholl & Muth, 1982) by roughly 80%. Interestingly, the facilitatory acetylcholine effect was similar in the absence and presence of physostigmine indicating that a maximum had been reached at 10 μM acetylcholine in the absence of the anticholinesterase.

Desensitization of presynaptic muscarinic receptors has to be considered as a further mechanism leading to a reduced extent of muscarinic inhibition upon prolonged, but not upon short exposure time (Figures 5 and 8). Fading of muscarinic responses with the exposure time has frequently been observed (for review see Fleming *et al.*, 1973), also in mammalian atrial myocardium (Furchgott *et al.*, 1960; Nilius, 1983). The fading of muscarinic effects was explained by desensitization of muscarinic cardiac receptor mechanisms. Desensitization is apparently not confined to non-neuronal muscarinic mechanisms. Recently, desensitization of muscarinic receptor-mediated cyclic GMP formation by cultured mouse neuroblastoma cells was observed (Richelson, 1978; for review see McKinney & Richelson, 1984). Similarly, specific desensitization of muscarinic inhibition of stimulated adenylate cyclase activity in homogenates from neuroblastoma-glioma hybrid cells was reported

(Green & Clark, 1982). Moreover, desensitization was also observed when muscarinic receptor-induced increase in phosphatidylinositol turnover in mouse neuroblastoma cells was studied (Cohen *et al.*, 1983). In experiments carried out with rat hippocampal synaptosomes, the field stimulation-evoked overflows of [^{3}H]-acetylcholine or [^{3}H]-5-hydroxytryptamine were investigated (Raiteri *et al.*, 1983). The muscarinic inhibition by acetylcholine 0.5 μM of [^{3}H]-acetylcholine release was decreased after paraoxon pretreatment, but under the same conditions, the muscarinic inhibition of [^{3}H]-5-hydroxytryptamine release was unchanged. The authors concluded that muscarinic autoreceptors may undergo desensitization, but not muscarinic heteroreceptors.

The time-dependent loss of muscarinic inhibition of evoked [^{3}H]-noradrenaline overflow (Figure 8) is compatible with the idea of a slow desensitization of presynaptic heteroreceptors located at adrenergic nerves of guinea-pig atria. Thus, a fundamental difference between desensitization of muscarinic and nicotinic receptors becomes apparent: while presynaptic nicotinic responses fade within seconds or minutes and no response at all is observed subsequently (Löffelholz, 1970a,b), the desensitization of presynaptic muscarinic mechanisms observed upon exposure to reasonably high (but not excessive) acetylcholine concentrations in the guinea-pig atria, is a rather slow process ($t_{\frac{1}{2}} > 31$ min; Figure 8). Thus, muscarinic agonists may stay active even upon prolonged constant presence. Such a small and moderate degree of desensitization may not always be detected, may not be present in all animal species, or may be irrelevant in tissues with a high receptor reserve where desensitized receptors may be functionally replaced by spare receptors.

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