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MECHANISM OF ACTION OF MUSCARINE ON THE LONGITUDINAL MUSCLE OF THE GUINEA-PIG ISOLATED ILEUM

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1 DL-Muscarine elicited a contraction of the ileal longitudinal muscle of the guinea-pig and the contraction was characterized by an after-response.

2 Physostigmine (2.0×10^{-8} M) potentiated the contraction of the longitudinal muscle elicited by DL-muscarine.

3 Hemicholinium-3 (HC-3) caused a rightward shift of the dose-response curve to DL-muscarine on the ileal longitudinal muscle of the guinea-pig ileum.

4 β-Bungarotoxin (10 μg/ml) significantly ($P < 0.025$) reduced the contraction elicited by DL-muscarine (2.5×10^{-8} M) suggesting presynaptic release of acetylcholine as an indirect mechanism of action of DL-muscarine.

5 Morphine (1.0×10^{-8} M) significantly ($P < 0.05$) reduced the contractions elicited by DL-muscarine (2.5×10^{-8} M) further suggesting presynaptic release of acetylcholine as an indirect mechanism of action of DL-muscarine.

6 A subthreshold dose of DL-muscarine (2.0×10^{-10} M) potentiated the effect of acetylcholine (2.5×10^{-8} M) and the potentiation was blocked by β-bungarotoxin.

Introduction

Although muscarine is one of the oldest known autonomic agents, pharmacodynamic studies of muscarine are scant. Prior to 1977, muscarine was not commercially available in a pure form to allow rigorous studies of the agent. We therefore designed and developed new furan analogues of muscarine for pharmacodynamic studies with the ultimate aim of elucidating its mechanism of action (Ochillo, Chaturvedi, Sastry, & Kau, 1976; Ochillo, 1977; Ochillo, Chaturvedi & Sastry, 1977a; Ochillo, Kau, Sen & Sastry, 1977b; Ochillo, 1978; Dennis, Nguyen & Ochillo, 1979; Benjamin & Ochillo, 1979; Smith & Ochillo, 1979).

Fraser (1957) reported that the duration of action of muscarine and acetylcholine (ACh) in mice was similar. This observation stimulated our interest since ACh is rapidly hydrolyzed by the cholinesterases in the body while muscarine is resistant to cholinesterase hydrolysis (Waser, 1961); similarly, furan analogues of muscarine are all resistant to cholinesterase hydrolysis (Ochillo *et al.*, 1976; Ochillo, 1977; Ochillo, Chaturvedi & Sastry, 1978a).

Since natural muscarine is now available commercially and also synthetically in pure form as DL-muscarine (by courtesy of Professor Eugster, Zurich), we decided to investigate further the pharmacodynamics of DL-muscarine on the longitudinal muscle of the guinea-pig isolated ileum. Our working hypothesis was that muscarine acts directly at the postsynaptic receptors and indirectly through presynaptic release of ACh. The results of our investigation support the hypothesis. From these studies, it was concluded that the presynaptic action of muscarine is partly responsible for the similarity of the duration of action of muscarine and ACh.

Methods

Preparations and solutions

Male guinea-pigs, weighing 250 to 300 g, used in this series of experiments were purchased from Camm Research Institute (Wayne, New Jersey). The guinea-pigs were killed by cervical dislocation or decapitation on the day of the experiment. The abdomen was opened and the ileum was carefully removed, cleaned and kept in a modified Tyrode solution of the following composition (mM): NaCl 128·3, KCl 2·68, $CaCl_2$ 1·80, NaH_2PO_4 0·36, $MgCl_2$ 0·49, $NaHCO_3$ 23·8 and glucose 11·10. The solution was gassed with 95% O_2 and 5% CO_2. The longitudinal muscle of the ileum was dissected according to the method of Ochillo, Rowell & Sastry (1978b).

A piece of the longitudinal muscle approximately 2.0 cm long was tied at both ends and suspended in a 20 ml bath containing Tyrode solution. One end of the muscle was fastened to an F-60 microdisplacement myograph transducer (Narco Biosystems, Houston, Texas) to record isometric contractions which were displayed on a Narco Biosystem DMP-4A physiograph.

Drugs

Acetylcholine chloride (ACh), β-bungarotoxin, calcium chloride, glucose, hemicholinium-3 (HC-3), magnesium

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chloride, monosodium phosphate, DL-muscarine, noradrenaline (norepinephrine), physostigmine, potassium chloride, sodium bicarbonate and sodium chloride were all obtained from Sigma Chemical (St. Louis. Mo.). Morphine sulphate and naloxone hydrochloride were obtained from Endo Laboratories, Inc. (Garden City, New York).

Procedures

The preparation was left to equilibrate for 20 to 30 min at 37°C before subjecting it to a tension of 0.5 g and then allowing it to equilibrate for an additional 30 min. During this period, the Tyrode solution was changed every 10 min. Each preparation was challenged with a dose of ACh (1.0×10^{-6} M) which was predetermined to be high enough to elicit maximum contraction of each preparation. The preparation was exposed to ACh for 2 min after which the preparation was washed with Tyrode solution. The tracing of the contraction of each preparation was allowed to come back to the baseline after each wash (approximately 7 min) before the next challenge with ACh. The procedure was repeated until the elicited contractions were consistent.

The effect of muscarine and acetylcholine on the longitudinal muscle of the guinea-pig isolated ileum The preparation was challenged with a fixed dose of muscarine (1.25×10^{-8} M) and the elicited contraction observed; termination of the action was by washing with Tyrode solution. The procedure was repeated with a dose of ACh (9.75×10^{-8} M) instead of muscarine.

The influence of physostigmine on the dose-response curve of muscarine Starting with the lowest concentration of muscarine that elicited a contraction of each preparation, the preparation was challenged with the dose and exposure to muscarine was terminated after 2 min by washing with Tyrode solution. After the wash, the tracing of the contraction for each preparation was allowed to come back to the baseline before the next dose of muscarine. The procedure was repeated with increasing concentrations of muscarine until there was no further increase of the muscle contraction. The dose-response curve was constructed for each preparation in the absence and presence of physostigmine (2.0×10^{-8} M), added to the Tyrode solution. The dose-response curve was plotted semi-logarithmically and the ED_{50} was determined graphically.

The influence of HC-3 on the dose-response curve of muscarine. The preparation and the procedure was followed as in Experiment 2 above, except that the dose-response curve was constructed in the absence and presence of HC-3 (1.6×10^{-5} M), added to the bathing medium. The dose-response curve was plotted semi-logarithmically and the ED_{50} was determined graphically.

The influence of β-bungarotoxin on the contraction of the ileal longitudinal muscle elicited by a fixed dose of muscarine We have shown previously that the action of muscarine at the presynaptic site is relatively greater below the ED_{50} of this preparation (Ochillo & Tsai, 1979). We therefore selected a dose of muscarine (2.5×10^{-8} M) which would elicit a contraction of each preparation approximately 30 to 60% of maximum. The contraction was elicited by the dose of muscarine in the absence and in the presence of β-bungarotoxin (β-BTX, 10 μg/ml) and the reduction in the contraction elicited by the same dose of muscarine in the presence of β-BTX was measured.

A similar procedure was carried out with β-BTX and ACh instead of muscarine for each preparation.

The influence of morphine on the contraction of the ileal longitudinal muscle elicited by a fixed dose of DL-muscarine A concentration of muscarine (2.5×10^{-8} M) eliciting approximately 30 to 60% of the maximum contraction elicited by ACh (2.0×10^{-7} M) was selected. Each preparation was challenged with the selected dose of muscarine and the contraction was recorded. The preparation was washed several times with Tyrode solution before exposure to morphine (1.0×10^{-8} M). Morphine alone did not elicit any contraction of the preparation. The preparation was then challenged with the selected dose of muscarine in the presence of morphine and the contraction noted and expressed as a percentage of the maximum contraction elicited by ACh (2.0×10^{-7} M). The challenge of the preparation with muscarine in the presence of morphine was repeated in the presence of naloxone (4.0×10^{-8} M).

The influence of noradrenaline on the contraction of the ileal longitudinal muscle elicited by a fixed dose of DL-muscarine A dose of muscarine (7.5×10^{-9} M) that elicited a contraction of each preparation approximately 30 to 60% of maximum was selected; the size of this contraction was noted and the preparation was washed free of muscarine and then pretreated with noradrenaline (1.0×10^{-6} M) for 5 min before challenging the preparation again with the selected dose of muscarine. The elicited contractions were measured and compared by Student's *t* test.

The influence of subthreshold concentration of muscarine on the contraction of the ileal longitudinal muscle elicited by a fixed dose of acetylcholine A concentration of ACh (2.5×10^{-8} M) eliciting approximately 30 to 60% of the maximum contraction of each prep-

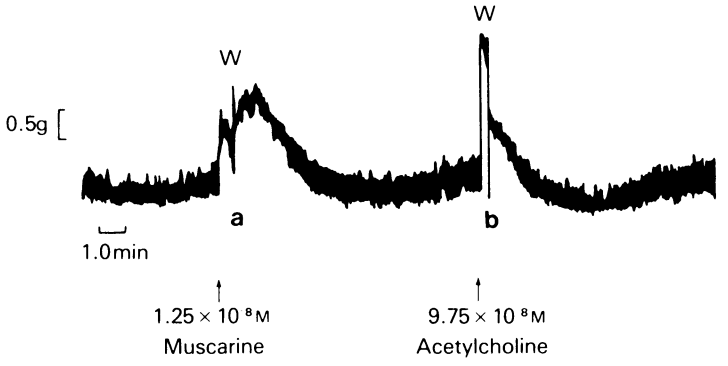

Figure 1 The effect of muscarine and acetylcholine (ACh) on the longitudinal muscle of the guinea-pig ileum. Both agents elicited a contraction of the muscle but the contraction elicited by muscarine (a) was characterized by an after-response, which was not observed with ACh as agonist (b).

aration was selected. Each preparation was washed free of the ACh several times with Tyrode solution before being challenged several times with a subthreshold concentration of muscarine (2.0×10^{-10} M), the preparation being washed with drug-free Tyrode solution after each challenge. Muscarine did not elicit any muscle contraction, confirming our assumption that the concentration selected was indeed subthreshold. The preparation was then challenged again with the dose of ACh (2.5×10^{-8} M) in the presence of subthreshold concentration of muscarine (2.0×10^{-10} M) and the contraction noted. After the challenge, the preparation was washed with Tyrode solution and the procedure was repeated in the presence of subthreshold concentrations of muscarine and β-BTX (10 μg/ml). The elicited contractions were measured each time and expressed as a percentage of the maximum contraction elicited by ACh alone (2.0×10^{-7} M).

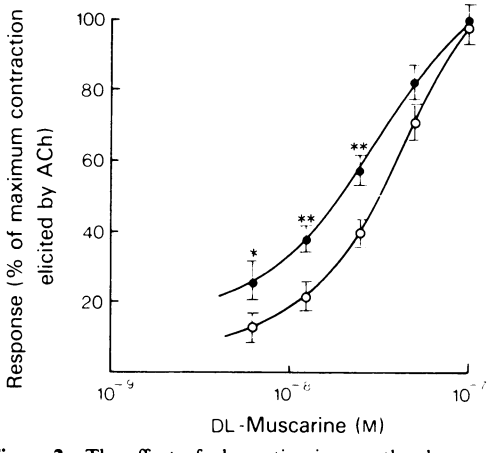

Figure 2 The effect of physostigmine on the dose-response curve to muscarine of the longitudinal muscle of the guinea-pig ileum. The response is a percentage of the maximum contraction elicited by acetylcholine (2×10^{-7} M). Physostigmine caused a leftward shift in the dose-response curve. Response to muscarine (○), ED_{50} $3.30 \pm 0.19 \times 10^{-8}$ M; response to muscarine plus physostigmine (●), ED_{50} $1.99 \pm 0.17 \times 10^{-8}$ M; $n = 6$ for each point. The shift appears to be greater at low concentration than at high concentration of muscarine. Although the concentration of physostigmine selected would only block the hydrolysis of acetylcholine partially, the hypersensitivity induced on this preparation by physostigmine was minimum at this dose.
$^{*}P < 0.02$; $^{**}P < 0.001$.

Results

The effect of muscarine and acetylcholine on the longitudinal muscle of the guinea-pig isolated ileum

The results of this experiment are presented in Figure 1. Muscarine elicited a contraction of the preparation and, on termination of its action by washing, there was an after-response. However, the after-response did not follow the contractions elicited by ACh, even after washing. The results suggest that the after-response is unique to muscarine. Furthermore, the after-response was blocked by atropine (1.0×10^{-6} M) and potentiated by physostigmine (2.0×10^{-8} M).

The influence of physostigmine on the dose-response curve of muscarine

Treatment of the preparation with physostigmine potentiated the activity of muscarine on this preparation as determined by a decrease in the ED_{50} (Figure 2). Since physostigmine has no effect on muscarine (Waser, 1961) and the dose of physostigmine used partially protects ACh from cholinesterase hy-

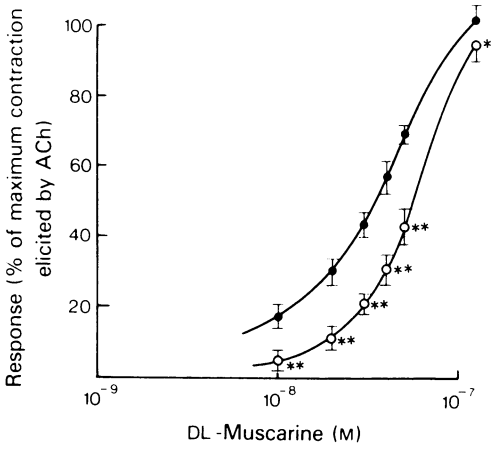

Figure 3 The effect of hemicholinium-3 (HC-3) $1{\cdot}6 \times 10^{-5}$ M on the dose-response curve to muscarine of the ileal longitudinal muscle of the guinea-pig. The response is a percentage of the maximum elicited by acetylcholine (2×10^{-7} M). HC-3 caused a rightward shift in the dose-response curve. Response to muscarine (●), ED_{50} $3{\cdot}43 \pm 0{\cdot}14 \times 10^{-8}$ M; response to muscarine plus HC-3 (○), ED_{50} $5{\cdot}51 \pm 0{\cdot}41 \times 10^{-8}$ M; $n = 7$ for each point. The shift appears to be a greater at low than at the high muscarine concentrations. The plotted values are means (vertical lines show s.e. mean) which were compared for statistical significance by use of Student's t test; $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}005$.

drolysis, the potentiation implicates ACh in the mechanism of action of muscarine in this preparation.

The influence of HC-3 on the dose-response curve to muscarine

The HC-3 treatment caused a rightward shift in the dose-response curve to muscarine (Figure 3). The shift is highly significant below ED_{50} ($3.43 \pm 0.14 \times 10^{-8}$ M) of muscarine ($P < 0.005$) and is significant at a high dose of muscarine ($P < 0.05$). Since HC-3 inhibits presynaptic ACh synthesis which in turn reduces ACh release, this shift in the dose-response curve is suggestive of the involvement of presynaptic ACh release in the mechanism of action of muscarine in this preparation.

The influence of β-bungarotoxin on the contraction of the ileal longitudinal muscle elicited by a fixed dose of DL-muscarine

The results presented in Figure 4a indicate that β-BTX treatment significantly ($P < 0.005$) reduced the muscle contraction elicited by a fixed dose of muscarine (2.5×10^{-8} M). Since β-BTX specifically blocks presynaptic ACh release, these results suggest that muscarine, in addition to acting at postsynaptic muscarinic receptors, also acts at a presynaptic site through the release of ACh. However, the same concentration of β-BTX had no effect on a similar dose of ACh on the same preparation (Figure 4b).

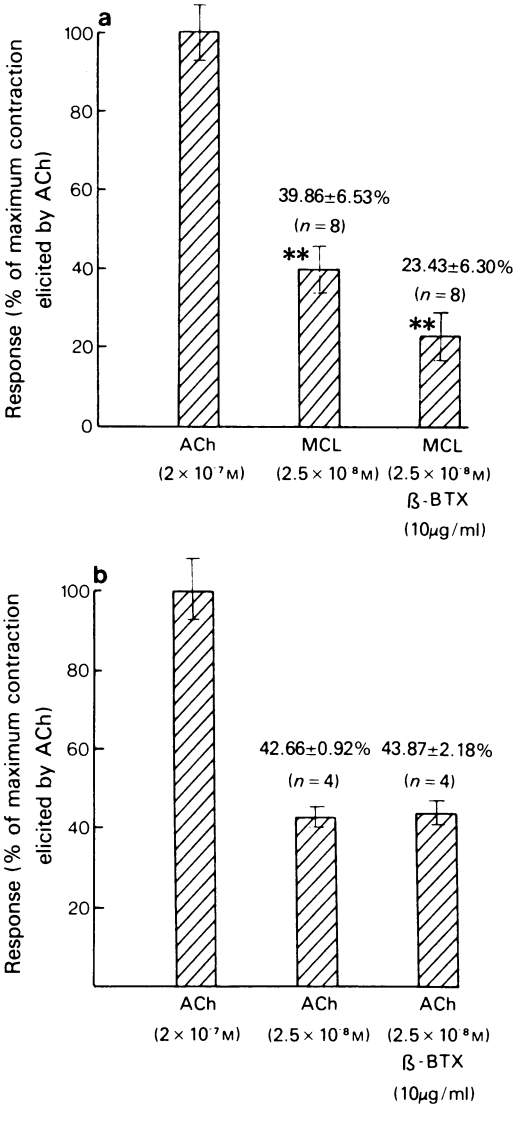

Figure 4 (a) The influence of β-bungarotoxin (β-BTX) on the contractions of the ileal longitudinal muscle of the guinea-pig elicited by a fixed dose ($2{\cdot}5 \times 10^{-8}$ M) of muscarine (MCL). The response is a percentage of the maximum contraction elicited by acetylcholine ($2{\cdot}0 \times 10^{-7}$ M) (ACh). β-Bungarotoxin (10 μg/ml) depressed the contractions elicited by muscarine. The plotted values (vertical lines show s.e. mean) which were compared by Student's t test: $^{**}P < 0{\cdot}005$. (b) The influence of β-BTX on the contraction of longitudinal muscle of the guinea-pig ileum elicited by ACh.

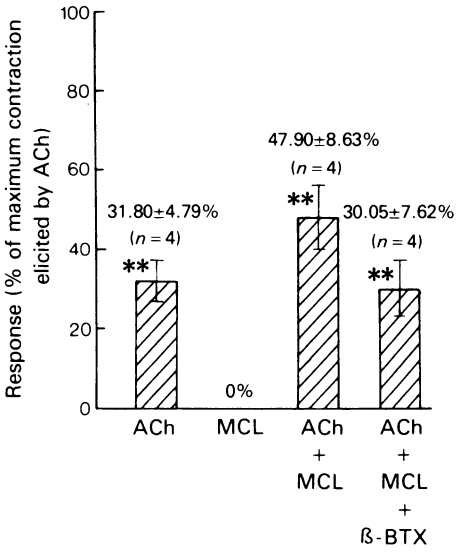

Figure 5 The effect of subthreshold concentration of muscarine (MCL) ($2{\cdot}0 \times 10^{-10}$ M) on the contraction of longitudinal muscle of the guinea-pig ileum elicited by acetylcholine (ACh) $2{\cdot}5 \times 10^{-8}$ M. Responses are shown as a percentage of the maximum contraction to ACh (2×10^{-7} M). The subthreshold concentration of muscarine significantly (**$P < 0.05$) increased the contractions elicited by ACh. The increase was abolished by pretreatment with β-bungarotoxin (β-BTX, 10 μg/ml). The plotted values are means (vertical lines show s.e. mean) which were compared by Student's t test.

The influence of morphine on the contraction of the ileal longitudinal muscle elicited by a fixed dose of DL-*muscarine.*

It was found that morphine (10^{-11} to 10^{-8} M) depressed the contraction to DL-muscarine. For example, morphine (1.0×10^{-8} M) reduced the contraction of the longitudinal muscle elicited by muscarine (2.5×10^{-8} M) from 62.20 ± 8.23 to 44.19 ± 3.72 ($n = 7$) percent of the maximum contraction elicited by 2.0×10^{-7} M ACh and this was naloxone-reversible.

The influence of noradrenaline on the contraction of the ileal longitudinal muscle elicited by a fixed dose of DL-*muscarine.*

Likewise, noradrenaline (1.0×10^{-6} M) reduced the contraction elicited by muscarine (7.5×10^{-9} M) from 59.8 ± 6.18 to 43.25 ± 5.34 ($n = 5$) percent of the maximum contraction due to ACh (2.0×10^{-7} M).

The influence of subthreshold concentration of muscarine on the contraction of the ileal longitudinal muscle elicited by a fixed dose of acetylcholine

Figure 5 shows that subthreshold concentration of muscarine (2.0×10^{-10} M) potentiated the contraction of the preparation elicited by ACh (2.5×10^{-8} M). The potentiation was completely blocked by β-BTX (10 μg/ml).

Discussion

Studies of the activities of any cholinergic agent on the ileal longitudinal muscle of the guinea-pig should consider the effects of the agent on the components of the cholinergic system, i.e. ACh, choline acetyltransferase, cholinesterase and the cholinoceptor. According to the information in the published literature, muscarine does not have any effect on the enzymes responsible for the metabolism of ACh (Waser, 1961). However, muscarine has been reported to have some effects on the release of ACh at the presynaptic cholinergic sites (Bass, Sastry & Owens, 1979; Ochillo & Tsai, 1979). The results of our investigation strongly support our working hypothesis that muscarine acts in part through the release of ACh at the presynaptic sites.

The after-response effect of muscarine (Figure 1) was suggestive of the agent releasing an endogenous substance which elicited further response and was rapidly hydrolyzed. We also observed that the after-response was blocked by atropine (1.0×10^{-6} M) and was potentiated by physostigmine (2.0×10^{-8} M). The first observation suggested the involvement of muscarinic receptor in the response since atropine is a specific muscarinic receptor antagonist. Since muscarine has no effect on any of the enzymes responsible for the metabolism of ACh and, physostigmine specifically inhibits cholinesterase leading to the protection of ACh from hydrolysis, the second observation indicates that muscarine probably acts on this preparation, in part, through the release of ACh. The released ACh would stimulate the cholinoceptors and, in the absence of physostigmine, would be hydrolyzed by cholinesterase; hence the similarity in the duration of action of muscarine and ACh.

The possibility of the after-response being a consequence of changing the bath fluid and/or being caused by ACh was ruled out by challenging the preparation with a dose of ACh, the contraction being terminated by washing with Tyrode solution. The after-response was not observed (Figure 1). This observation also ruled out the possibility of the effects of the exogenously applied ACh persisting after changing the Tyrode solution.

The data presented in Figure 2 indicate that physostigmine treatment causes a greater leftward shift

towards the foot of the dose-response curve of muscarine than it does above the ED_{50}. As was pointed out above, the potentiation of agonist effect in this preparation which is caused by physostigmine usually involves the inhibition of cholinesterases leading to the accumulation of ACh. Since these enzymes have no direct effect on the hydrolysis of muscarine (Waser, 1961), the observed potentiation is probably due to the sparing effect of physostigmine on the ACh released by muscarine.

In the course of this series of experiments, we observed that the concentration of physostigmine (3.85×10^{-7} M) which would completely inhibit cholinesterases also sensitizes the preparation. Since increased sensitization of the preparation would confuse the potentiation we were trying to estimate, we titrated the dose of physostigmine to a concentration giving partial cholinesterase inhibition without any noticeable sensitization of the preparation. The observed potentiation can be attributed to the sparing effects of physostigmine on the ACh released by muscarine.

Hemicholinium-3 (HC-3) is a competitive antagonist of choline reuptake, which is the rate limiting step in the presynaptic ACh synthesis and release. (Bhatnagnar, Lam & McColl, 1964; Chenier, Lam & McColl, 1969). HC-3 caused a rightward shift of the dose-response curve to muscarine from the region of low to high muscarine concentration. Here too, the shift was greater towards the foot of the dose-response curve for muscarine than above the ED_{50}. The data provide additional support for the hypothesis that muscarine acts in part through the presynaptic release of ACh.

The agent, β-BTX, a substance derived from the venom of *Bungarus multicinctus*, which acts presynaptically to block ACh release (Chang & Lee, 1963; Chen & Lee, 1970; Chang, Chen & Lee, 1973; Kelly & Brown, 1974) was used to elucidate further the mechanism of action of muscarine in this preparation. β-BTX had no effect on the contractions elicited by exogenously applied ACh (Figure 4b). However, the agent significantly depressed the response that was elicited in the same preparation by muscarine (Fig. 4a). The effects of β-BTX on the contraction of the preparation elicited by muscarine further suggest that part of the mechanism of action of muscarine is through the release of ACh at the presynaptic site.

Since morphine has been reported to inhibit the release of ACh in this preparation (Paton, 1957; Cox & Weinstock, 1966, Paton & Zar, 1968; Sawynok & Jhamandas, 1977), the observation that morphine in pharmacological concentrations reduced the contraction of the longitudinal muscle elicited by muscarine and the fact that the effect was naloxone-reversible further support our working hypothesis.

Similarly, catecholamines have been reported to inhibit the release of ACh from this preparation (Paton & Vizi, 1969; Kosterlitz, Lydon & Watt, 1970). Our data with noradrenaline are, therefore, in good agreement with the data of other investigators and provide additional support for our working hypothesis.

The observation that a subthreshold concentration of muscarine potentiated the effect of a fixed dose of ACh provides further support for the working hypothesis. The success in blocking the potentiation by β-BTX and morphine provide additional lines of evidence in support of the hypothesis that muscarine acts in part at the presynaptic site by releasing ACh. The presynaptically-released ACh then potentiated the effect of the fixed dose of ACh at the postsynaptic receptors.

The evidence presented in this investigation is consistent with the hypothesis although the hypothesis is at variance with some of the published literature in this area. For example, some investigators have found that the stimulation of the presynaptic muscarinic receptors with a muscarinic agonist leads to the inhibition of electrically-evoked release of ACh (Kilbinger & Wagner, 1975; Dzieniszewski & Kilbinger, 1978). Sawynok & Jhamandas (1977) reported that oxotremorine, a muscarinic agonist, reduced atropine-induced ACh release from guinea-pig ileum. However, we are unaware of any investigation of the possibility of muscarine acting on presynaptic muscarinic receptors. Furthermore, there is no experimental evidence indicating that the stimulation of these ACh release-modulating receptors by ACh or muscarine would lead to the same pharmacological effects as are observed when postsynaptic muscarinic receptors are stimulated. On the basis of the results of our investigation, we propose that muscarine acts on two types of receptors, one at the presynaptic membrane and the other at the postsynaptic membrane.

Argument has been presented suggesting that the stimulation (or occupation) of the presynaptic muscarine receptors leads to an inhibitory action while the stimulation of the post-synaptic muscarinic receptors leads to stimulatory action (Bass *et al.*, 1979; Westfall, 1977). The argument supports our proposal that, indeed, there are in this preparation two types of muscarinic receptors.

We believe that our finding will help in the interpretation of data on muscarine which have been difficult to understand. For example, muscarine is supposed to be devoid of any nicotinic activity and yet Douglas & Poisner (1965) found that muscarine caused a release of catecholamines from the adrenal medulla. We have made a similar observation with one of the furan analogues of muscarine (Ochillo *et al.*, 1977a). Also, we have found a furan analogue of muscarine (Ochillo *et al.*, 1977b) and muscarine (unpublished) to stimulate nicotinic receptors of the

rectus abdominis muscle preparation of *Rana papiens*. The similarity in the duration of action of ACh and muscarine observed in mice by Fraser (1957) can be explained on the basis of our hypothesis.

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