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A MUSCARINIC MECHANISM INHIBITING THE RELEASE OF NORADRENALINE FROM PERIPHERAL ADRENERGIC NERVE FIBRES BY NICOTINIC AGENTS

BY

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The positive inotropic and chronotropic effects of acetylcholine and nicotine, observed on atropinized hearts of dogs, cats, rabbits and guinea-pigs, have been explained by the the finding that under these experimental conditions an adrenaline-like substance is released into the perfusates (Hoffmann, Hoffmann, Middleton & Talesnik, 1945). By means of chemical and biological assay procedures, it was later shown that noradrenaline is the stimulant amine released from rabbit isolated hearts by acetylcholine (Richardson & Woods, 1959) and the nicotinic drug, dimethylphenylpiperazine (DMPP) (Lindmar & Muscholl, 1961); in the experiments on noradrenaline release after acetylcholine, atropine 10^{-6} g/ml.) was applied. From the results obtained on isolated heart preparations it has generally been assumed that the only action of atropine is to block myocardial muscarinic receptors and thus unmask the stimulant nicotinic effects of acetylcholine or nicotine (Hoffmann *et al.*, 1945; Giotti, 1954; Lee & Shideman, 1959; Holtz, 1960). In keeping with this idea is the observation that DMPP, which lacks conspicuous muscarinic action, stimulates the heart (Chen, Portman & Wickel, 1951) and releases noradrenaline from it independently of the presence of atropine (Lindmar & Muscholl, 1961; Lindmar, Muscholl & Sprenger, 1967). It is interesting that the concentration of atropine needed to unmask the nicotinic effects of choline esters on guinea-pig atria was found to be critical (Barnett & Benforado, 1966); however, the reasons for this are not known.

Different sites from which nicotinic drugs may release noradrenaline have been proposed. It has been shown by elaborate denervation techniques that in the dog and cat virtually all the cardiac noradrenaline is contained in postganglionic adrenergic fibres (Cooper, 1966); this observation excludes the possible existence of sympathetic ganglia or chromaffin tissue stores in the heart, which previously have often been considered as potential points of attack of nicotinic agents. Recently the hypothesis of a depolarization of terminal adrenergic fibres by nicotinic drugs (Furchgott, 1960) has gained support. In the rabbit heart, membrane-stabilizing local anaesthetics such as amethocaine, *l*-cocaine and *d*-cocaine inhibited the release of noradrenaline after DMPP just as readily as did hexamethonium (Lindmar & Muscholl, 1961). Electrophysiological examination of antidromically conducted impulses arising near the nerve endings provided the direct evidence that injection of acetylcholine and nicotine excited postganglionic adrenergic fibres of the

cat spleen (Ferry, 1963) and the rabbit heart (Cabrera, Torrance & Viveros, 1966). Thus it is reasonable to regard the adrenergic nerves as the source of noradrenaline released by nicotinic drugs into the perfusion fluid of the isolated heart.

In the course of an investigation of the ionic requirements of the acetylcholine-induced noradrenaline output from the rabbit heart (Löffelholz, 1967), atropine was omitted from the perfusion fluid in one experiment. The unexpected finding that the noradrenaline release was greatly diminished initiated the present study, in which it was found that the peripheral sympathetic nerve fibres, in addition to the well-known excitatory nicotinic receptors for noradrenaline release, contain inhibitory muscarinic receptors. Some of the results have been presented as a short communication (Löffelholz, Lindmar & Muscholl, 1967).

METHODS

Perfusion of the heart

Rabbits of either sex weighing about 1.7 kg were stunned by a blow on the neck and bled from the carotid arteries. The heart was immediately dissected and perfused according to the Langendorff technique. By means of a two-way stopcock the normal Tyrode solution could be quickly replaced by any test solution. Perfusion was carried out at a pressure of 60 cm H_2O and a temperature of 34° C. The perfusion medium was Tyrode solution (concentrations in g/l.: NaCl 8.0; KCl 0.2; $CaCl_2$ 0.2; $MgCl_2$ 0.1; $NaHCO_3$ 1.0; NaH_2PO_4 0.05; glucose 1.0); it contained ascorbic acid 10 mg/l. and was continuously gassed with a mixture of 95% oxygen and 5% carbon dioxide. The apex of the heart was attached to a force-displacement transducer (Grass model FT.03) and the contractions were recorded on a Sanborn model 296 recorder.

Usually the hearts were perfused for 60 min before a drug was applied, but atropine was sometimes added to the Tyrode solution at the start. Unless otherwise stated, in any one heart only one effect of a drug or drug combination on noradrenaline output was studied. In all, 135 rabbit hearts were used.

In eight additional experiments the isolated hearts from guinea-pigs weighing approximately 900 g were perfused in the same way as the rabbit hearts. The effect of acetylcholine was studied as early as 30 min after the perfusion had started.

Estimation of noradrenaline

The venous outflow of the heart was collected into graduated cylinders containing an appropriate amount of N-H_2SO_4 in order to adjust the pH to about 3. Noradrenaline was determined fluorimetrically by a modification of the trihydroxyindole method after adsorption on, and elution from, alumina (Lindmar & Muscholl, 1964; 1965). The recovery of 1 μg of noradrenaline added to 100 ml. of Tyrode solution was repeatedly tested; the quantities of endogenous noradrenaline released into the perfusates were not corrected for the rate of recovery of added noradrenaline which in forty-one experiments ranged from 70 to 93%. The amount of adrenaline released from the hearts was too small to be estimated precisely by the differential fluorimetric method used; the concentration of adrenaline in the rabbit heart is only 2% of that of noradrenaline (Muscholl, 1959).

In the isolated rabbit heart, a constant infusion of acetylcholine (Löffelholz, 1967) or DMPP (Lindmar, Löffelholz & Muscholl, 1967) released noradrenaline into the perfusates only during the first 2 min; the drugs were therefore administered for 2 min and the perfusates collected during this period.

Removal of noradrenaline by the heart

The effect of atropine or acetylcholine on the removal by the heart of noradrenaline added to the perfusion fluid at a concentration of 20 ng/ml. was tested as previously described (Lindmar & Muscholl, 1964, 1965). The percentage of noradrenaline disappearing from the perfusion medium

during a single passage through the heart was calculated by comparing the amount of amine recovered from the perfusate with the amount infused. Perfusion with noradrenaline was carried out for 20 min; during the first 2 min the perfusate was discarded and it was then collected during the next 18 min in four periods of 4.5 min. Perfusion with atropine (10^{-6} g/ml.) was started 60 min before the perfusion of noradrenaline and maintained throughout the whole period. Acetylcholine (3.8×10^{-5} g/ml.), because of its short-lived effect on noradrenaline release from sympathetic nerves (Löffelholz, 1967) was not administered until the perfusion with noradrenaline was started and two control samples had been collected. The removal of noradrenaline during the first 2 min of perfusion with acetylcholine and during two following periods of 4.5 min each was measured.

Incomplete chemical recovery was corrected in the following way. Samples of the perfusion medium containing noradrenaline were drawn from the perfusion apparatus immediately before and after the infusion period. The two recoveries were measured individually, and the amounts of noradrenaline estimated in the perfusates after passage through the heart were adjusted by taking into account the mean of the two recovery figures obtained in the same experiment.

Removal of acetylcholine by the heart

The method was similar to that used to study the removal of noradrenaline. Tyrode solution containing acetylcholine (3.8×10^{-5} g/ml.) was perfused for 10.5 min. During the initial 30 sec, the perfusate was discarded. Thereafter it was collected in five portions corresponding to 2 min each. Acetylcholine was determined in 2 ml. aliquots of the perfusate by the hydroxamic acid method of Hestrin (1949) as modified by Pilz (1958). Two minutes after addition of 0.5 ml. of the alkaline hydroxylamine solution to the sample, however, a mixture of 0.5 ml. of citrate buffer (pH 1.4) and 1.0 ml. of 2 N-HCl was added rather than the buffer alone. This kept the pH between 1.0 and 1.4, which is essential for the stability of the coloured complex formed after addition of 1 ml. of the ferric salt solution. The optical density of the ferric-acethydroxamic acid complex was measured exactly 2 min after its formation by means of the Zeiss Elko III colorimeter with filter S 49 E.

The percentage of acetylcholine removed was calculated from the amount of acetylcholine recovered in the perfusates. For experimental periods up to 20 min the acetylcholine dissolved in Tyrode solution was stable. The effect of atropine (10^{-5} g/ml.) or neostigmine (10^{-6} g/ml.) on the removal of acetylcholine was tested by perfusing these drugs 3–4 min before, and during, the administration of acetylcholine. Neither atropine nor neostigmine interfered with the estimation of acetylcholine.

Isolated atria

Rabbit atria were suspended in Tyrode solution vigorously bubbled with 5% carbon dioxide in oxygen. The capacity of the organ bath was 70 ml. and the bath temperature 34° C. The spontaneous contractions were recorded with a force-displacement transducer on a Hellige Helcoscriptor. Acetylcholine, methacholine and pilocarpine were injected into the bath and left in contact with the auricles for 3 min. Tests were repeated every 25 min. The maximum alterations of rate and force of contraction observed during the 3 min period were expressed as percentage difference from the control reading taken immediately before a drug was added.

Drugs

These were acetylcholine chloride and neostigmine methyl sulphate (Deutsche Hoffmann-La Roche A.G.); atropine sulphate and pilocarpine hydrochloride (C. H. Boehringer Sohn, Ingelheim); 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP, Parke, Davis & Co.); methacholine chloride (Schuchard) and (−)-noradrenaline hydrochloride (Farbwerke Hoechst A.G.). Concentrations of drugs refer to the salts except those for noradrenaline which refer to the base.

Statistical analysis

When the significance of data was evaluated Student's t test was used. Mean values ± S.E. of means are given throughout the paper; n is the number of estimations.

RESULTS

Effect of atropine on the release of noradrenaline evoked by acetylcholine and DMPP

At the end of the 1 hr equilibration period, the mean spontaneous rate of the 135 perfused rabbit hearts was 129 beats/min (range 80–168) and the coronary flow was 33 ml./min (range 20–54). In eight experiments the spontaneous noradrenaline output was 3.3 ± 1.0 ng/min.

If acetylcholine in a concentration of 3.8×10^{-5} g/ml. was perfused for 2 min the noradrenaline output of the heart rose to 138 ± 42 ng during this period ($n = 7$). In five experiments the Tyrode solution contained atropine (10^{-6} g/ml.) from the beginning of the perfusion. Atropine did not affect heart rate, coronary flow or spontaneous output of noradrenaline. In the presence of atropine, however, the noradrenaline output after acetylcholine was increased ten-fold compared with the output in the absence of atropine (Table 1). Similarly, in the perfused guinea-pig heart the noradrenaline release evoked by acetylcholine was increased ten-fold if atropine was present in the perfusion fluid (Table 1). The spontaneous noradrenaline output of the guinea-pig heart was below the threshold of the method; during the equilibration period the heart rate ranged from 141 to 168 beats/min (mean 155) and the coronary flow from 9 to 29 ml./min (mean 16).

These findings suggested the possibility that the noradrenaline release by acetylcholine, which is supposedly mediated through activation of nicotinic receptors, is at the same time depressed by the muscarinic activity of acetylcholine. If atropine facilitated the noradrenaline release caused by acetylcholine by inactivating an inhibitory muscarinic mechanism, then release by a nicotinic drug devoid of muscarinic actions should be unaffected by the presence of atropine. This hypothesis was tested by comparing the effects of different concentrations of atropine on the noradrenaline release caused by the nicotinic drug, DMPP, with the release elicited by acetylcholine. As shown in Fig. 1, concentrations of atropine, increasing from 10^{-9} to 10^{-6} g/ml., gradually enhanced the noradrenaline output of the hearts after acetylcholine (3.8×10^{-5} g/ml.). In contrast, the noradrenaline release caused by DMPP (10^{-5} g/ml.) was not affected by atropine in concentrations of 10^{-7} and 10^{-6} g/ml. A further increase in the concentration of atropine (3×10^{-6}, 10^{-5} g/ml.) resulted in a dose-dependent depression of the noradrenaline release induced by either acetylcholine or DMPP.

The alterations of the heart rate observed in the experiments shown in Fig. 1 were expressed as percentage differences from the control values and plotted against the atropine concentrations (Fig. 2). In the absence of atropine, acetylcholine in a concentration of 10^{-9} g/ml. caused cardiac arrest. Increased concentrations of atropine gradually blocked the negative chronotropic action of acetylcholine until, at concentrations of 3×10^{-6} and 10^{-5} g/ml., a small rise in heart rate above the control value was noted. In the presence of atropine (10^{-7}–3×10^{-6} g/ml.) the administration of acetylcholine was followed by a greater release of noradrenaline into the perfusate than was seen after the administration of DMPP (Fig. 1); however, the increase in heart rate after DMPP was always much larger than after acetylcholine. This may have been because the negative chronotropic effect of acetylcholine was never fully abolished by the concentrations of atropine employed, with the result that there was a smaller rise in heart rate than might have been expected from the relatively large amount of noradrenaline released into the perfusate.

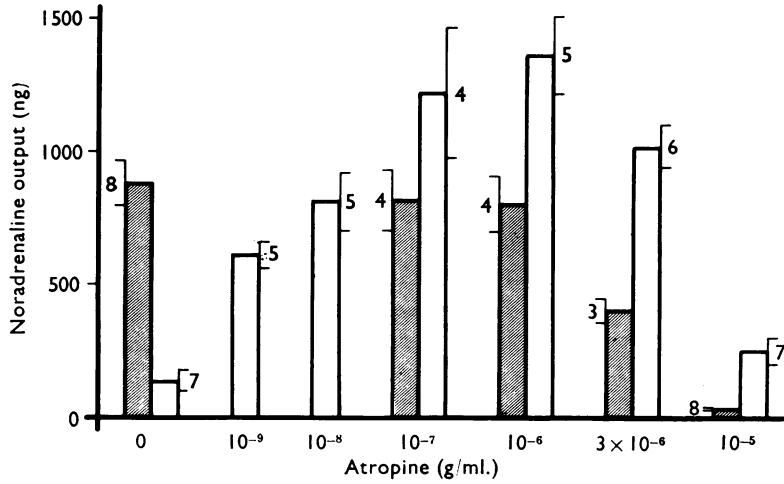

Fig. 1. Rabbit perfused heart. The effects of atropine on the noradrenaline output induced by DMPP or acetylcholine. Abscissa, concentration of atropine (g/ml.) in the perfusion fluid 15–60 min before and during administration of DMPP or acetylcholine. Ordinate, noradrenaline output (ng) during the 2 min period of DMPP or acetylcholine administration. Shaded columns, DMPP (10^{-5} g/ml.); open columns, acetylcholine (3.8×10^{-5} g/ml.). Vertical bars at the tops of the columns indicate S.E. of mean and the figures beside the columns the number of experiments from which the means were obtained. All values were obtained on hearts not previously exposed to drugs (" first " effects).

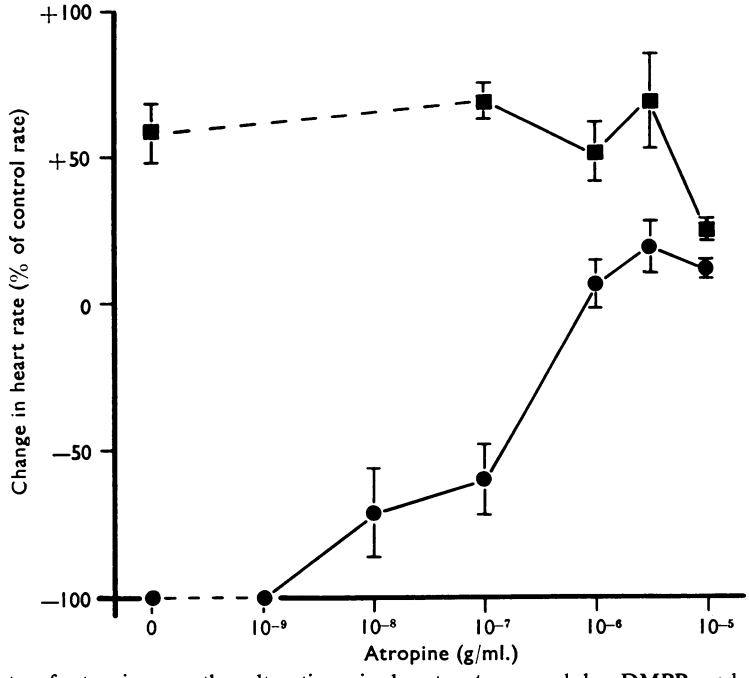

Fig. 2. Effects of atropine on the alterations in heart rate caused by DMPP and acetylcholine. Abscissa, as in Fig. 1. Ordinate, mean change in heart rate expressed as percentage of control rate. ■, DMPP; ●, acetylcholine. Vertical bars indicate S.E. of mean. The concentrations of drugs, length of infusion and number of experiments are the same as in Fig. 1.

In the experiments shown in Figs. 1 and 2, the results were indistinguishable whether atropine was administered 15 or 60 min before acetylcholine was applied. The coronary flow was moderately decreased in response to the standard doses of acetylcholine (by $17 \pm 4.2\%$) and DMPP (by $28 \pm 6\%$). In the presence of atropine (10^{-9} g/ml.), acetylcholine did not significantly alter the flow rate. At all the higher concentrations of atropine (10^{-8}–10^{-5} g/ml.) acetylcholine decreased the coronary flow by approximately the same percentage (mean decrease $28 \pm 1.4\%$, $n=27$). Similarly, in the nineteen experiments with atropine (10^{-7}–10^{-5} g/ml.) plus DMPP the flow rate was reduced by $37 \pm 4\%$. Thus the decrease in coronary flow was not noticeably related to the concentrations of the drugs used or to the amount of noradrenaline released into the perfusates.

As shown in Fig. 1, increase of the atropine concentration from 10^{-6} to 3×10^{-6} and further to 10^{-5} g/ml. gradually depressed the noradrenaline release caused by acetylcholine or DMPP. The effects of different concentrations of acetylcholine (10^{-5}–3.8×10^{-4} g/ml.) were tested in the absence and in the presence of these higher concentrations of atropine (Fig. 3). The dose-response curve of acetylcholine in the presence of atropine (3×10^{-6} g/ml.) rises steeply until, at acetylcholine (3.8×10^{-5} g/ml.), it flattens. The dose-response curve obtained in the presence of 10^{-5} g/ml. of atropine has a similar shape but is shifted to the right. In contrast, an increase in the concentration of acetylcholine in the absence of atropine leads to only a comparatively small increase of the amount of noradrenaline released into the perfusate. In the presence of atropine the dose-response curve of acetylcholine is parallel to that of DMPP and has a similar maximum.

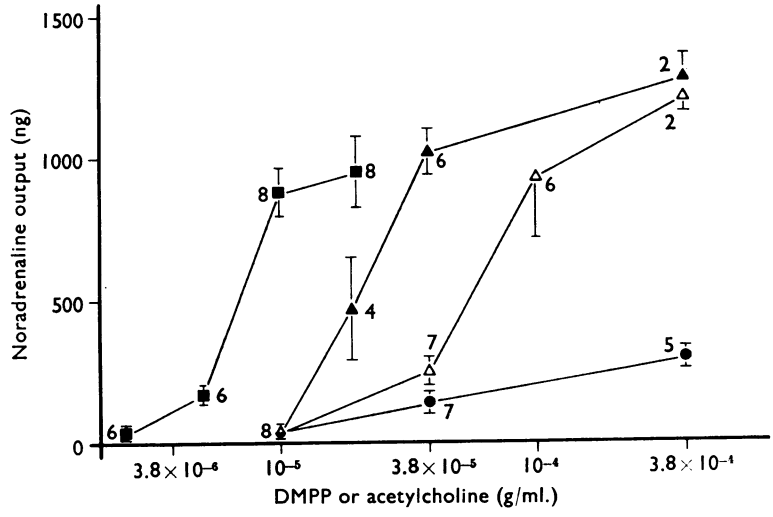

Fig. 3. Effects of DMPP, acetylcholine, or acetylcholine in the presence of atropine, on the noradrenaline output of the rabbit heart. Abscissa, concentration of DMPP or acetylcholine (g/ml.). Ordinate, noradrenaline output (ng) during the administration of DMPP or acetylcholine. ■, DMPP (the data for 2.5, 5 and 20×10^{-6} g/ml.) obtained from Lindmar *et al.*, 1967); ●, acetylcholine; ▲, acetylcholine in the presence of atropine (3×10^{-6} g/ml.); △, acetylcholine in the presence of atropine (10^{-5} g/ml.). Vertical bars indicate S.E. of mean and figures the number of experiments. All values were obtained as "first" effects (see Fig. 1). At 10^{-5} g/ml. of acetylcholine the noradrenaline output was independent of the presence of atropine; therefore the eight values were pooled.

Effects of muscarinic agents on the release of noradrenaline evoked by DMPP

We have shown that the effects of DMPP on noradrenaline release could be imitated by acetylcholine if its muscarinic action was abolished by atropine. Conversely, the addition of a muscarinic drug to DMPP would be expected to produce effects similar to those caused by the administration of acetylcholine alone.

Methacholine and pilocarpine were used as muscarinic agents and their effects compared with those of acetylcholine. As shown in Fig. 4, the dose-response curves for the amplitude of contractions run parallel, except for that part of the acetylcholine curve which corresponds to concentrations of 10^{-6}–10^{-5} g/ml. The potency of acetylcholine seems to be a little higher than that of methacholine, although not statistically significantly, and is much higher than that of pilocarpine. The negative chronotropic effects of the three drugs were related in a similar way, but the concentrations needed to produce a 50% decrease in atrial rate were about ten times higher than those needed for a comparable decrease in contractile force.

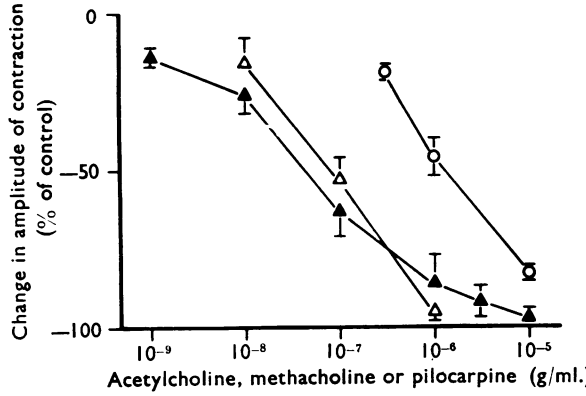

Fig. 4. Depression of the contractions of rabbit atria by acetylcholine, methacholine or pilocarpine. Abscissa, concentrations of drugs (g/ml.). Ordinate, change in amplitude of contractions, the maximum observed in a 3 min period and expressed as a percentage of the control value. ▲, Acetylcholine ; △, methacholine ; ○, pilocarpine. Each point is the mean of three experiments, the vertical bars indicating S.E. of means.

In the experiments shown in Fig. 5, different concentrations of acetylcholine, methacholine and pilocarpine were added to the perfusion fluid 2 min before addition of DMPP (10^{-5} g/ml.). As shown in Fig. 1 this concentration of DMPP, in the absence of any other drug, caused a noradrenaline output of 881 ± 84 ng. Addition of the muscarinic agents produced a dose-dependent inhibition of the noradrenaline release caused by DMPP. Acetylcholine was most effective; the concentration needed for a 50% inhibition of noradrenaline release was 3.4×10^{-7} g/ml. The corresponding values for methacholine and pilocarpine were 5.9×10^{-6} and 4.6×10^{-5} g/ml., respectively. The inhibitory effect of acetylcholine on noradrenaline release evoked by DMPP was clearly distinguishable from the excitatory action of acetylcholine causing a release of noradrenaline. For instance, a concentration of acetylcholine (10^{-5} g/ml.) which completely inhibited noradrenaline release by the standard dose of DMPP (Fig. 5) released only a minute

amount of noradrenaline (41 ± 12 ng, Fig. 3). Further, the concentration of acetylcholine (3.4×10^{-7} g/ml.) necessary for a 50% inhibition of noradrenaline release by DMPP was only 1/73 of the concentration (2.5×10^{-5} g/ml.) producing approximately half the maximum release of noradrenaline in the presence of atropine (3×10^{-6} g/ml.) (Fig. 3).

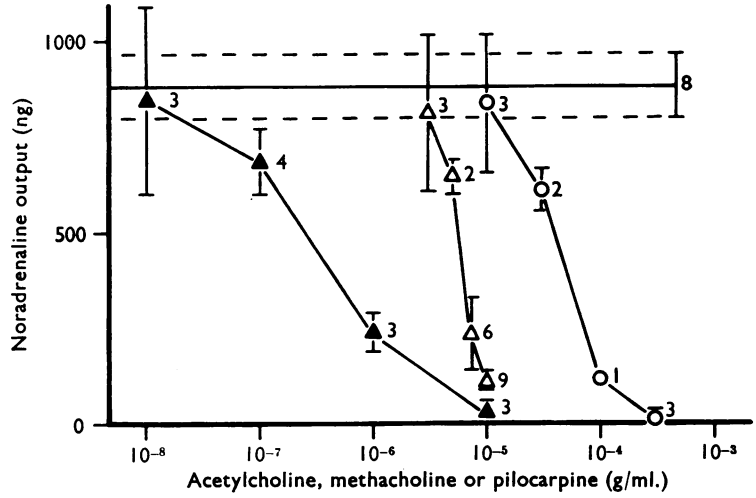

Fig. 5. Depressant effects of acetylcholine, methacholine or pilocarpine on DMPP-induced noradrenaline output by the rabbit heart. Abscissa, concentration of acetylcholine, methacholine or pilocarpine (g/ml.). Ordinate, noradrenaline output (ng) during administration of DMPP. Noradrenaline release was induced by DMPP (10^{-5} g/ml.), either alone (horizontal line at top of graph) or in combination with acetylcholine (▲), methacholine (△) or pilocarpine (○). These drugs were present in the perfusion fluid for 2 min before and during the administration of DMPP. Vertical bars indicate S.E. of mean and figures the number of experiments. The values were obtained as " first " effects (see Fig. 1).

The muscarinic agents were tested for their ability to cause noradrenaline release from the perfused rabbit heart. Acetylcholine (10^{-8}–10^{-6} g/ml.), methacholine (1–2×10^{-5} g/ml.) and pilocarpine (3×10^{-5}–3×10^{-4} g/ml.) did not raise the resting output of noradrenaline. These concentrations effectively inhibited the noradrenaline release caused by DMPP.

Antagonism by atropine of the inhibitory effect of methacholine

If the action of acetylcholine on the sympathetic nerve fibre consisted of a nicotinic component inducing noradrenaline release and a muscarinic component inhibiting it, then the small noradrenaline output observed after the combined administration of DMPP and a muscarinic drug should be greatly increased in the presence of atropine. As a muscarinic agent methacholine was preferred to pilocarpine because of its greater effectiveness.

For reasons of economy the next experiments were performed on perfused hearts in which one dose of DMPP and atropine or DMPP and methacholine had previously been given. All the effects shown in Fig. 6 are therefore " second " effects. However, a heart

previously exposed to atropine was never used to elicit a "second" effect with DMPP plus methacholine. The mean noradrenaline output during "second" effects was generally lower than that seen in "first" effects (for DMPP plus methacholine compare Figs. 5 and 6; for DMPP plus atropine compare Figs 1 and 6). It was found that addition of atropine (10^{-7} g/ml.) to the perfusion fluid completely antagonized the inhibitory action of methacholine on the noradrenaline release caused by DMPP (Fig. 6).

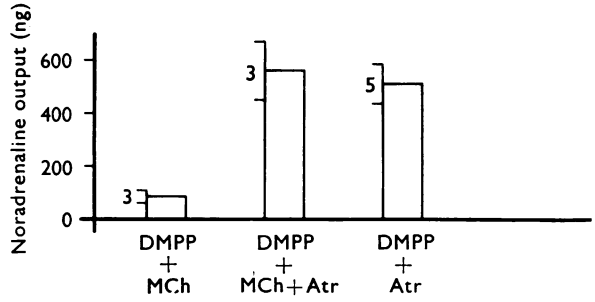

Fig. 6. Reversal by atropine of the depressant effects of methacholine on DMPP-induced release of noradrenaline. Abscissa, drugs used; DMPP (10^{-5} g/ml.); MCh, methacholine (7.4×10^{-6} g/ml.); Atr, atropine (10^{-7} g/ml.). Ordinate, noradrenaline output (ng) during administration of DMPP. Vertical bars indicate S.E. of mean and figures the number of experiments. Experimental procedure as in Fig. 5, except that hearts had been exposed to drugs once before ("second" effects). Atropine was present in the perfusion fluid 15 min before and during administration of DMPP and methacholine.

In another series of experiments DMPP (10^{-5} g/ml.) was administered simultaneously with methacholine (10^{-5} g/ml., $n=6$); atropine in a concentration of 10^{-6} g/ml. ($n=7$) did not fully reverse the depression of noradrenaline release by methacholine (number of controls = 8). Nevertheless, atropine increased the noradrenaline output by 88% above the level seen after DMPP plus methacholine ($P<0.02$).

Removal of acetylcholine by the heart

The experiments reported here provide strong evidence that atropine facilitates the release of noradrenaline after acetylcholine by antagonizing an inhibitory muscarinic mechanism. As an alternative hypothesis, however, it may be assumed that atropine, by delaying the removal of acetylcholine during its passage through the heart, increases the effective concentration of acetylcholine and thus potentiates its actions.

The mean value of acetylcholine removed determined by analysis of twenty-five perfusion samples obtained from five rabbit hearts was $13.6 \pm 1.0\%$ of the amount infused (3.8×10^{-5} g/ml.). The highest concentration of atropine used in the present investigation (10^{-5} g/ml.) was administered 3–4 min before and during acetylcholine perfusion. There was no significant effect on the removal of acetylcholine (Fig. 7). In contrast, neostigmine (10^{-6} g/ml.) significantly depressed the percentage of acetylcholine removed from the perfusion fluid, as would be expected from its cholinesterase-inhibiting activity. Moreover, atropine did not further decrease the removal of acetylcholine which had already been depressed by neostigmine.

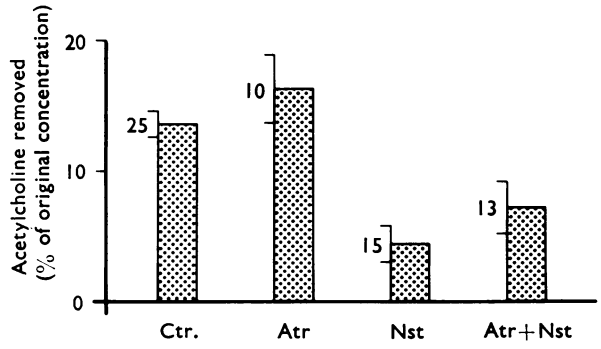

Fig. 7. Effects of atropine and neostigmine on the removal of acetylcholine from the fluid perfusing the rabbit heart. Abscissa, drugs used: Ctr, nil; Atr, atropine (10^{-5} g/ml.); Nst, neostigmine (10^{-6} g/ml.). Ordinate, acetylcholine removed from the perfusion fluid, expressed as a percentage of its original acetylcholine concentration (3.8×10^{-5} g/ml.). Vertical bars indicate S.E. of mean and figures the number of observations. From a single heart, perfusion fluid was collected for five control and experimental periods of 2 min duration.

Removal of noradrenaline by the heart

Another factor which may be responsible for the facilitation of noradrenaline release by atropine is a possible inhibition of re-uptake of noradrenaline already liberated from the sympathetic fibres. If part of this noradrenaline is prevented from being taken up again by the nerves, then a greater proportion of the amine could escape into the perfusion fluid. It was therefore decided to investigate whether atropine inhibited noradrenaline uptake by the perfused rabbit heart.

In three control experiments the mean removal of noradrenaline during its passage through the heart was $43 \pm 3.1\%$ of the amount infused (20 ng/ml., $n=6$). In two different hearts atropine (10^{-6} g/ml.) did not significantly alter the amount of noradrenaline removed from the perfusate ($47 \pm 1.8\%$, $n=8$). Hence the possibility was excluded that atropine increased the output of noradrenaline by interfering with its uptake.

Finally the question arose as to whether the output of noradrenaline caused by acetylcholine, in the absence of atropine, was kept at a low level by a large re-uptake of noradrenaline occurring at the same time. In the first 2 min of perfusion with acetylcholine (3.8×10^{-5} g/ml.), the removal of noradrenaline was apparently decreased because it fell from the control value of $43 \pm 3.1\%$ ($n=6$) to $21 \pm 4.6\%$ ($n=3$). In the next two perfusion periods of 4.5 min each the removal ($39 \pm 3.1\%$, $n=6$) did not significantly differ from the control value. The apparent decrease in the noradrenaline removal observed during the first 2 min of administration of acetylcholine can be explained in the following way. The dose of acetylcholine used caused an average release of 138 ng noradrenaline into the perfusate (Table 1). If this amount is deducted from the quantity of noradrenaline recovered from the perfusates during the first 2 min of administration of acetylcholine, the corrected removal was $38 \pm 5.8\%$. It would therefore seem that acetylcholine does not at any time affect the removal, and thus the uptake by the heart, of noradrenaline.

TABLE 1

FACILITATION BY ATROPINE OF THE NORADRENALINE RELEASE INDUCED BY ACETYLCHOLINE IN RABBIT AND GUINEA-PIG HEARTS

Acetylcholine ($3{\cdot}8 \times 10^{-6}$ g/ml.) was administered for 2 min. Atropine (10^{-6} g/ml.) was added to the perfusion medium 60 min (rabbit) and 30 min (guinea-pig) before acetylcholine. The figures represent noradrenaline release (ng, mean ±S.E.) into the perfusate during the 2 min period. Number of hearts in parentheses. Spontaneous noradrenaline output from rabbit heart 6·6 ng/2 min; from guinea-pig heart not detectable.

	Rabbit heart	Guinea-pig heart
Acetylcholine	138± 42 (7)	39±12 (4)
Atropine+acetylcholine	1,361±144 (5)	394±67 (4)

DISCUSSION

The present experiments have demonstrated that atropine greatly increases the amount of noradrenaline released from sympathetic neurones by drugs having both muscarinic and nicotinic actions. The most likely interpretation is that atropine blocks a mechanism mediated by muscarinic receptors which suppresses the release of noradrenaline resulting from stimulation of nicotinic receptors. The evidence that noradrenaline release from the rabbit heart after nicotinic drugs is evoked by excitation of postganglionic adrenergic nerves has been presented in the introduction.

DMPP was used as a predominantly nicotinic drug causing noradrenaline release unaffected by the presence of low concentrations of atropine. The view that the mechanism which inhibits the DMPP-induced release of noradrenaline from the adrenergic fibres is the result of stimulation of muscarinic receptors is supported by the following findings.

(1) The large amount of noradrenaline released after DMPP was gradually diminished and finally abolished when increasing concentrations of muscarinic drugs (acetylcholine, methacholine and pilocarpine) were added. Approximately the same concentrations of acetylcholine inhibited DMPP-induced noradrenaline release and atrial contractions. However, the concentrations of methacholine necessary to inhibit noradrenaline release were about 2 log units, and those of pilocarpine about 1 log unit, greater than the concentrations producing a graded negative inotropic effect.

(2) Acetylcholine, which possesses both nicotinic and muscarinic properties, became increasingly more effective in raising the noradrenaline output as its muscarinic action was antagonized by atropine.

(3) The action of acetylcholine was mimicked by that of a combination of DMPP and methacholine; that is, atropine increased the noradrenaline output after this drug combination, just as it increased the output after acetylcholine.

If the acetylcholine concentration is raised in the absence of atropine, the ensuing activation of the inhibitory mechanism prevents the noradrenaline release mediated by nicotinic receptors from rising to an extent similar to that evoked by raising the concentration of the nicotinic drug, DMPP (Fig. 3). On the other hand, the depression of noradrenaline release by doses of atropine exceeding 10^{-6} g/ml. (Fig. 1) is caused by an action on nicotinic receptors, for it is observed equally well with acetylcholine and DMPP. This is corroborated by the finding that the dose-response curves of acetylcholine in the presence of atropine (3×10^{-6} and 10^{-5} g/ml.) run parallel to the dose-response

curve of DMPP (Fig. 3). Thus very high concentrations of atropine, which block the muscarinic inhibitory mechanism, also antagonize competitively the action of acetylcholine and DMPP on the nicotinic receptors mediating noradrenaline release.

Although the site from which noradrenaline is liberated by nicotinic drugs is undoubtedly in the peripheral adrenergic fibres, the compounds modifying the noradrenaline output of the heart need not necessarily act on the same structures as the releasing agents. For instance, the drugs used affected autonomic myocardial receptors thereby producing alterations in the mechanical activity of the heart. Furthermore, they affected receptors of the vascular system, thereby causing changes in the coronary flow. These effects could possibly influence the process of noradrenaline release or alter the fraction of total noradrenaline liberated from the fibres and recovered from the perfusates. As stated in the RESULTS, however, the relatively small alterations in coronary flow observed under the various experimental conditions were not related to the quantities of noradrenaline recovered from the perfusates. Similarly, the mechanical activity of the heart did not *per se* affect the noradrenaline release ; a significant increase in noradrenaline output after acetylcholine was produced by atropine on the totally quiescent heart (10^{-9} g/ml.) and on the heart beating slowly (10^{-8} g/ml.), normally (10^{-6} g/ml.), or at an increased rate (3×10^{-6} g/ml.). On the other hand, the noradrenaline release after DMPP was not significantly altered by 3×10^{-6} g/ml. of methacholine although the hearts had stopped beating. When higher concentrations of methacholine were administered before DMPP, the hearts again stopped while the decrease in noradrenaline output varied directly with the dose of methacholine. Finally, in hearts beating normally, after a high concentration of atropine (10^{-5} g/ml.) followed by DMPP, the noradrenaline output was greatly depressed.

It has been reported that 10^{-6}–5×10^{-6} g/ml. of atropine delays the removal of acetylcholine from the perfusion fluid of the rabbit isolated heart (Genuit & Junker, 1943) and that an intravenous dose of atropine increases the acetylcholine release from both the cerebral cortex (Mitchell, 1963) and the caudate nucleus (Polak, 1965). In view of the possibility, suggested by results of Polak & Meeuws (1966) on brain cortex slices, that atropine inhibits the uptake by the heart of acetylcholine and thereby increases its effective concentration near the receptors, the removal of acetylcholine under the present experimental conditions was measured. During a single passage through the heart a significant amount of acetylcholine was removed and this remained unaltered in the presence of atropine (10^{-5} g/ml.), the highest concentration used in the present work. Similarly, the removal of noradrenaline, and consequently its uptake (Lindmar & Muscholl, 1964), was not affected by acetylcholine or atropine.

The question now arises as to how the opposing effects, nicotinic excitation and muscarinic inhibition, originate in the peripheral adrenergic neurone. In the splenic nerve of the cat, the antidromic discharge in the C-fibres caused by acetylcholine was not affected by an intravenous injection of atropine 1 mg (Ferry, 1963). The depolarization of vagal C-fibres produced by acetylcholine was, however, inhibited by atropine 10^{-4}–10^{-3} g/ml. (Armett & Ritchie, 1961). If the latter observation also applied to sympathetic cardiac nerves, it might explain the competitive inhibition, by high concentrations of atropine, of the noradrenaline release after both acetylcholine and DMPP. Similarly, the noradrenaline output of the perfused cat spleen caused by acetylcholine or DMPP was

inhibited by atropine (8×10^{-6} g/ml.) (Hertting & Widhalm, 1965). These findings corroborate the results of the present investigation, namely, that concentrations of atropine above 10^{-6} g/ml. block the nicotinic receptors which mediate noradrenaline release from the sympathetic fibres.

Cholinoceptive neurones, which are either excited by nicotinic drugs or inhibited by muscarinic drugs, have recently been found in the medulla and pons of the cat; furthermore, nicotinic and muscarinic excitatory receptors may occur in the same neurone (for references see Bradley, Dhawan & Wolstencroft, 1966). An excitatory response consisting of a significant noradrenaline release after administration of muscarinic drugs has, however, been ruled out for the heart by the present study. In spite of the evidence presented above, it would be difficult to envisage, in the adrenergic fibre, muscarinic receptors mediating an inhibition of the response to stimulation of the excitatory nicotinic receptors if there were not a precedent for such a situation in the sympathetic ganglion cell.

As Takeshige, Pappano, De Groat & Volle (1963) have shown, methacholine blocks transmission in the superior cervical ganglion of the cat and this block is associated with a phase of ganglionic hyperpolarization. Further pharmacological analysis has revealed that three distinctive cholinoceptive sites are present in sympathetic ganglia (Takeshige & Volle, 1964). The electrical phenomena which correspond to activation of these receptor sites are: first, an initial period of depolarization evoked by acetylcholine; second, a hyperpolarization evoked by acetylcholine or methacholine and, third, a late occurring depolarization evoked by acetylcholine or methacholine. It was suggested that the block of synaptic transmission by methacholine was caused by the activation of an atropine-sensitive inhibitory mechanism. From the present results it seemed that the adrenergic nerve fibre, like the ganglion cell, contains muscarinic inhibitory receptors but no muscarinic excitatory receptors.

It would be interesting to know whether the muscarinic mechanism operating in the peripheral adrenergic fibre fulfils a physiological role. Electron microscope studies by Thoenen, Tranzer, Hürlimann & Haefely (1966) provided evidence that cholinergic and adrenergic nerve fibres of the vas deferens are in intimate contact with each other and are enclosed by the same Schwann cell. It is tempting to speculate that in these circumstances an adrenergic response could be elicited if the cholinergic fibre were excited and released acetylcholine which, in turn, released noradrenaline. Yet the latter response would normally be prevented by the inhibitory muscarinic mechanism which is activated at concentrations of acetylcholine much lower than those needed for noradrenaline release. However, cholinergic stimulation may result in an adrenergic response if atropine or other drugs blocking the muscarinic inhibitory mechanism are applied.

If this interpretation is correct it might explain some findings hitherto obscured by the supposition that atropine blocks muscarinic receptors of effector organs only. In a recent review article summarizing the evidence for the "cholinergic link" hypothesis, Burn (1967) mentioned investigations by various authors using five different tissues (cat nictitating membrane; taenia of the guinea-pig caecum; rabbit heart; dog retractor penis; dog hind leg). As predicted by the hypothesis, the administration of a cholinesterase inhibitor potentiated the response to postganglionic sympathetic nerve stimulation

at low frequencies. It is noteworthy that in all cases hyoscine or atropine was used to exclude the direct action of acetylcholine. In the light of the present findings it seems that atropine, apart from blocking muscarinic receptors on the muscle, facilitates adrenergic responses if they are brought about by a nicotinic action of acetylcholine. Thus the administration of atropine-like drugs may have caused, rather than merely unmasked, the increase in the response to nerve stimulation observed in the presence of anticholinesterases.

SUMMARY

1. Rabbit isolated hearts were perfused with Tyrode solution. The noradrenaline contained in the perfusates was adsorbed on alumina, eluted, and measured fluorimetrically. Heart rate, contractile amplitude and coronary flow were recorded.

2. High concentrations of acetylcholine increased the resting output of noradrenaline only little. This output was greatly enhanced in the presence of atropine (10^{-9}–10^{-6} g/ml.). In contrast, the large amount of noradrenaline released by DMPP was unaffected by concentrations of atropine up to 10^{-6} g/ml.

3. Concentrations of atropine above 10^{-6} g/ml. competitively depressed the noradrenaline output after both acetylcholine and DMPP.

4. The noradrenaline release caused by DMPP was gradually inhibited, and finally abolished, by increasing concentrations of acetylcholine, methacholine, and pilocarpine. The order of potency of muscarinic drugs resembled that observed for their negative inotropic and chronotropic actions on rabbit atria.

5. The inhibitory action of methacholine on the noradrenaline release caused by DMPP was reversed by atropine.

6. Atropine did not alter the amount of acetylcholine removed from the perfusion fluid during a single passage through the heart. Neither atropine nor acetylcholine affected the amount of noradrenaline removed.

7. It is concluded that the peripheral adrenergic nerve fibre contains inhibitory muscarine receptors in addition to the well-known excitatory nicotinic receptors mediating noradrenaline release. Because the muscarinic receptors are stimulated by much lower concentrations of acetylcholine than the nicotinic receptors, a substantial noradrenaline release can be produced by acetylcholine only if its muscarinic action is blocked.

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