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RELEASE-MODULATING ACETYLCHOLINE RECEPTORS ON CHOLINERGIC NEURONES OF THE GUINEA-PIG ILEUM

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1 Twitch responses of the guinea-pig ileum to electrical transmural stimulation (0.2 Hz) were smaller after a dose of acetylcholine (ACh) than before it. The magnitude of the post-ACh inhibition of twitch was dose-dependent.

2 The post-ACh inhibition of twitch could not be explained in terms of post-junctional desensitization and was not modified by guanethidine, thymoxamine, propranolol or naloxone. Inhibition of twitch also followed high frequency stimulation (10 Hz) but this inhibition, unlike that following ACh, was partially antagonized by naloxone.

3 Hexamethonium (C_6) in concentrations known to block contractions to nicotine, potentiated the post-ACh inhibition of twitch and modified the pattern of recovery. An initial rapid phase followed by a slower phase was converted by C_6 to an initial slow phase followed by a more rapid rate of recovery.

4 The C_6-sensitive (nicotinic) component of twitch recovery after ACh was also dose-dependent and contributed greatly to the rapid recovery during the first minute after ACh washout, whereas during the same period the C_6-resistant inhibition remained relatively constant; thereafter both components declined. The C_6-resistant inhibition was considered to be due to the activation of prejunctional muscarinic receptors.

5 5-Hydroxytryptamine (5-HT) and nicotine also caused inhibition of twitch but the pattern of response differed from those due to ACh, the maximum inhibition usually being produced 1 min after recommencing stimulation. High doses of 5-HT produced inhibitory responses similar to those following ACh, whereas nicotine produced a characteristic triphasic pattern of response.

6 It is concluded that ACh acts on at least two prejunctional receptors subserving a modulatory role on transmitter release, a nicotinic receptor whose activation enhances ACh output and a muscarinic receptor whose activation leads to an inhibition of transmitter secretion.

Introduction

The guinea-pig isolated ileum suspended in Krebs solution releases acetylcholine (ACh) spontaneously into the surrounding fluid. This ACh has its origin in the intramural nerve plexus (Johnson, 1963; Paton & Zar, 1968). Johnson (1963) found that when the ACh from such ileal segments was collected over different periods the total amount recovered increased, but the rate of spontaneous release fell progressively, as the collection period was increased.

The possibility arises that the ACh accumulation in the synaptic cleft is itself inhibiting the further output of the transmitter and, in support of this suggestion, oxotremorine has been shown to cause an atropine-sensitive reduction in spontaneous (Kilbinger & Wagner, 1975) and electrically evoked (Kilbinger, 1977) output of ACh from guinea-pig ileum. Kilbinger & Wagner (1975) postulated that oxotremorine was acting on a prejunctional receptor responsible for feedback control of ACh release from the ileum. Furthermore, initial experiments by Fosbraey & Johnson (1978) showed that electrical transmural stimulation of the guinea-pig ileum immediately after a dose of ACh gave smaller twitch responses than were obtained before the ACh. This phenomenon has now been examined in detail, and it would seem to provide additional evidence that extracellular ACh itself can modulate the release of transmitter ACh.

Methods

All experiments were carried out on ileum isolated from male guinea-pigs weighing over 250 g. Segments 2 to 3 cm in length and proximal to the terminal 15 cm, were freed of mesentery and suspended in 15 ml organ baths of Krebs solution at 37°C; longitudi-

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nal muscle contractions were recorded by means of a Grass FTO3C force-displacement transducer and amplifier connected to a Smith's Servoscribe twin channel pen recorder. The Krebs solution was constantly bubbled with 5% CO_2 and 95% O_2. The tissue was attached to an electrode assembly from which one platinum wire passed through the ileal lumen and a second wire, parallel to the first, was just free of the serosal surface. The ileum could be stimulated transmurally by means of square wave impulses of 0.5 ms duration at a frequency of 0.2 Hz and a potential difference between the electrodes of 7 to 8 V. The potential difference and current were continually monitored by means of an oscilloscope to ensure that a constant stimulus strength was maintained.

Each impulse resulted in a twitch-like contraction. Consecutive impulses soon resulted in constant responses from a steady baseline and there was little or no spontaneous activity. After 15 min recording of constant twitch responses (approximately 80% maximal twitch response), the stimulator was switched off and a dose of ACh, chosen at random from a previously constructed dose-response curve, was added and left in contact with the tissue for 30 s before being washed out by overflow. Electrical stimulation was then resumed immediately. The twitch responses were at first smaller than before the dose of ACh and, in order to quantify this phenomenon, the inhibition of the first post-ACh twitch was calculated as a percentage of the pre-ACh twitch height. The height of contraction of the tissue to added ACh was expressed as a % maximal response. The experiments were repeated with different, randomly allocated doses of ACh (range 0.85 nM to 68.4 μM) and nicotine, 5-hydroxytryptamine (5-HT) or high frequency (10 Hz) stimulation for 30 s were substituted for ACh in similar experiments. Injections of Krebs solution served to control for the effects of changing the bath fluid. The effects of ACh and nicotine, in doses insufficient to contract the ileum, were also examined on the twitch responses to electrical stimulation. The use of submaximal stimulation parameters was a precaution against the effects of added drugs on twitch being masked by supramaximal pulses. Although the effects of ACh on twitches induced by supramaximal pulses were similar to those described in this paper, the effects of nicotine and 5-HT were more difficult to obtain.

The effects of various antagonist drugs on the post-ACh inhibition of twitch were investigated in paired experiments in which one tissue was bathed with Krebs containing the antagonist.

In some experiments the mesenteric vasculature and sympathetic periarterial nerves were left intact. This enabled the preparations to be stimulated electrically in two ways; transmurally as before and periarterially; periarterial nerve stimulation caused the intestine to relax. In the present experiments this relaxation was measured as an inhibition of the twitch responses to transmural stimulation.

Drugs

The following drugs were used: acetylcholine chloride (BDH); guanethidine sulphate (Sigma); hexamethonium bromide (Koch-Light); 5-hydroxytryptamine creatinine sulphate (BDH); naloxone hydrochloride (Endo); nicotine hydrogen tartrate (BDH); (−)-noradrenaline hydrogen tartrate (Sigma); (±)-propranolol hydrochloride (Sigma); thymoxamine hydrochloride (Warner). All drugs were dissolved in Krebs solution of the following composition (mM): NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11.5 and stored on ice. Noradrenaline oxidation was prevented by means of ascorbic acid (5.7 mM).

Results

Effect of exogenous acetylcholine on the responses of the ileum to electrical stimulation

The characteristic action of ACh on the twitch response to electrical stimulation is shown in Figure 1a. When electrical stimulation was inducing constant twitch responses, the stimulator was switched off and ACh was added to the bath. After 30 s contact, during which there was a contraction to the added ACh, the bath fluid was replaced and stimulation was restarted immediately. At first the twitches were smaller than before the ACh, but recovered to the control height in two phases, a rapid phase over 10 to 20 twitches and a slower phase taking a further 60 to 80 twitches to recover the last 10% of the pre-ACh height (Figure 1a). Occasionally the initial inhibition of twitch following the washout of added ACh was followed by an increase in twitch height which exceeded the pre-ACh level (Figure 1b). This response was usually obtained with low doses of ACh but a transient 'hump' during the first minute after ACh was also sometimes obtained after higher doses, superimposed on the markedly inhibited post-ACh twitch responses. This latter response was characteristically obtained with high doses of nicotine (see Figure 9b).

Relationship between doses of acetylcholine and post-acetylcholine inhibition of electrically induced twitch

ACh caused a dose-dependent inhibition of the twitch response to electrical stimulation over the concentration range 1.7 nM to 68.4 μM (Figure 2). In experiments on matched pairs of ileal segments, one tissue received the same volume of Krebs solution as the

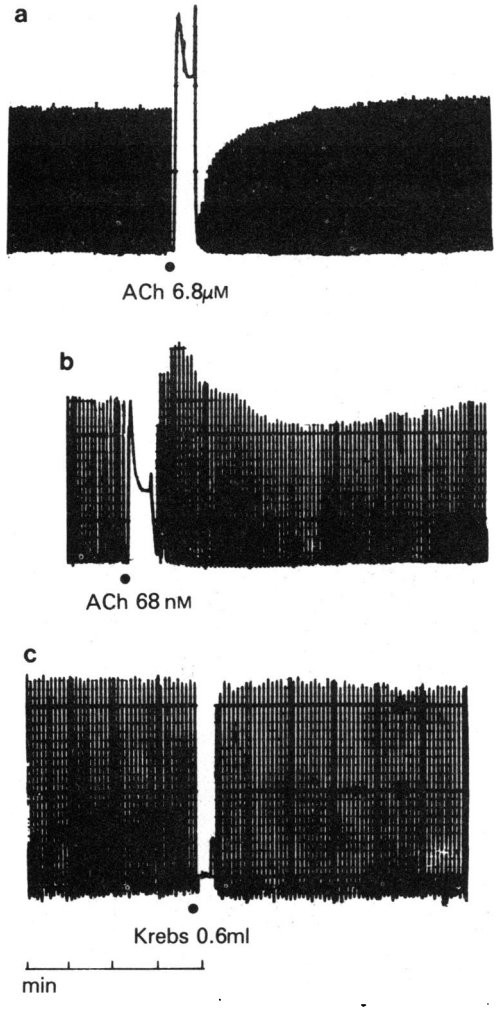

Figure 1 The effects of exogenous acetylcholine (ACh) on the twitch response of the longitudinal muscle of guinea-pig ileum to transmural electrical stimulation. The stimulator was turned off during the 30 s drug contact time and switched on again immediately after washing out the ACh. (a) Characteristic inhibition of post-drug twitch caused by 6.8 μM ACh. Note that most of the recovery to pre-ACh twitch height occurred during the first minute. (b) The inhibition of twitch following a small dose of ACh (68 nM) was followed by a marked increase which in this case exceeded the pre-ACh level. (c) Control response to the injection of 0.6 ml Krebs solution showing almost no inhibition after the bath fluid was replaced.

volumes in which the ACh was given to the other tissue (0.1 to 0.8 ml). Krebs solution caused very little inhibition (usually $<10\%$) of the twitch response when stimulation was restarted (Figure 1c) and the

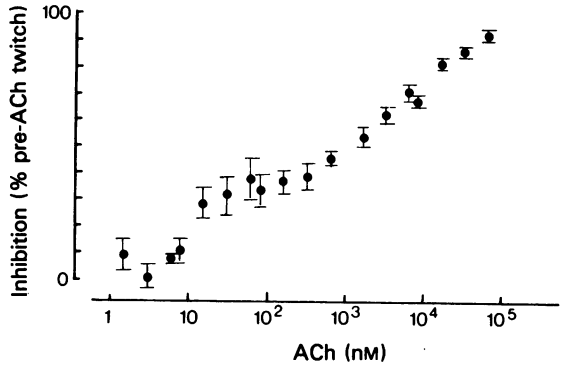

Figure 2 The relation between the inhibition of the first post-acetylcholine (ACh) twitch response to transmural electrical stimulation as a percentage of the pre-ACh twitch height (mean; vertical lines show s.e. mean) and concentration of ACh in the bathing fluid (nM, log scale).

inhibition was not volume-dependent. The inhibitions caused by doses of ACh exceeding 10 nM were significantly different ($P < 0.001$) from the responses following Krebs solution, indicating that the ACh inhibition was not a consequence of changing the bath fluid by overflow.

Relationship between contraction of the ileum to exogenous acetylcholine and inhibition of electrically induced twitch

The height of contraction of the ileum to ACh during the 30 s contact time with the tissue was directly related to the subsequent inhibition of the first twitch response to electrical stimulation. Values relating the % inhibition of the first post-ACh twitch and the % maximal contraction of the ileum for 108 separate doses of ACh are presented in Figure 3. The two parameters were highly correlated ($r = 0.88$).

One explanation of the twitch inhibition suggested by this relationship was that immediately following the contraction to ACh the postsynaptic ACh receptors had become desensitized. This was challenged by the repeated administration of the same dose of ACh (the dose that would cause approximately 50% maximal contraction) immediately after washing out the previous dose and also by giving a very small dose of ACh (sufficient to induce a 10% maximal contraction) immediately after a large dose. A typical experiment is illustrated in Figure 4. Four consecutive doses of ACh (40 nM) did not result in tachyphylaxis of the contraction (Figure 4a) and the subsequent inhibition of twitch height was greatly potentiated and recovery was protracted. The effect of four consecutive doses of ACh (40 nM) given at 30 s intervals on the magnitude

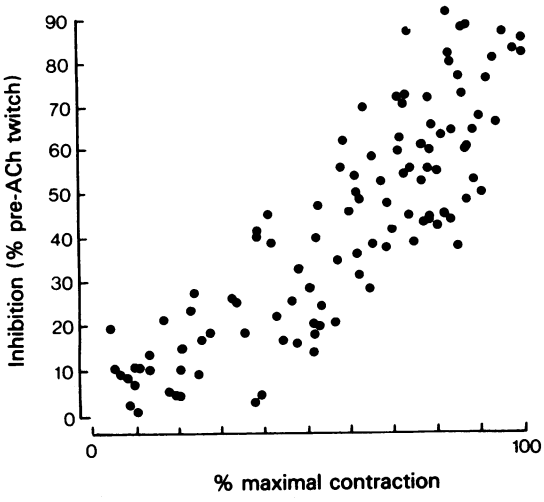

Figure 3 Relationship between % inhibition of the first post-acetylcholine (ACh) twitch response (ordinate scale) and % maximal contraction of the ileum for 108 different doses of ACh. The correlation coefficient (r) = 0.88.

and duration of twitch inhibition could be mimicked by a single dose of ACh (40 nM) left in contact with the tissue for 2 min (4 × 30 s, Figure 4b) or a larger dose (160 nM) left in contact for 30 s (Figure 4c). Doses of ACh that caused very small but definite contractions still exerted their effects when given immediately after a large dose of ACh (Figure 4d).

Influence of sympatholytic drugs on the acetylcholine-induced inhibition of twitch

When the adrenergic neurone blocker, guanethidine, was added to the Krebs solution (final bath concentration 10 μM) and left in contact for 30 min, the relaxation caused by periarterial nerve stimulation was abolished but the response to added ACh and the post-ACh inhibition were unaffected. Noradrenaline (NA) still caused its characteristic relaxation in the presence of guanethidine. Thymoxamine (1 μM), a selective postjunctional α-adrenoceptor antagonist (Drew, 1976) partially inhibited the relaxant responses to NA but did not affect the responses to ACh. Propranolol (1 μM) affected neither the responses to NA nor to ACh.

Effect of naloxone on the post-acetylcholine inhibition of twitch

Inhibition of twitch response to low frequency stimulation has been found to follow an interpolated period of high frequency stimulation (10 Hz; Puig, Gascón, Craviso & Musacchio, 1977); this inhibition was largely reversed by naloxone. In the present experiments in which one tissue was bathed with Krebs containing naloxone (0.01, 0.1 and 1 μM) whilst the other served as a control, there was no difference in the degree of post-ACh inhibition of twitch or of the height of contraction whereas inhibition of twitch following stimulation for 30 s at 5 or 10 Hz was partially reversed by naloxone (Figure 5).

Effect of ganglion blockade on the contractions of the ileum to acetylcholine and on post-acetylcholine inhibition of twitch

Hexamethonium (C_6) abolishes the contractile response of the ileum to nicotine (Brownlee & Johnson, 1963) but has no effect on the contractile response to ACh. In the present experiments a nicotine-blocking concentration of C_6 (0.49 mM) also had no significant effect on the contractions to ACh but significantly increased the post-ACh inhibition of twitch. The pattern of recovery of the twitch was also affected by C_6. The initial rapid recovery was delayed for about 1 min and the overall outline traced by the twitches during recovery was converted from a convex to a concave pattern (Figure 6). Thus in the absence of C_6, the post-ACh inhibition of twitch is a composite response consisting of a C_6-sensitive (nicotinic) enhancement of twitch recovery superimposed on a C_6-resistant inhibition. Thus, the use of C_6 enables these two components to be separated.

The kinetics of recovery from inhibition of twitch in the presence and absence of C_6 are given for three doses of ACh in Figure 7. The areas enclosed by each pair of lines represent the nicotinic component of twitch recovery in the absence of C_6. The higher the dose of ACh, the more important is the nicotinic component of the post-ACh twitch recovery.

The C_6-sensitive nicotinic component (a – b of Figure 6) of the post-ACh twitch recovery has been plotted against the % inhibition of twitch in the presence of C_6 ('a' of Figure 6) in Figure 8. During the first minute the nicotinic component increases dramatically (presumably as the concentration of ACh in the biophase is rapidly decreasing), whereas the inhibition in the presence of C_6 remains relatively constant. Thereafter there is a simple linear relationship between the two parameters (r = 0.996) the three graphs becoming superimposed (slope function = 0.783).

Effect of exogenous 5-hydroxytryptamine and nicotine on the responses of the ileum to electrical stimulation

When nicotine or 5-HT replaced ACh, a dose-related decrease in post-drug twitch height was also observed but, whereas with ACh the first twitch was the one

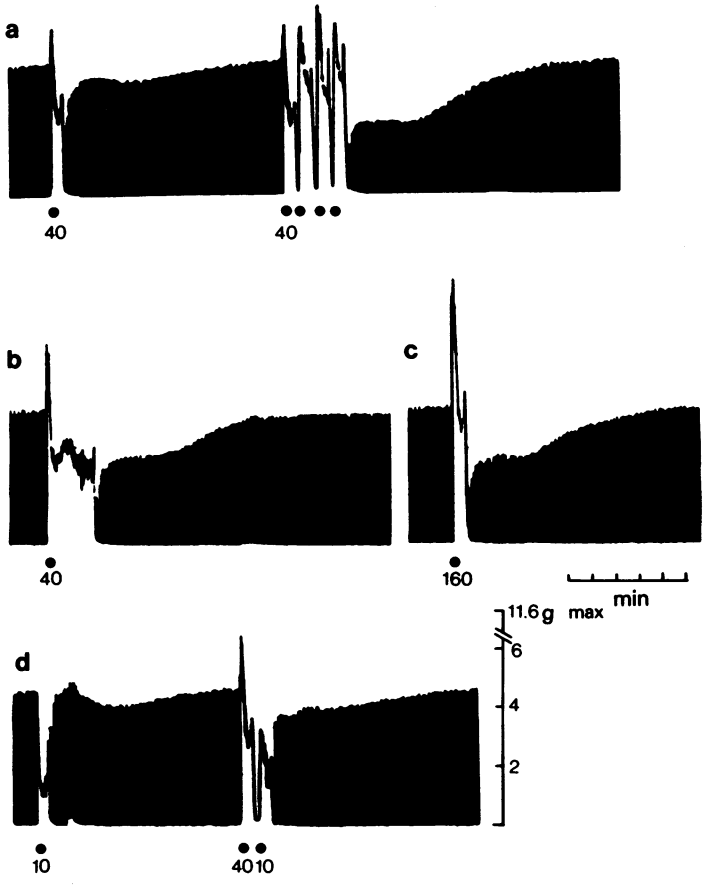

Figure 4 Lack of effect of acetylcholine (ACh) on post-synaptic membrane sensitivity. For explanation of individual panels see text. Dots indicate point of administration of stated doses of ACh (nM).

inhibited the most (Figure 1), after nicotine or 5-HT the early post-drug twitches were often very large and with low doses sometimes exceeded the pre-drug level. Thereafter, there was a progressive inhibition reaching a maximum after approximately 1 min (Figure 9a) to be followed by a gradual recovery during the subsequent 3 to 5 min. High doses of nicotine characteristically had triphasic effects on the twitch response (Figure 9b) consisting of an initial inhibitory component preceding a rapid partial reversal of the inhibition followed by a second inhibitory phase before gradual recovery. Very large doses of nicotine or 5-HT could suppress post-drug twitch response completely whereas complete suppression was very rare with ACh (Figure 10).

Small doses of ACh or nicotine insufficient to contract the ileum caused a marked increase in the twitch response. Potentiation caused by nicotine was prevented by treatment of the ileum with C_6 (0.49 mM) and the potentiation caused by ACh was reduced by at least 50%.

Discussion

The twitch responses of the isolated ileum of the guinea-pig to a train of square wave electrical pulses, when interrupted by the addition of ACh to the bathing medium for 30 s, are inhibited during the 10 min interval following the washout of the ACh (Fosbraey & Johnson, 1978). In the present experiments this inhibition of electrically induced twitch was found to be related to the dose of ACh preceding it and the addition of Krebs solution to the organ bath had no effect on twitch height. The degree of post-ACh inhibition was simply related to the height of

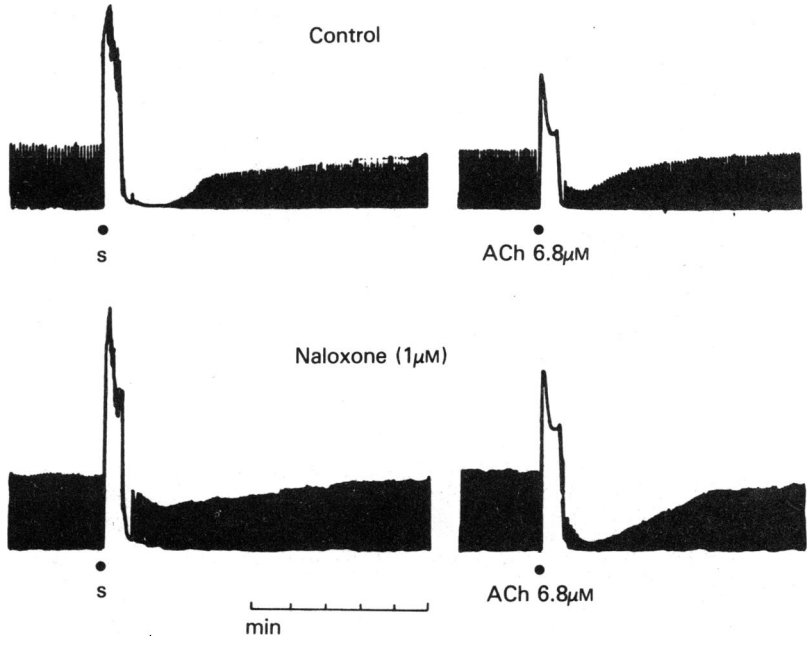

Figure 5 Inhibition of twitch responses to low frequency electrical stmulation (0.2 Hz) by an interpolated 30 s period of high frequency stimulation (10 Hz, S; left hand panel) and by acetylcholine (ACh 6.8 μM, right hand panel) in the presence (lower records) and absence (upper records) of naloxone (1.0 μM). The inhibition of twitch following stimulation at 10 Hz was partially reversed by naloxone whereas the post-ACh inhibition was not.

contraction of the ileum to exogenous ACh indicating that ACh had similar potencies at the receptors responsible for both phenomena. However, the time courses for the two effects were quite different. The contraction (postjunctional effect) caused by the addition of ACh to the organ bath was terminated immediately on exchanging the Krebs solution, whereas the post-ACh inhibition of twitch took some 10 min to be reversed completely.

The twitch response to electrical transmural stimulation of the guinea-pig ileum is known to result from the increased release of ACh from the myenteric plexus (Paton & Zar, 1968). Thus, inhibition of twitch height to exogenous ACh might be due to one of the following: (a) the desensitization of the postjunctional muscarinic receptors for ACh; (b) release of NA from adrenergic nerve terminals by ACh; (c) the direct inhibition of the output of transmitter ACh from the myenteric plexus or the release of another substance which inhibits ACh output.

The inhibition could not be explained on the basis of desensitization of the postjunctional receptor as no decrease in contraction height occurred when the same dose of ACh was added repeatedly immediately after washing out the preceding dose, nor was the contraction produced by a small dose of ACh reduced when given immediately after washing out a large dose.

The post-ACh inhibition was unaffected by the adrenoceptor antagonists thymoxamine and propranolol and the adrenergic neurone blocker, guanethidine. Thus, there was no evidence to suggest that the post-ACh inhibition of twitch was caused by the release of endogenous NA.

The possibility that the exogenous ACh, persisting after the exchange of the bath fluid, was acting on neuronal opiate receptors to inhibit the output of ACh was excluded by the finding that naloxone (0.01, 0.1 and 1 μM) did not modify the inhibitory response in concentrations that readily reverse morphine-induced inhibition of the twitch at low stimulus frequencies (Kosterlitz & Watt, 1968). However, it was of interest to find that the inhibition of twitch following high-frequency stimulation was greatly attenuated by naloxone, an observation also made by Puig *et al.* (1977) and Oka & Sawa (1979) who attributed the naloxone-sensitive inhibition to the release of an endogenous opiate receptor ligand. High frequency stimulation also releases transmitter ACh which, by acting like added ACh, may contribute to the sub-

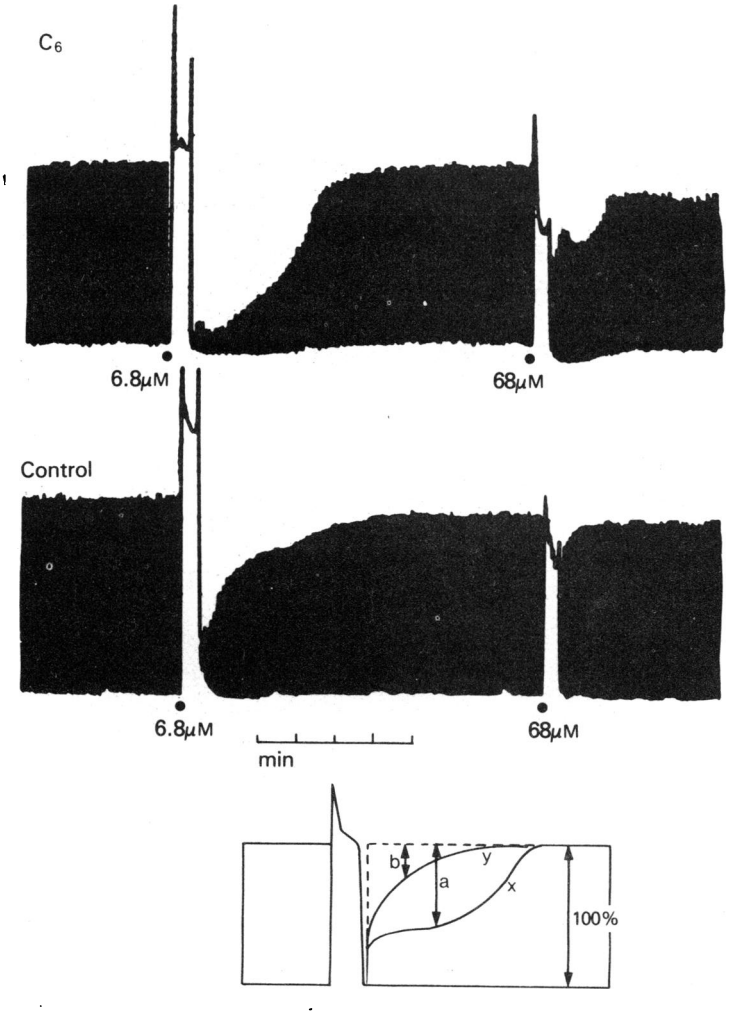

Figure 6 The effects of two doses of acetylcholine (ACh) on twitch height in the presence (upper record) and absence (lower record) of hexamethonium (C_6, 0.49 mM). Note that the overall outline traced by the twitches during recovery was converted from a convex to a concave pattern by C_6. This effectively delayed the rapid phase of recovery following ACh wash-out. The diagram shows super-imposition of the patterns of recovery of the twitch responses in the presence (x) and absence (y) of C_6: a = % inhibition of twitch in the presence of C_6; b = % inhibition of twitch in the absence of C_6; a − b = C_6-sensitive nicotinic component.

sequent inhibition of twitch. This probably explains why naloxone did not completely reverse the inhibition of twitch following high frequency stimulation.

The presence of guanethidine and hexamethonium ruled out contributions due to NA or nicotinic receptor stimulation. Since exogenous ACh-evoked inhibition of twitch is not sensitive to naloxone, this indicates that ACh released on high frequency electrical stimulation is unlikely to play a role in the release of the endogenous opiate receptor ligand.

Thus, the possibility remains that the post-ACh inhibition of twitch results from the activation of release-modulating cholinoceptors. This suggestion is consistent with the observation of Fosbraey & Johnson (1978) that inhibition of the electrically-evoked transmitter [^{3}H]-ACh release can be achieved by superfusion with Krebs solution containing exogenous ACh and that this inhibition can be reversed by atropine. Evidence for the existence of such receptors in the myenteric plexus of the guinea-pig ileum

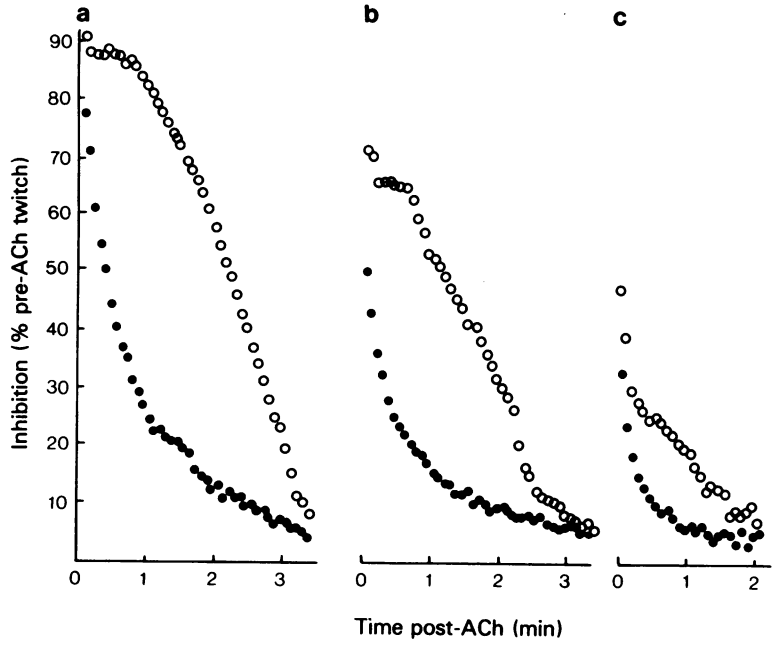

Figure 7 The time course of recovery from inhibition of the post acetylcholine (ACh) twitch response in the presence (○) and absence (●) of C_6 for three doses of ACh: (a) 6.8 μM, (b) 0.68 μM, (c) 0.068 μM. The areas enclosed by each pair of lines represents the nicotinic contribution to twitch recovery i.e. ∫ (a − b) from Figure 6.

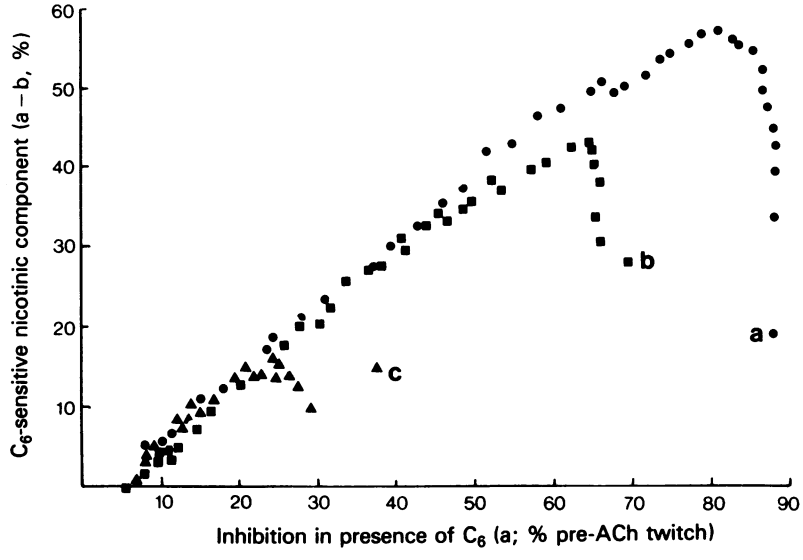

Figure 8 Hexamethonium (C_6)-sensitive nicotinic components (a − b in Figure 6, %, ordinate scale) plotted against % inhibition of twitch response in the presence of C_6 (a in Figure 6, abscissa scale) for each twitch during the recovery from three doses of acetylcholine (ACh) (doses as in Figure 7). Each graph begins at the labelled point (a, b or c) then proceeds to the origin. The right hand side of each graph demonstrates the marked increase in the nicotinic component during the first minute with little or no change in the post-ACh inhibition in the presence of C_6. After this time the two parameters are simply related ($r = 0.996$) and all three graphs become superimposed.

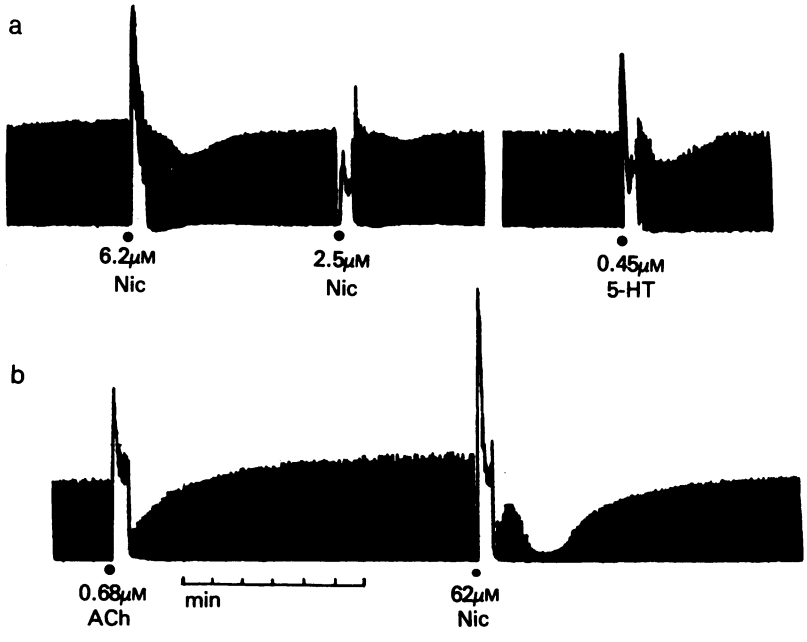

Figure 9 The effects of nicotine and 5-hydroxytryptamine (5-HT) on the twitch responses of guinea-pig ileum to transmural electrical stimulation. The stimulator was turned off during the 30 s drug contact time and switched on again after washing out the drug. (a) Responses to two low doses of nicotine (Nic, 6.2 and 2.5 μM) and one of 5-HT (0.45 μM). Note how the heights of the twitch responses immediately after washing out the drugs were the same as or greater than the pre-drug levels and thereafter were progressively inhibited reaching a maximal inhibition after about 1 min. (b) The characteristic triphasic response to a high dose of nicotine (62 μM) on the twitch response of the ileum to transmural electrical stimulation. A response to a dose of acetylcholine (ACh, 0.68 μM) giving approximately equal inhibitory effect on the first post-ACh twitch is given for comparison.

has also been presented by Kilbinger & Wagner (1975), Kilbinger (1977) and Dzieniszewski & Kilbinger (1978), who showed that the muscarinic agonist, oxotremorine, would inhibit the spontaneous, electrically-evoked and DMPP-induced release of ACh. Sawynok & Jhamandas (1977) found that oxotremorine would not inhibit the electrically-evoked release of ACh from the guinea-pig ileum but it would reduce the increased output of ACh caused by atropine. These investigators also showed that morphine would not reduce the atropine-induced enhancement of ACh release, a finding consistent with the present experiments in which naloxone would not reverse the post-ACh inhibition of twitch.

Since ACh released from the guinea-pig ileum has its origin in the nervous tissue, the inhibitory cholinoceptors are most likely to reside within the myenteric plexus. Additional evidence for the existence of muscarinic receptors on cholinergic neurones is provided by Szerb & Somogyi (1973) who showed that physostigmine, neostigmine, echothiophate or oxotremorine would greatly depress the electrically evoked release of [^{3}H]-ACh from rat cerebral cortical slices; these effects could be prevented by atropine. Release-modulating receptors for ACh have also been identified on sympathetic nerve endings in various tissues (Löffelholz & Muscholl, 1969; Fozard & Muscholl, 1972; Vanhoutte & Shepherd, 1973) but, in the heart, muscarinic agonists were found to have lower affinities for these receptors than for the postjunctional receptors subserving inhibition of atrial tension.

Kilbinger (1977) found that C_6 did not significantly modify the electrically-evoked output of ACh, yet ACh is known to be the classical ganglionic transmitter and ganglionic stimulants like nicotine and DMPP act on C_6-sensitive cholinoceptive receptors to contract the ileum primarily by releasing ACh (Brownlee & Johnson, 1963; 1965). In the absence of electrical stimulation, the contractile responses caused by the action of exogenous ACh on muscarinic receptors of smooth muscle will not be accompanied by a neuronal component, which in the present experiments, is revealed on the twitch responses only after the bath fluid has been changed and the stimulation

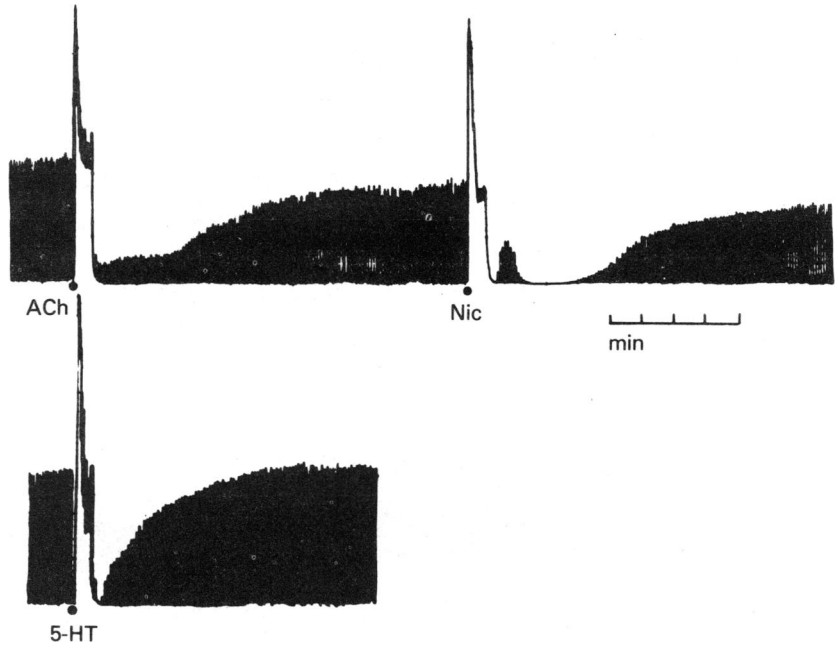

Figure 10 Complete twitch suppression by a high dose of nicotine (Nic 62 μM); this could not be obtained in this experiment with high doses of acetylcholine (ACh 0.68 μM). The lower record is from a different experiment showing complete suppression of twitch response with 5-hydroxytryptamine (5-HT 5.7 μM).

resumed. In this situation the contractile effects of the added ACh are immediately reversed but the inhibitory effect on neuronal responses to electrical stimulation persists for several minutes. The use of C_6 has enabled a division of this neuronal response to ACh into two components, a C_6-sensitive enhancement of post-ACh twitch height and the more dominant C_6-resistant inhibition of twitch. It seems likely that these two components result from the activation of nicotinic and muscarinic neuronal cholinoceptors respectively. The effects of ACh on these two components of the recovery of post-ACh twitch have been quantified. Within the first minute of washing out ACh, the contribution due to stimulation of nicotinic receptors increases markedly while the inhibition due to the activation of muscarinic receptors remains constant, thereafter both the nicotinic enhancement and the muscarinic inhibition diminish and the decreases of both components are simply related.

There are at least two possible explanations why the initial post-ACh nicotinic component increases relative to the muscarinic component at a time when the concentration of ACh in the biophase is presumably declining rapidly. The first would follow from the assumption that in the presence of high extracellular concentration of ACh the twitch response results from the combined effects of transmitter ACh released by electrical stimulation and ACh release evoked by the activation of nicotinic receptors (evoked release theory). The second possible explanation assumes that exogenous ACh does not itself release transmitter ACh but merely modulates the release evoked by electrical stimulation (release modulation theory).

Evoked release theory

This requires that the responses to both ACh-evoked and electrically-evoked ACh release may be suppressed by the simultaneous activation of muscarinic release-modulating receptors. Let us suppose that both prejunctional muscarinic receptors and the nicotinic receptors are equally sensitive to ACh, but that the ease with which muscarinic receptor activation inhibits the output of ACh due to nicotinic receptor activation differs from the ease with which muscarinic receptor activation inhibits the output of ACh due to electrical stimulation. Dzieniszewski & Kilbinger (1978) found that the muscarinic agonist, oxotremorine, would reduce DMPP-induced ACh release but not that due to high $[K^+]$ and it seems likely that the nicotinic receptor activation by ACh will also

approximate more closely to physiologically evoked ACh release than will gross electrical stimulation and thus be more susceptible to the effects of muscarinic inhibition. If this were the case one would expect the component of twitch response due to electrical stimulation to recover from the effects of muscarinic inhibition more rapidly than the twitch component due to nicotinic stimulation. Since the reverse situation is true and the nicotinic component to twitch increases immediately after ACh washout while the muscarinic effect is gradually wearing off, this must indicate that the nicotinic release receptors are more sensitive to ACh than are the muscarinic receptors. Alternatively, on washing out ACh from the tissue the concentration of ACh is maintained higher around the nicotinic receptor than it is around the muscarinic receptor and thus as the muscarinic effect declines the nicotinic agonist effect becomes manifest until, with further decline in the concentration of ACh in the biophase, a point is reached beyond which the results of both nicotinic and muscarinic receptor occupancy are reversed together (Figure 8).

Release modulation theory

However, these experiments provide no evidence that exogenous ACh is itself releasing transmitter ACh throughout the recovery period as no contraction of the ileum occurs after ACh is washed out in the absence of electrical stimulation. Furthermore, even high doses of ACh did not result in a C_6-sensitive component of the contractile response. Thus, the evidence supports the second explanation that electrically evoked transmitter ACh release can be enhanced by the actions of exogenous ACh on a release-modulating nicotinic receptor. Furthermore, if the nicotinic receptors are more sensitive to ACh than muscarinic receptors then a low dose of ACh should activate nicotinic receptors predominantly and, assuming that the two receptor populations are similar in size, then the post-ACh twitch response might even exceed the pre-ACh level. This does sometimes occur as can be seen in Figure 1b in which a small concentration of ACh caused a small post-ACh inhibition of twitch followed by a greatly enhanced twitch height. Doses of ACh or nicotine insufficient to cause a contraction of the ileum will, when added to the transmurally stimulated preparation, potentiate the twitch response. C_6 will abolish the potentiation caused by nicotine and greatly reduce but not abolish that caused by ACh indicating that nicotinic modulatory receptors are sensitive to doses of ACh below the threshold for contraction. The residual response to ACh after C_6 represents summation on postsynaptic receptors of the exogenous ACh with that released on electrical stimulation. From the simple, linear relationship between post-ACh inhibition and contraction to ACh shown in Figure 3 it follows that subcontractile doses of ACh do not result in the activation of the prejunctional inhibitory muscarinic receptors and thus the potentiation of twitch height by these very low doses is not complicated by an inhibitory modulation of transmitter release.

Experiments on the modulation by oxotremorine and atropine of ACh release evoked by electrical stimulation of guinea-pig ileum have largely been performed in the presence of physostigmine (Kilbinger, 1977) and the question has arisen whether the ACh released under physiological conditions in the absence of cholinesterase inhibition can reach inhibitory muscarinic receptors before being hydrolysed. The present experiments have shown that even after concentrations of ACh as low as 1.7 nM it is possible to demonstrate modulation and also with the more physiological situation of the release of ACh by 5-HT, it is possible to demonstrate alteration of post-drug twitch height in the absence of cholinesterase inhibition.

The magnitude of the post-ACh inhibition of twitch depended not only on the dose of ACh but also on the time for which this ACh was kept in contact with the ileum. It is possible that ACh by depolarizing the nerve endings inhibits calcium entry normally evoked by electrical stimulation and thereby reduces the transmitter release.

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References

BROWNLEE, G. & JOHNSON, E.S. (1963). Site of the 5-hydroxytryptamine receptor on the intramural nervous plexus of the guinea-pig isolated ileum. *Br. J. Pharmac. Chemother.*, **21**, 306–322.

BROWNLEE, G. & JOHNSON, E.S. (1965). The release of acetylcholine from the isolated ileum of the guinea-pig induced by 5-hydroxytryptamine and dimethylphenylpiperazinium, *Br. J. Pharmac. Chemother.*, **24**, 689–700.

DREW, G.M. (1976). Effects of α-adrenoceptor agonists and antagonists on pre- and postsynaptically located α-adrenoceptors. *Eur. J. Pharmac.*, **36**, 313–320.

DZIENISZEWSKI, P. & KILBINGER, H. (1978). Muscarinic modulation of acetylcholine release evoked by dimethylphenylpiperazinium and high [K^+] from guinea-pig myenteric plexus. *Eur. J. Pharmac.*, **50**, 385–392.

FOSBRAEY, P. & JOHNSON, E.S. (1978). Presynaptic inhibi-

tion of ACh release from cholinergic neurones in the myenteric plexus of the guinea-pig ileum. *Br. J. Pharmac.*, **62**, 466P.

FOZARD, J.R. & MUSCHOLL, E. (1972). Effect of several muscarinic agonists on cardiac performance and the release of noradrenaline from sympathetic nerves of the perfused rabbit heart. *Br. J. Pharmac.*, **45**, 616–629.

JOHNSON, E.S. (1963). The origin of the acetylcholine released spontaneously from the guinea-pig ileum. *Br. J. Pharmac. Chemother.*, **21**, 555–568.

KILBINGER, H. (1977). Modulation by oxotremorine and atropine of acetylcholine release evoked by electrical stimulation of the myenteric plexus of guinea-pig ileum. *Naunyn-Schmiedebergs Arch. Pharmac.*, **300**, 145–151.

KILBINGER, H. & WAGNER, P. (1975). Inhibition by oxotremorine of acetylcholine resting release from guinea-pig ileum longitudinal muscle strips. *Naunyn-Schmiedebergs Arch. Pharmac.*, **287**, 47–60.

KOSTERLIZ, H.W. & WATT, A.J. (1968). Kinetic parameters of narcotic agonists and antagonists, with particular reference to N-allylnoroxymorphone (naloxone). *Br. J. Pharmac. Chemother.*, **33**, 266–276

LÖFFELHOLZ, K. & MUSCHOLL, E. (1969). A muscarinic inhibition of noradrenaline release evoked by postganglionic sympathetic nerve stimulation. *Naunyn-Schmiedebergs Arch. Pharmac.*, **265**, 1–15.

OKA, T. & SAWA, A. (1979). Calcium requirements for electrically-induced release of an endogenous opiate receptor ligand from the guinea-pig ileum. *Br. J. Pharmac.*, **65**, 3–5.

PATON, W.D.M. & ZAR, M.A. (1968). The origin of acetylcholine released from the guinea-pig ileum and longitudinal muscle strips. *J. Physiol.*, **194**, 13–33.

PUIG, M.M., GASCÓN, P., CRAVISO, G.L. & MUSACCHIO, J.M. (1977). Endogenous opiate receptor ligand: electrically induced release in the guinea-pig ileum. *Science, N.Y.*, **195**, 419–420.

SAWYNOK, J. & JHAMANDAS, K. (1977). Muscarinic feedback inhibition of acetylcholine release from the myenteric plexus of guinea-pig ileum and its status after chronic exposure to morphine. *Can. J. Physiol. Pharmac.*, **55**, 909–916.

SZERB, J.C. & SOMOGYI, G.T. (1973). Depression of acetylcholine release from cerebral cortical slices by cholinesterase inhibition and by oxotremorine. *Nature, New Biol.*, **241**, 121–122.

VANHOUTTE, P.M. & SHEPHERD, J.T. (1973). Venous relaxation caused by acetylcholine acting on sympathetic nerves. *Circulation Res.*, **32**, 259–267.

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