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Differentiation between the actions of acetylcholine and tetramethylammonium on the isolated taenia of the guinea-pig caecum by hemicholinium-3

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Summary

1. Contractions of the isolated taenia of the guinea-pig caecum produced by acetylcholine and TMA were examined in the presence of various antagonists and anticholinesterases.

2. Hemicholinium-3 (HC-3) (50–400 μg/ml) inhibited contractions or relaxations produced by TMA but not contractions produced by acetylcholine. The inhibition was rapid in onset and readily reversible. Contractions produced by transmural stimulation were unaffected by HC-3 but responses produced by nicotine were inhibited.

3. Low concentrations of hyoscine and benzhexol inhibited responses to acetylcholine to a greater extent than those to TMA.

4. Morphine, raised concentrations of Mg^{++} or reduced concentrations of Ca^{++} inhibited contractions produced by TMA and by acetylcholine to a similar extent.

5. Edrophonium, in concentrations which preferentially inhibit acetylcholinesterase, increased contractions produced by acetylcholine and converted responses to nicotine or transmural stimulation into contractions or biphasic responses with a marked contraction phase but did not increase contractions produced by TMA.

6. *Iso*-OMPA, in concentrations which preferentially inhibit butyrylcholinesterase, had no effect on responses to acetylcholine, nicotine, transmural stimulation or TMA.

7. HC-3 inhibited contractions produced by TMA in the presence of anticholinesterases but had little effect on contractions produced by acetylcholine.

8. These results suggest that TMA produces contractions by acting directly on receptors of the smooth muscle. An analysis of possible reasons for HC-3 (in the concentrations used) acting as an antagonist of TMA but not of acetylcholine indicates that the findings do not necessarily contradict the interpretation that both agonists act on the same receptor.

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Introduction

Contractions of the taenia of the guinea-pig caecum produced by acetylcholine or tetramethylammonium (TMA) appear to be due to an action on 'muscarinic' receptors on the smooth muscle because they are inhibited by hyoscine and not by non-depolarizing ganglion blocking agents, tetrodotoxin, cinchocaine or cocaine (Hobbiger, Mitchelson & Rand, 1969). In addition, high concentrations of TMA occasionally produce a biphasic response consisting of a relaxation followed by a contraction. High concentrations of acetylcholine produce a similar response in the presence of hyoscine. The relaxation is due to an action on 'nicotinic' receptors of ganglion cells since it is inhibited by ganglion blocking drugs, tetrodotoxin and local anaesthetics (Akubue, 1966; Hobbiger *et al.*, 1969).

The experiments reported here show that the contractions produced by TMA are inhibited by hemicholinium (HC-3) whereas those produced by acetylcholine are not. Additional investigations involving the acetylcholine antagonists, hyoscine and benzhexol, selective inhibition of acetylcholinesterase and butyrylcholinesterase, nicotine, transmural stimulation, and modifications of acetylcholine release failed to provide evidence that the differential antagonism by HC-3 towards TMA and acetylcholine is a manifestation of the two agonists combining with different receptors.

Methods

Isolated tissue experiments

Adult guinea-pigs of either sex weighing 300–800 g were killed by a blow on the head and a taenia was dissected from the caecum. Preparations consisting of a length of 2–3 cm (unstretched) were suspended in McEwen's solution (McEwen, 1956) in a thermostatically controlled organ bath (10 ml capacity) at 37° C. The bath fluid was gassed with oxygen containing 5% carbon dioxide. Responses were recorded on a smoked drum with an isotonic frontal writing lever producing approximately a 6-fold magnification and exerting a tension of 1 g.

Agonists were added to the organ bath in volumes of 0·1–0·5 ml and remained in contact with the tissue for 30–60 s unless otherwise stated. The tissue was then washed by overflow for 10 s during which time 40–50 ml of the McEwen's solution passed through the organ bath. The interval between additions of agonists was 4 min or more. Antagonists were added to the organ bath initially 10 min before their effect on an agonist was tested but with hyoscine or benzhexol this interval was 15 minutes. Thereafter, antagonists were added immediately after each washout of the agonist. A procedure similar to that used for antagonists was adopted for the anticholinesterase edrophonium. When *iso*-OMPA, an irreversible inhibitor of cholinesterase was used, the anticholinesterase was left in contact with the tissue for 10 min, after which time the preparations were washed several times. Responses to agonists were then redetermined without further addition of *iso*-OMPA.

For transmural stimulation the lower end of the taenia was passed through bipolar electrodes (Burn & Rand, 1960) which did not hinder movement of the preparation. A Palmer square-wave electronic stimulator was used for stimulation. The frequency of stimulation was varied from 1 to 100 Hz, the pulse width was maintained at 0·1 ms to avoid direct stimulation of the smooth muscle (Bennett,

Burnstock & Holman, 1966), the voltage was supramaximal and the duration of stimulation ranged from 10 to 40 s with intervals of not less than 3 min between periods of stimulation.

The composition of McEwen's solution (g/l.) was: NaCl, 7·6 ; KCl, 0·42 ; $CaCl_2$, 0·24 ; $NaHCO_3$, 2·1 ; NaH_2PO_4, 0·143 ; glucose, 2·0 ; sucrose, 4·5.

To investigate the effect of Mg^{++} a Krebs solution was used. The composition of Krebs solution (g/l.) was: NaCl, 6·92 ; KCl, 0·35 ; $CaCl_2$, 0·28 ; $NaHCO_3$, 2·1 ; KH_2PO_4, 0·16 ; $MgSO_47H_2O$, 0·29 ; glucose, 2·0.

As the taenia may show considerable fluctuations in tone, dose-response curves obtained for agonists were corrected for changes in tone by the method described previously (Hobbiger *et al.*, 1969).

Selection of inhibitors of cholinesterases

Cholinesterase activities were determined in the Warburg apparatus at 37° C in an atmosphere of nitrogen containing 5% carbon dioxide. The source of butyrylcholinesterase was guinea-pig plasma and of acetylcholinesterase was haemolysed guinea-pig erythrocytes. Both types of cholinesterase are present in the taenia, but in concentrations lower than those in the blood. Acetylcholine chloride, final concentration 10 mM, was used as substrate. All dilutions were made with 25 mM $NaHCO_3$ and the final volume of the reaction mixture was 3 ml. The first reading was taken 5 min after adding the substrate from the sidearm to the enzyme in the main compartment and thereafter the manometers were read at 10 min intervals.

Enzyme activities were calculated from the volumes of carbon dioxide produced from 5 to 35 min after addition of substrate to the enzyme and corrected for non-enzymic hydrolysis of substrate, enzyme blanks and variations in temperature and atmospheric pressure.

Edrophonium inhibited acetylcholinesterase preferentially ; it reduced the activity of this enzyme by 50% (I_{50}) at a concentration of 4·6 μg/ml whereas the I_{50} for butyrylcholinesterase was 288 μg/ml. Inhibition by edrophonium had the characteristics of a readily reversible reaction and thus was inversely related to substrate concentration. In manometric experiments, relatively large substrate concentrations are used as compared with the concentrations of acetylcholine used in the organ bath. Consequently, the I_{50} under non-competitive conditions, which can be calculated from the observed I_{50}, is a better measurement of the inhibition obtained in isolated organs. Calculations of the I_{50} under non-competitive conditions gave values of 0·2 μg/ml and 58 μg/ml for acetylcholinesterase and butyrylcholinesterase, respectively.

Iso-OMPA inhibits butyrylcholinesterase preferentially (Aldridge, 1953) ; the I_{50} for this enzyme was 3·1 μg/ml whereas 10 μg/ml had no effect on acetylcholinesterase activity. Inhibition by *iso*-OMPA was progressive with time and was arrested but not reversed by substrate. Thus the observed I_{50} values themselves can be taken as a guide to the inhibition obtained in isolated organs. HC-3 was a weak reversible inhibitor of butyrylcholinesterase (observed I_{50}, 4 mg/ml) and was recorded with no effect on acetylcholinesterase in concentrations of 2 mg/ml.

Drugs

The drugs used were acetylcholine chloride (B.D.H.), AHR-602 (N-benzyl-3-pyrrolidyl acetate methobromide) (Robins), carbamylcholine chloride (B.D.H.),

histamine acid phosphate (B.D.H.), McN-A-343 [4-(*m*-chlorophenyl-carbamoyloxy)-2-butynyltrimethylammonium chloride] (McNeil), nicotine hydrogen tartrate (B.D.H.), propionylcholine chloride (Koch-Light), tetramethylammonium iodide (B.D.H.), benzhexol hydrochloride (Lederle), hemicholinium-3 bromide (Aldrich), hyoscine hydrobromide (Allen & Hanbury), morphine sulphate (B.D.H.), tetrodotoxin (Sankyo), edrophonium chloride (Roche), *iso*-OMPA (tetramono*iso*propyl pyrophosphortetramide). All concentrations given in the text refer to the concentration of the above compounds in the organ bath.

Results

Hemicholinium-3 (HC-3)

The tone of preparations was not affected by HC-3 in concentrations up to 400 μg/ml but sometimes the amplitude of spontaneous movements of preparations with a low tone was increased.

HC-3 (50–400 μg/ml) did not inhibit contractions produced by acetylcholine even when alterations in tone were taken into consideration (Fig. 1). However, the dose-

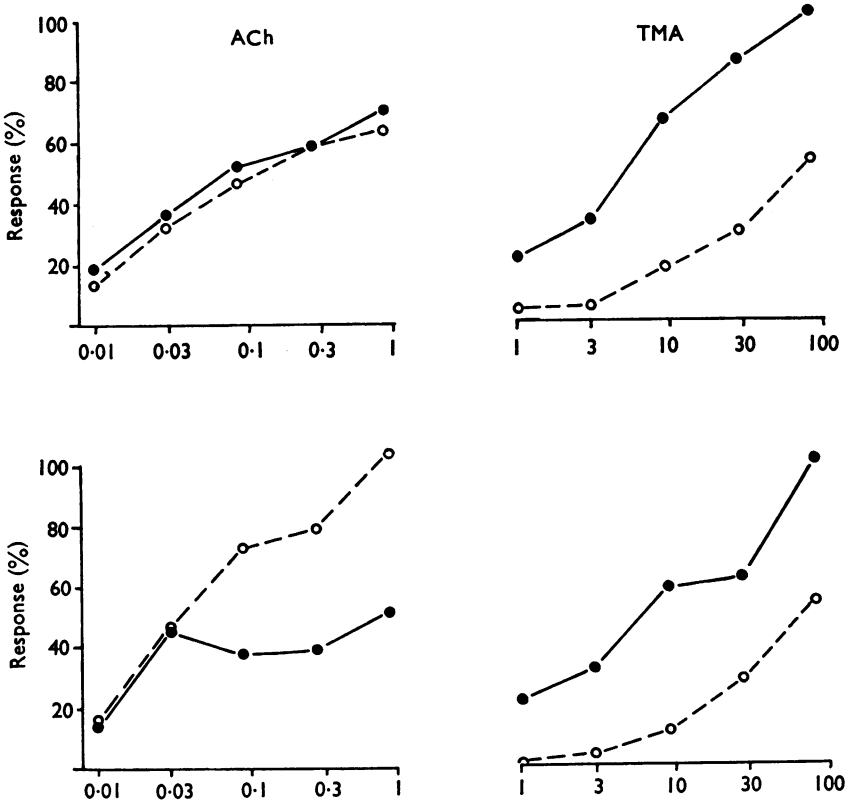

FIG. 1. Corrected (upper) and recorded (lower) dose-response curves for acetylcholine (ACh) and TMA in the absence (●——●) and presence (○- - -○) of hemicholinium (200 μg/ml). Each point is the mean of three experiments. Responses to ACh were obtained from the same preparations as those to TMA and are expressed as a percentage of the response to the highest concentration of TMA. The abscissa expresses the concentrations of agonists (μg/ml) on a logarithmic scale. In order to eliminate modifications in height of contractions arising from fluctuations in tone of the preparation contraction heights were corrected for changes in tone by the method described by Hobbiger *et al.* (1969).

response curve for contractions produced by TMA was shifted progressively to the right by increasing concentrations of HC-3 (50–200 μg/ml). Figure 1 shows the inhibition obtained with 200 μg/ml. The effect of HC-3 on contractions produced by TMA developed immediately and could be reversed readily by washing. The response to TMA was also inhibited by HC-3 when the agonist was added before HC-3. For example, TMA (9 μg/ml) was allowed to remain in contact with a preparation for 6 minutes. The contraction produced by TMA reached a maximum in 1 min and there was only slight 'fading' of the response throughout the next 5 minutes. However, if HC-3 (200 μg/ml) was added 1 min after TMA when the response to TMA had fully developed there was a rapid relaxation of the preparation to the initial level of tone. HC-3 (200 μg/ml) also inhibited contractions produced by TMA in the presence of tetrodotoxin (0·1 μg/ml).

When TMA produced a biphasic response, the relaxation phase was abolished and the contraction phase was markedly reduced by HC-3 (200 μg/ml).

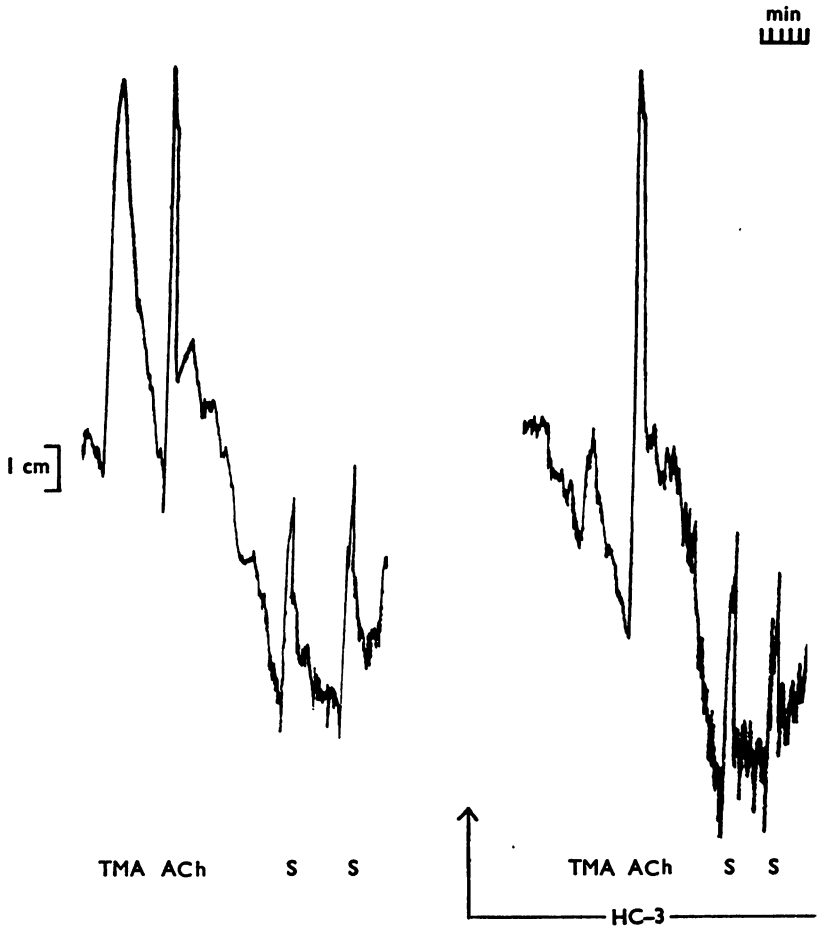

FIG. 2. Effect of hemicholinium (HC-3; 200 μg/ml) on responses to TMA (10 μg/ml), acetylcholine (ACh; 0·8 μg/ml) and to transmural electrical stimulation of the taenia at 20 Hz for 40 seconds (S). Interval between time marks, 1 minute. Vertical scale, 1 cm on kymograph tracing.

Responses to nicotine, whether relaxation or contraction, were abolished by HC-3 (200 μg/ml). Contractions produced by transmural stimulation were not affected by HC-3 (200 μg/ml) (Fig. 2). Relaxations produced by transmural stimulation were either not affected by HC-3 (200 μg/ml) or were altered to a biphasic response of relaxation followed by contraction.

Contractions produced by histamine were not affected by HC-3 (200 μg/ml) indicating that the contractility of the muscle was not affected.

Morphine

Contractions produced by acetylcholine or TMA were reduced only slightly by morphine (1–100 μg/ml) (Fig. 3). This reduction was largely due to an increase in the tone of preparations produced by morphine. After corrections for changes in tone were made the reduction in sensitivity was not greater than 1·5-fold for either agonist. Contractions produced by nicotine, however, were markedly reduced although not abolished by morphine (1–100 μg/ml) (Fig. 3).

Increase in Mg^{++}

A 4-fold increase in the Mg^{++} concentration of Krebs solution caused a slight fall in the tone of the preparation and slightly inhibited contractions produced by acetyl-

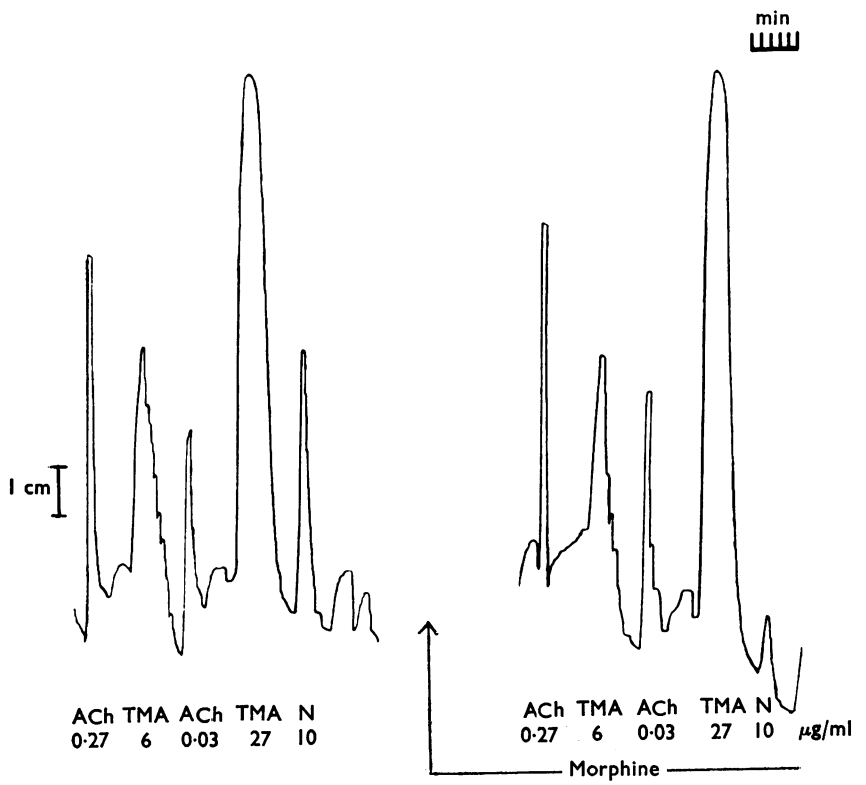

FIG. 3. Effect of morphine (1 μg/ml) on contractions produced by acetylcholine (ACh), TMA, and nicotine (N). The numbers indicate concentrations (μg/ml). Interval between time marks, 1 minute. Vertical scale, 1 cm on kymograph tracing.

choline or TMA, the change in sensitivity being not greater than 1·5-fold for both agonists. A 10-fold increase in the Mg^{++} concentration caused a further shift to the right of the corrected dose-response curves for acetylcholine and TMA ; this effect was greater with acetylcholine.

Reduction of Ca^{++}

A reduction in the Ca^{++} concentration of McEwen's solution by 90% caused comparable shifts to the right of the corrected dose-response curves for acetylcholine and TMA, sensitivity decreasing by a factor of 1·5–2 for both agonists.

Hyoscine

The dose-response curve for acetylcholine was shifted to the right by hyoscine (0·001 μg/ml) to a greater extent than that for TMA (Fig. 4). Increases in the

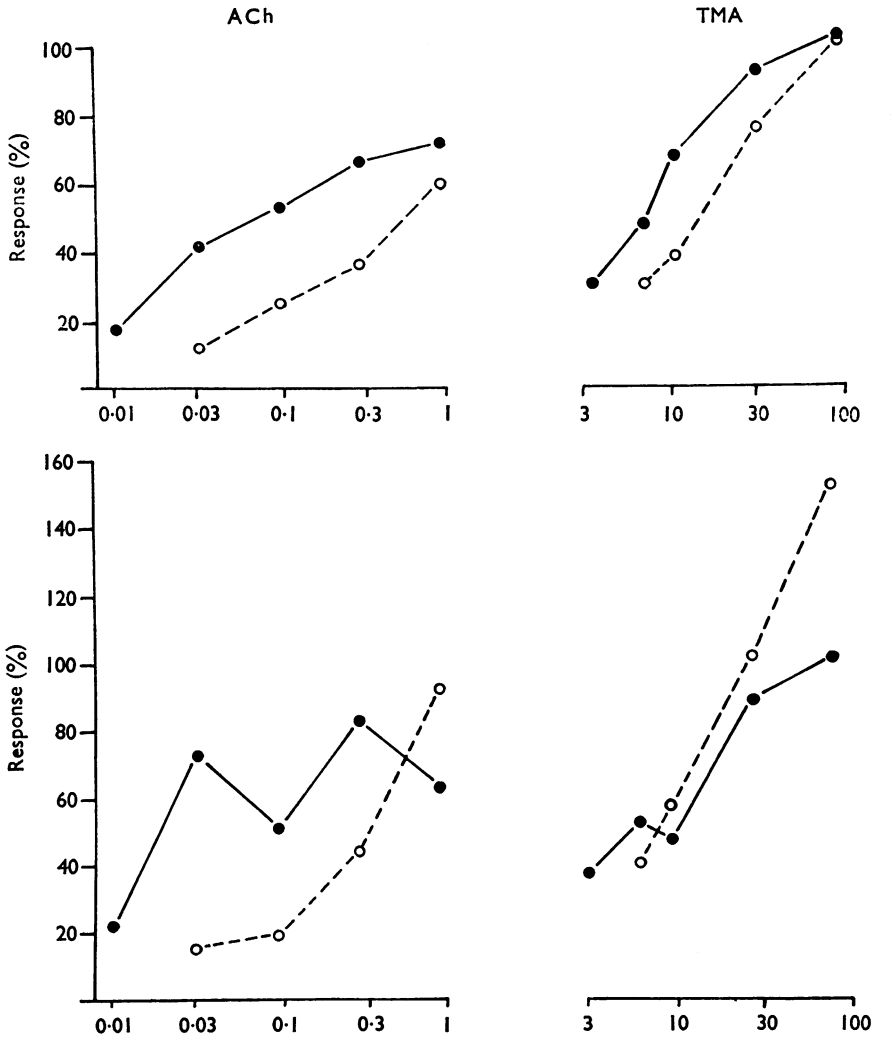

FIG. 4. Corrected (upper) and recorded (lower) dose-response curves for acetylcholine (ACh) and TMA in the absence (●——●) and presence (○- - -○) of hyoscine (0·001 μg/ml). Each point represents the mean of three experiments. Other details as in Fig. 1.

concentration of hyoscine (0·003 or 0·01 μg/ml) caused further progressive shifts of the dose-response curves.

A relaxation phase appeared in the response to high concentrations of TMA (27–81 μg/ml) in the presence of hyoscine.

Benzhexol

Benzhexol (0·003–0·03 μg/ml) produced effects similar to hyoscine on the dose-response curves for acetylcholine and TMA. As TMA produced biphasic responses in the presence of benzhexol too, additional experiments with benzhexol were performed in the presence of tetrodotoxin (0·1 μg/ml) which abolished the relaxation. This procedure did not alter the effect of benzhexol on the dose-response curves.

Edrophonium

Contractions produced by acetylcholine were augmented by edrophonium (1–10 μg/ml) (Fig. 5). In preparations with a low tone the increase in the response to acetylcholine was marked, but often edrophonium increased the tone and then the recorded augmentation was less pronounced. Responses to TMA were not affected by edrophonium when tone changes were taken into consideration (Fig. 5).

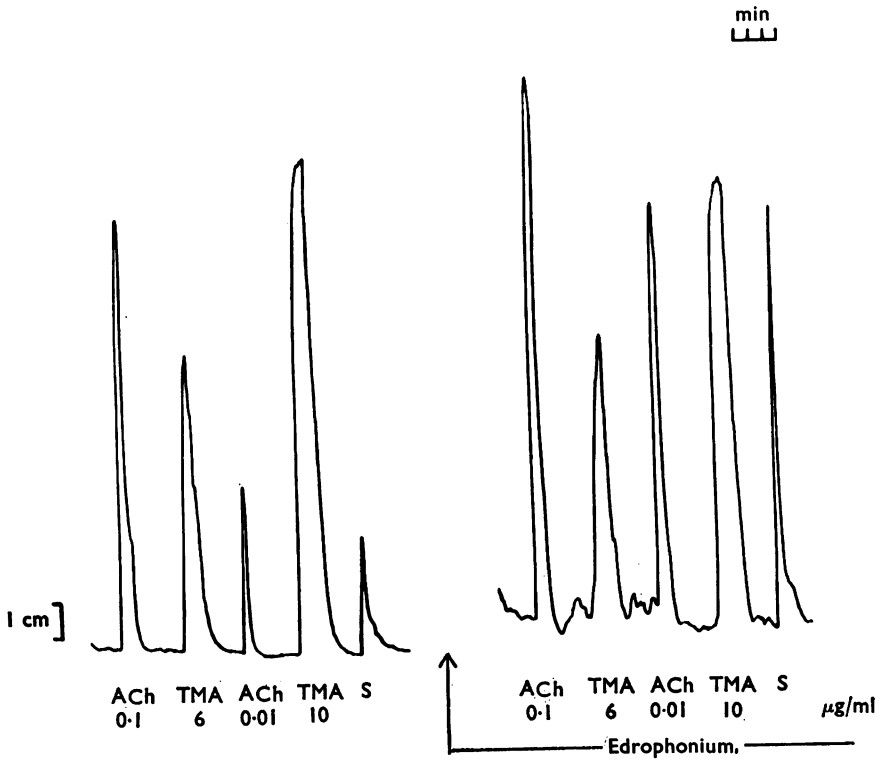

FIG. 5. Effect of edrophonium (1 μg/ml) on responses to transmural electrical stimulation (S) at 20 Hz for 15 s, acetylcholine (ACh) and TMA. The numbers indicate concentrations (μg/ml). Interval between time marks, 1 minute. Vertical scale, 1 cm on kymograph tracing.

With nicotine as the agonist, edrophonium (1–10 μg/ml) enhanced the contraction phase of biphasic responses and converted relaxations into biphasic responses. Edrophonium (1–10 μg/ml) also reversed the effect of transmural stimulation from a relaxation to a contraction. The extent of the reversal and the size of the contraction produced was related to the frequency of stimulation. Low frequency stimulation in the presence of edrophonium often elicited an initial transient relaxation followed by contraction whereas higher frequencies caused only contraction.

Iso-*OMPA*

Iso-OMPA (5–50 μg/ml) had no effect on the tone of the preparation and produced no change in the responses to acetylcholine or TMA. The relaxations pro-

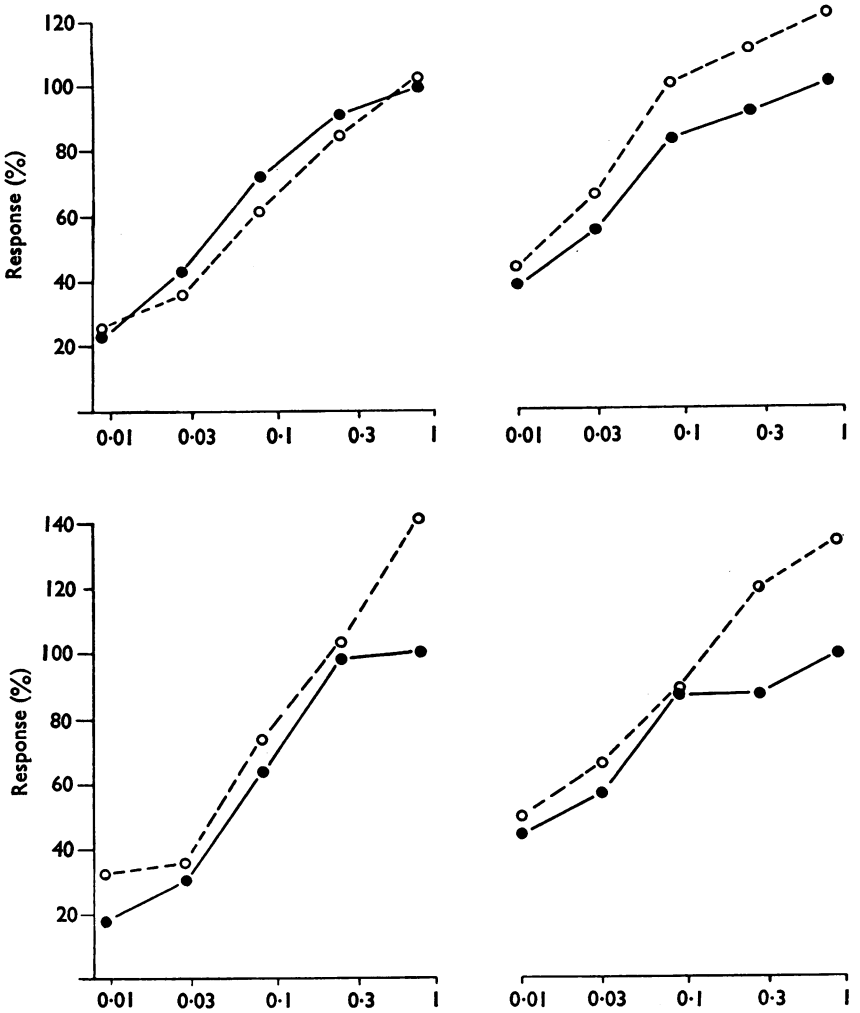

FIG. 6. Corrected (upper) and recorded (lower) dose-response curves for acetylcholine in the absence (●——●) and presence (○- - -○) of hemicholinium (200 μg/ml). The curves on the left side were obtained when edrophonium (1 μg/ml) was present throughout the experiment and those on the right side were obtained in a different series of experiments after pretreatment with *iso*-OMPA (50 μg/ml for 10 min). Responses are expressed as a percentage of the response obtained with the highest concentration of acetylcholine. Each point represents the mean of three experiments. Other details as in Fig. 1.

duced by nicotine or by transmural stimulation were not affected by *iso*-OMPA (1–50 μg/ml).

HC-3 in the presence of anticholinesterases

Dose-response curves (corrected for changes in tone) for acetylcholine in the presence of edrophonium (1–10 μg/ml) were not affected or were shifted only slightly to the right by HC-3 (200 μg/ml) (Fig. 6). When the taenia was treated with *iso*-OMPA (50 μg/ml) for 10 min or if such treatment was followed by the addition of edrophonium (10 μg/ml) to the organ bath, HC-3 (200 μg/ml) shifted the dose-response curve for acetylcholine slightly to the left (Fig. 6).

On the other hand, HC-3 (200 μg/ml) inhibited contractions produced by TMA in the presence of edrophonium (10 μg/ml) or after treatment with *iso*-OMPA (50 μg/ml) to the same extent as it did in the absence of the anticholinesterases.

HC-3 on contractions produced by other agonists

HC-3 (200 μg/ml) was tested on contractions of the taenia produced by a number of other cholinomimetic drugs which act on receptors on smooth muscle and which are inhibited by hyoscine (Hobbiger *et al.*, 1969 ; Mitchelson, 1967). Contractions of the taenia produced by propionylcholine were unaffected by HC-3 but contractions produced by carbamylcholine, McN-A-343 or AHR-602 were inhibited by HC-3.

Discussion

Acetylcholine and TMA produce contractions of the taenia which are inhibited by hyoscine but not by hexamethonium or tetrodotoxin (Hobbiger *et al.*, 1969) ; this observation suggests that their site of action is on ' muscarinic ' receptors at the nerve-smooth muscle junction. The experiments reported in this paper show that HC-3 in concentrations of up to 400 μg/ml inhibited contractions produced by TMA but not those produced by acetylcholine. In the taenia, therefore, HC-3 has no atropine-like action (that is, antiacetylcholine activity) which has been found in other tissues (Bertolini, Greggia & Ferrari, 1967 ; György, Pfeifer & Kenyeres, 1970).

HC-3 inhibited contractions and relaxations produced by nicotine and also relaxations produced by higher concentrations of TMA. These effects of HC-3 are analogous to those obtained with hexamethonium (Hobbiger *et al.*, 1969) and suggest that HC-3 may also have a blocking action on ' nicotinic ' receptors of ganglion cells. A ganglion blocking action for HC-3 has been noted by Beck (quoted by Leaders & Pan, 1967).

In an attempt to explain the difference between the actions of HC-3 on responses to acetylcholine and to TMA the following possibilities must be considered: (1) TMA causes the release of cholinergic transmitter from excitatory nerve endings and HC-3 inhibits this action ; (2) HC-3 has both an anticholinesterase activity and an atropine-like activity ; (3) two types of muscarinic receptor are present ; (4) both acetylcholine and TMA combine with the same receptor but receptor occupancy by the agonist is not determined solely by an uncomplicated equilibrium between agonist, antagonist and receptor and for this reason HC-3 affects the action of agonists to a different extent.

With reference to the first possibility it has been suggested that TMA, like carbamylcholine, acts on preganglionic nerve endings to release transmitter (Volle & Koelle, 1961 ; Koelle, 1962 ; McKinstry, Koenig, Koelle & Koelle, 1963 ; McKinstry, 1965 ; McKinstry & Koelle, 1967). In addition György *et al.* (1970) concluded that HC-3 blocked the response of the rat bladder to carbamylcholine by preventing carbamylcholine from releasing tissue stores of acetylcholine. The contractions of the taenia produced by TMA and acetylcholine were not affected by tetrodotoxin (Hobbiger *et al.*, 1969) and were inhibited to a similar extent by a reduction in Ca^{++} or an increase in Mg^{++}. If TMA were acting by releasing acetylcholine from nerve terminals a selective blockade of its action by these agents should have been observed. Moreover, the response to TMA was not affected by edrophonium whereas contractions produced by nerve stimulation, nicotine and acetylcholine were increased. The time course of the blockade by HC-3 of contractions produced by TMA differed from the well known slow onset of blockade of cholinergic nerve stimulation. First, the onset of blockade of responses to TMA was immediate and blockade occurred when there was no effect on nerve stimulation. Second, the blockade was readily reversible by washing and this is in contrast to the gradual and often partial return of responses to nerve stimulation after blockade by HC-3 (Rand & Ridehalgh, 1965).

The second possibility to be considered is that HC-3 causes a blockade of the 'muscarinic' receptor and also inhibits cholinesterases. Thus, TMA would be affected only by the blockade of the 'muscarinic' receptor whereas acetylcholine would be influenced by both actions and remain more effective. However, this possibility does not appear likely in view of the observation that HC-3 has only low anticholinesterase activity and the action of acetylcholine, unlike that of TMA, is not reduced by HC-3 after inhibition of cholinesterases by edrophonium, *iso*-OMPA or a combination of both.

The most likely interpretation concerning the site of action of acetylcholine and TMA is that they act upon the 'muscarinic' receptor. This receptor is thought to contain an anionic site together with at least two other binding sites (electrophilic site) (Barlow, 1964 ; Bebbington, Brimblecombe & Rowsell, 1966). Although the anionic site appears to play a major role in the binding of the cationic group in TMA and choline esters (Burgen, 1965) the electrophilic site is also important for the action of acetylcholine. Schild (1960) found that he could inhibit the effect of acetylcholine but not that of TMA on the guinea-pig ileum by the use of diazotized sulphanilic acid or polyethyleneimine. He concluded from this that these antagonists blocked the electrophilic site but left the anionic site unaffected.

The observation that hyoscine or benzhexol did not inhibit TMA to as great an extent as acetylcholine is similar to the findings of Clark & Raventos (1937) using atropine, although the difference obtained with atropine was not as marked. Atropine-like drugs probably combine with the 'muscarinic' receptor at sites other than the anionic site (Burgen, 1965). It is therefore reasonable to assume that hyoscine inhibits contractions produced by acetylcholine to a greater extent than contractions produced by TMA, because the former combines with the anionic and electrophilic sites of the receptor whereas the latter only combines with the anionic site. In the case of HC-3, contractions produced by TMA could be more affected than those produced by acetylcholine because HC-3 is likely to combine predominantly with the anionic site.

The possibility that access of acetylcholine to the receptor with which TMA combines is prevented by the presence of cholinesterases is ruled out since HC-3 did not have a different effect in the presence of anticholinesterases. Burgen & Spero (1968) have suggested that there may be two types of 'muscarinic' receptor in the longitudinal muscle in the small intestine of the guinea-pig and that combination with one type accounts for contractions by acetylcholine and carbamylcholine whereas combination with the other type accounts for contractions by TMA. The results reported in this paper suggest that the effect of HC-3 on contractions produced by carbamylcholine is similar to its effect on contractions by TMA and thus it seems unlikely that the differential inhibition towards acetylcholine and TMA by HC-3 can be explained on the basis of the two receptors postulated by Burgen & Spero.

Carbamylcholine, McN-A-343 and AHR-602 are three cholinomimetic drugs which act on the taenia, like TMA. They all produce a contraction which reaches a maximum slowly and the response is inhibited by hyoscine but not by hexamethonium or tetrodotoxin (Hobbiger *et al.*, 1969). HC-3 inhibited the contraction produced by carbamylcholine, McN-A-343 and AHR-602, an observation which suggests that they also act on the taenia in a different manner from acetylcholine.

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