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ACTIONS OF VARIOUS MUSCARINIC AGONISTS ON MEMBRANE POTENTIAL, POTASSIUM EFFLUX, AND CONTRACTION OF LONGITUDINAL MUSCLE OF GUINEA-PIG INTESTINE

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1 Depolarizations were recorded intracellularly in smooth muscle from the taenia of the guinea-pig caecum in response to the iontophoretic application of acetylcholine, carbachol, oxotremorine-M, methylfurmethide, hexyl trimethylammonium and tetramethylammonium (TMA).

2 No differences between the iontophoretic responses to agonists stable to cholinesterase were detected.

3 The latency and time to peak of acetylcholine-induced depolarizations were less than those to stable agonists, and the response was briefer and less complex in shape. These differences were reduced, or disappeared, upon inhibition of cholinesterase.

4 The rate of loss of ^{42}K and changes in length were measured in superfused strips weighing about 10 mg of separated longitudinal muscle of guinea-pig ileum.

5 Acetylcholine, carbachol, methylfurmethide, butyltrimethylammonium and TMA contracted the muscle and increased the rate of loss of ^{42}K.

6 Contrary to previous reports, no evidence of a selective action of any of these agonists on ^{42}K loss was detected. TMA appeared to be a partial agonist in evoking ^{42}K loss, although it produced a maximum contraction.

7 The maximum ^{42}K efflux produced by acetylcholine was about 40% of the maximum evoked by application of carbachol or methylfurmethide. If cholinesterase was inhibited, the ^{42}K efflux evoked by maximally effective concentrations of acetylcholine was comparable to that evoked by a stable agonist.

8 These results are consistent with the idea that the muscarinic agonists used interact in an essentially similar way with muscarinic receptors to produce their effects on membrane potential, permeability, and tension.

Introduction

Studies of the binding of radioactively labelled muscarinic agonists to longitudinal smooth muscle of ileum, or to other tissues rich in muscarinic receptors, have suggested that two types of specific muscarinic binding site exist. Potent agonists, such as acetylcholine, carbachol, methylfurmethide, oxotremorine-M or (+/−)-*cis*-methyldilvasene bind to intact smooth muscle (Young, 1974; Taylor, Cuthbert & Young, 1975; Ward & Young, 1977), its homogenates (Beld & Ariens, 1974; Yamamura & Snyder, 1974; Snyder, Chang, Kuhar & Yamamura, 1975; Kloog, Sachs, Korczyn, Heron & Sokolovsky, 1979) or homogenates of cerebral cortex (Burgen, Hiley & Young, 1974b; Snyder *et al.*, 1975; Hulme, Burgen & Birdsall, 1975; Birdsall, Burgen, Hiley & Hulme, 1976; Kloog & Sokolovsky, 1977; Birdsall, Burgen & Hulme, 1978a), but the way this binding varies with concentration of agonist is not consistent with the idea that there is a single homogeneous class of specific muscarinic binding sites to which these agonists bind according to the Law of Mass Action. Hill plots of the binding are not straight lines with slopes of unity, as is the case with muscarinic antagonists (Hulme *et al.*, 1975; Taylor *et al.*, 1975; Birdsall & Hulme, 1976; Birdsall *et al.*, 1976; Ward & Young, 1977; Hulme, Birdsall, Burgen & Mehta, 1978; Kloog *et al.*, 1979) but are curves with a variable slope which may be as little as 0.35. This finding has been explained as being due to the presence of two (or more) types of specific muscarinic binding site for which strong agonists may have very different affinities (Hulme *et al.*, 1975; Birdsall & Hulme, 1976; Birdsall *et al.*, 1978a).

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Given that there are two or more types of specific muscarinic binding site in smooth muscle and other tissues, the question arises whether any, some, or all of these binding sites are the muscarinic receptors by which the physiological responses of the tissue are mediated. Potent agonists apparently differ in their affinities for the two binding sites by a factor of a hundred or more while weak or partial agonists, such as tetramethylammonium (TMA) or hexyltrimethylammonium (hexyl TMA) show little or no selectively for these two postulated types of binding site. It seemed of interest, therefore, to examine the responses to the iontophoretic application of various muscarinic agonists which had been reported to show various degrees of selectivity for these two types of binding site. The iontophoretic method of application of agonists to smooth muscle (Purves, 1974; Bolton, 1976) was chosen because of the superior temporal resolution of this method, which seemed most likely to reveal any differences existing in the kinetics of the reactions of these various agonists with the muscarinic receptor.

If different types of receptor exist for which some muscarinic agonists show very different affinities, then the possibility arises that these different receptor types may be linked in different ways to the physiological responses of the tissue. Indeed, Burgen & Spero (1968; 1970) have described experiments in which some muscarinic agonists, such as acetylcholine, were much more potent in producing contraction (some 1000 times) than in increasing ^{86}Rb or ^{42}K efflux from smooth muscle. Other agonists, were found to be more nearly equipotent in their effects on contraction and permeability, for example the EC_{50} efflux: EC_{50} contraction ratio for methylfurmethide was 5. Carbachol was some 330 times more potent on contraction than on permeability. Burgen & Spero (1970) also described how the increased efflux of ^{86}Rb produced by carbachol was insensitive to variation in the calcium concentration of the bathing solution while the contractile response was extremely sensitive, raising $[Ca^{2+}]$ from 2.5 mM to 5.0 mM, or lowering it to 1.25 mM, reducing the EC_{50} contraction 40 to 100 fold. Because of the importance of these observations of differential effects on ionic efflux and contraction, we decided to repeat the experiments.

Methods

Electrophysiology

Taeniae were taken from the apical end of the caecum of recently killed guinea-pigs (200 to 600 g body weight). Longitudinal strips, 0.2 to 0.5 mm wide, about 5 mm long and fusiform in shape, were cut from the edge of a taenia. These were introduced into a form of double sucrose-gap apparatus (Bolton, 1975). This apparatus enables the membrane potential in a 'nodal' region of the strip to be altered by passing current between the 'node' and the ends of the muscle depolarized in 154 mM KCl, across the two sucrose-gaps connected in parallel. To create a node of active tissue whose membrane potential can be manipulated in this way, the muscle strip was first drawn through two chambers, about 0.75 mm wide, through which high resistance (>8 MΩ.cm) deionized sucrose flowed at 36°C creating two cuffs about the muscle strip. These cuffs separated the nodal region of the strip from the depolarized ends of the muscle. The node was perfused with previously gassed physiological salt solution at 36°C, composition shown below. In some experiments tetrodotoxin 2×10^{-7} M was added to this solution.

The membrane potential of cells in the node was recorded intracellularly with glass microelectrodes filled with 3 M KCl having a resistance of about 50 MΩ. Muscarinic agonists were applied by iontophoresis to the surface of the muscle from double-barrelled iontophoretic pipettes with spacer (del Castillo & Katz, 1957). These were constructed from 1 mm borosilicate glass tubing with internal capillary which, after pulling, enables them to be filled from the wide end with drug solution. Two short lengths of this tubing with a glass spacer rod (about 1 mm diameter) between them were cemented together with fast setting epoxy resin. They were then subjected to a first pull of 7 mm, after softening by heating with a filament in an electrode puller, during which the three tubes were sealed together by rotating one end about 270° with respect to the other. After allowing to cool, a second, longer straight pull produced double-barrelled pipettes. These were filled with solutions (usually 1 mg/ml) of different muscarinic agonists and the tip of the pipette brought close (10 to 25 μm) to the point of membrane potential recording. The pipette was lowered towards the surface of the muscle until a depolarizing response to drug release could be recorded. Normally this evokes action potential discharge and the resulting contraction dislodges the microelectrode. To prevent this, the membrane was hyperpolarized by passing a rectangular pulse of inward current into the 'node' (see Figures 2–4). Normally, the membrane responses to two agonists were recorded during the same electrotonic potential and the order of the pulses was reversed from time to time when making comparison to avoid bias.

Radioisotope experiments

Guinea-pigs of either sex (300 to 700 g) were killed by a blow on the head. The ileum was removed and strips of longitudinal muscle, about 3 mm wide and 2 cm long weighing about 10 mg, were separated from

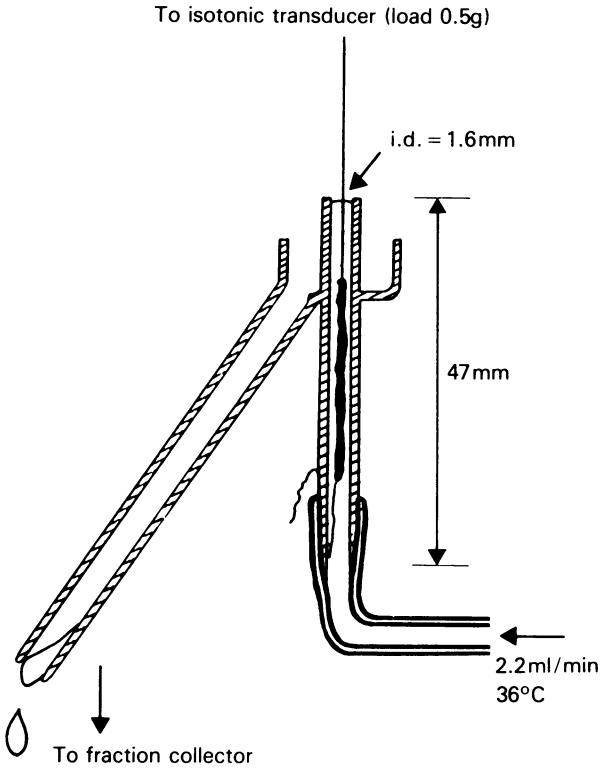

Figure 1 Diagram of perfusion chamber showing the muscle loaded with ^{42}K being perfused with non-radioactive physiological salt solution; i.d. = internal diameter. Volume of perfusion chamber = 0.1 ml.

the bulk of underlying circular muscle, as described by Bolton (1972).

The physiological salt solution had the following composition (mM): NaCl 120, KCl 5.9, $NaHCO_3$ 15, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.5 and glucose 11.5. The solution was gassed with 95% O_2, 5% CO_2 and its pH was about 7.2.

Loading procedure: the loss of potassium from strips of longitudinal muscle was followed by the use of ^{42}K. In a few experiments, ^{86}Rb was used as a substitute for this. Intracellular potassium was first exchanged for ^{42}K by incubating the muscle in normal solution made with ^{42}KCl and having an activity of 4 μCi/ml. Where loading was with ^{86}Rb, tissues were incubated in normal solutions to which 5.6 mM $^{86}RbCl$ had been added, giving an activity of 5 to 10 μCi/ml. For both isotopes loading was for 3 to 5 h at 37°C in these solutions gassed with 95% O_2, 5% CO_2; in the case of ^{42}K, the solution contained tetrodotoxin (2×10^{-7} M), while the Mg^{2+} concentration in rubidium-containing solutions was 2.4 mM. Following loading, strips were dipped in warm, non-radioactive physiological salt solution to remove excess radioactive ions and transferred to the apparatus shown in Figure 1. They were perfused with warm physiological salt solution (36 ± 0.5°C) at a rate of 2.2 ml/min. As the solution passes over the muscle the radioactive isotope (^{42}K or ^{86}Rb) will exchange for unlabelled potassium. The effluent was collected every minute or half minute and levels of radioactive isotope estimated by gamma counting. The effluent collected in the first 10 min was discarded. Subsequent washout of radioactivity could generally be described by a single exponential (cf. Casteels, 1969). The length of the muscle was measured isotonically under a load of 0.5 g. To obtain dose-response curves for shortening and for efflux of radioisotope, drugs were applied in ascending order of concentration by substituting a drug-containing solution for the indicated periods.

Treatment of results The efflux rate constant was calculated by dividing the counts in the perfusate by the average number of counts contained in the muscle during the collection period (Durbin & Jenkinson, 1961), viz:

$$\text{efflux rate constant } (\text{min}^{-1}) = \frac{\text{counts in perfusate}}{\text{average tissue counts} \times \text{collection time (min)}}.$$

At the end of the experiment, the muscle strip was removed and counted and the counts in the tissue during the experiment obtained by summation. Efflux rate constants were calculated using a computer programme kindly supplied by Dr A. F. Brading. The increase in efflux rate constant caused by applying a drug was obtained by subtracting the basal efflux rate constant averaged over the 5 min before drug application from the peak increase recorded during application of the drug.

For each strip of muscle, absolute increases in efflux rate constant and muscle shortening were calculated as a percentage of the maximum obtainable on each strip and these normalized responses averaged over several experiments.

Drugs

The following drugs were used: acetylcholine chloride, carbachol chloride, tetramethylammonium chloride (TMA) (BDH), edrophonium chloride (Roche), neostigmine methyl sulphate, tetrodotoxin (Sigma). Oxotremorine-M bromide and propyl benzilylcholine mustard hydrochloride were gifts from Dr E. C. Hulme. Butyltrimethylammonium iodide (butyl TMA), hexyltrimethylammonium iodide (hexyl TMA), and methylfurmethide iodide were kindly supplied by Dr E. W. Gill.

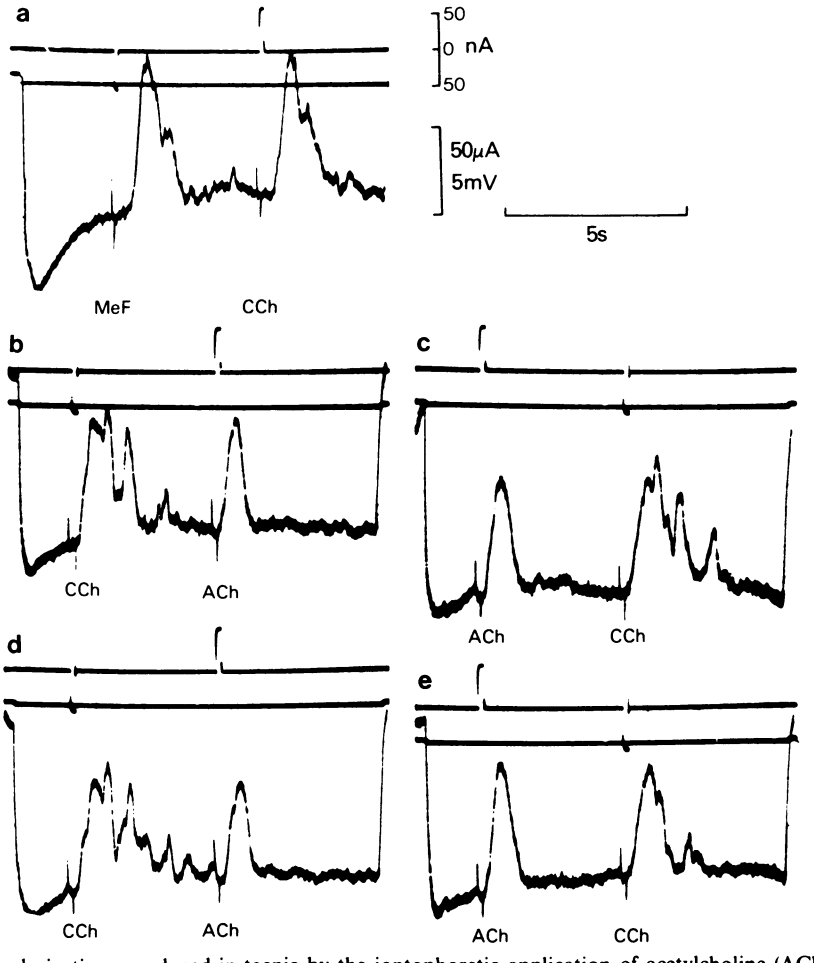

Figure 2 Depolarizations produced in taenia by the iontophoretic application of acetylcholine (ACh), carbachol (CCh) or methylfurmethide (MeF). The membrane was first hyperpolarized by passing current across the sucrose gaps (as indicated by the step in the middle trace which is the sucrose-gap current monitor) before applying brief pulses of agonists (as indicated by the upper trace which is the iontophoretic current monitor, direction of displacement of trace indicates barrel in use). The calibrations adjacent to panel (a) apply to all panels and are (upper) iontophoretic current monitor, (lower) sucrose-gap current monitor and membrane potential. Intracellular record at 36°C.

Statistics

Student's *t* test was applied either to pair differences, or to differences between the means of assumed independent samples (Snedecor & Cochran, 1967).

Results

Responses evoked by iontophoretic application of agonists

When the node width in the double sucrose-gap is 0.5 to 0.6 mm, as in these experiments, action potentials are often not discharged spontaneously by taenia muscle and the membrane potential is stable and hyperpolarized, values of 60 to 80 mV being commonly recorded (cf. Kuriyama & Tomita, 1970; Bolton, Tomita & Vassort, 1981; Figure 4). If the iontophoretic pipette is brought up close to the surface of the muscle, pulses (2.5 nC) of acetylcholine or carbachol generally elicited depolarizing responses of 1 to 10 mV. These responses showed a latency, 200 to 700 ms, and rose to a peak within a second or so (Bolton, 1976 and Figures 2 and 3).

Differences in affinity of agonists for the muscarinic receptor may be reflected in different time courses of the response to iontophoretic application, as the affin-

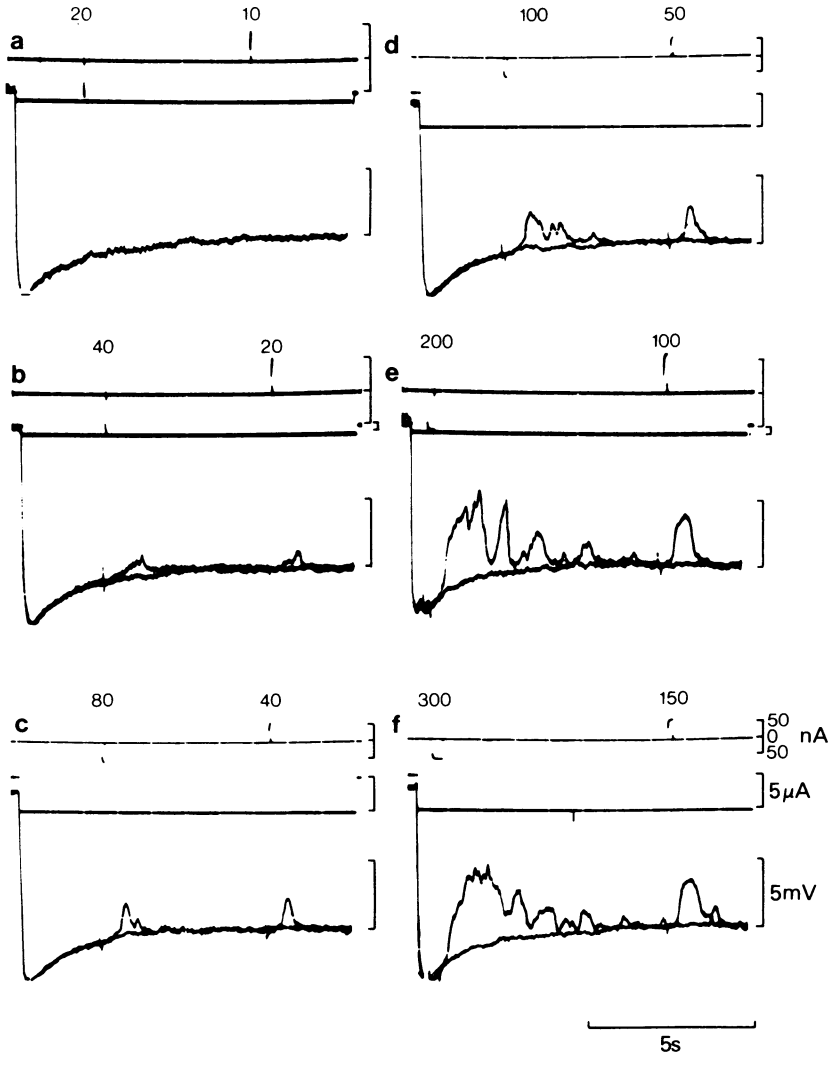

Figure 3 Effect on the membrane potential of taenia of increasing the amounts of acetylcholine (right hand response in each panel) or carbachol (left hand response) released iontophoretically. The amount of drug released was varied by changing the duration (shown in ms over each panel) of a fixed-strength releasing pulse. Other details as Figure 2.

ity constant is the ratio of the forward and backward rate constants. Also, some strong agonists (acetylcholine, carbachol) are believed to have very different affinities (100 fold difference, or more) for two types of muscarinic binding sites (= receptors?) while other, weaker, agonists (hexyl TMA, TMA) are believed to have very similar affinities for the two postulated types of receptor. For this reason, the latency and time to peak of responses to a number of muscarinic agonists were compared in an attempt to detect differences in the kinetics of interaction of various agonists with the muscarinic receptor.

In any experiment, only two agonists were applied. A pair of responses were elicited during a hyperpolarizing electrotonic potential. This was repeated a number of times, the order of the pulses being reversed to avoid bias during the series. A hyperpolarizing pulse was applied because, at the resting membrane potential, depolarization evoked by application of muscarinic agonist generally elicited action potential discharge and contraction (Figures 2, 3 and 4); even if the electrode was not dislodged, the position of the iontophoretic pipette with respect to the muscle was liable to be changed during contraction.

Latency of the response was measured to the first detectable depolarization from the end of the iontophoretic pulse. Time to peak from this point to the peak of the response, or first peak if the response showed several peaks (see Figures 2 and 3). It was often difficult to obtain responses to TMA, or hexyl TMA, as large releasing currents were required and some iontophoretic pipettes would not pass these. Generally, the duration of the releasing pulse was the same for both agonists and the peak responses were matched in size by varying the iontophoretic current, although with weak agonists this was seldom possible.

No differences could be detected between agonists stable to cholinesterase, whether these were strong or weak. Some representative results are shown in Table 1 for carbachol, oxotremorine-M, methylfurmethide, TMA and hexyl TMA. Despite considerable differences in potency, the mean latency and time to peak for these agonists were not significantly different (Figure 2a).

In contrast, the responses to acetylcholine were consistently of shorter latency and time to peak than those to a stable agonist whether it was strong or weak (Table 1). The difference was small and is not always obvious from single records (Figure 2b–e) but was consistently found upon averaging measurements made upon a number of responses. It seemed likely that the briefer effects of acetylcholine reflected the activity of cholinesterase in the tissue, but in view of the reported heterogeneity of binding of muscarinic agonists, it seemed important to establish this with certainty.

It was noticeable that the acetylcholine response tended to be of a simple monophasic shape, even when the iontophoretic current was increased considerably beyond that just needed to elicit a response (Figure 3). In contrast, stable agonists tended to produce somewhat longer-lasting responses which, in many experiments, showed a number of peaks on their descending phase, or were of generally complex shape (Figure 2 b–e, and Figure 3 c–f) especially if higher iontophoretic currents were used. However, this was by no means invariably the case (Figure 4). It is also clear from Figure 3 that the dose-peak-response curve for acetylcholine differs from that for carbachol.

Two types of experiment were done to investigate these differences. In one an anticholinesterase, edrophonium or neostigmine, was placed in one barrel of the iontophoretic pipette and releasing currents to 1 to 20 nA applied for several seconds. These increased the size of responses to acetylcholine released from the other barrel. In other experiments, responses to iontophoretic acetylcholine were compared to those to iontophoretic carbachol and edrophonium or neostigmine introduced in the bathing solution at a concentration of 4×10^{-6} M, or sometimes 2×10^{-5} M.

The application of an anticholinesterase in the bathing solution depolarized the membrane, sometimes substantially (Figure 4). This depolarization was of rapid onset which makes a direct action on muscarinic receptors more likely (see Cox & Lomas, 1972) than an indirect action on these via the accumulation of acetylcholine released within the tissue. Edrophonium was preferred to neostigmine, because of the faster recovery from depolarization. In the presence of edrophonium, or neostigmine, the response to iontophoretic acetylcholine was usually potentiated while

Table 1 Latency and time to peak of depolarizing responses to iontophoretic muscarinic agonists

		Latency (ms)	*Time to peak* (ms)
ACh / CCh	$n = 143$	219	555
		250	660
	diff.	31 ± 6 $P < 0.001$	105 ± 9 $P < 0.001$
ACh / C_6TMA	$n = 67$	302	409
		378	506
	diff.	76 ± 7 $P < 0.001$	97 ± 7 $P < 0.001$
CCh / Mefur	$n = 76$	421	832
		440	840
	diff.	19 ± 10 NS	8 ± 18 NS
CCh / TMA	$n = 10$	636	840
		548	850
	diff.	88 ± 47 NS	10 ± 37 NS
Oxo-M	$n = 12$	321	710
C_6TMA	$n = 17$	359	727
	diff.	38 ± 46 NS	17 ± 79 NS

Two agonists were compared in each experiment by the use of double-barelled iontophoretic pipettes. The number of responses (n) measured is given. The differences (diff.) are expressed as mean ± s.e. mean; NS indicates $P > 0.05$.

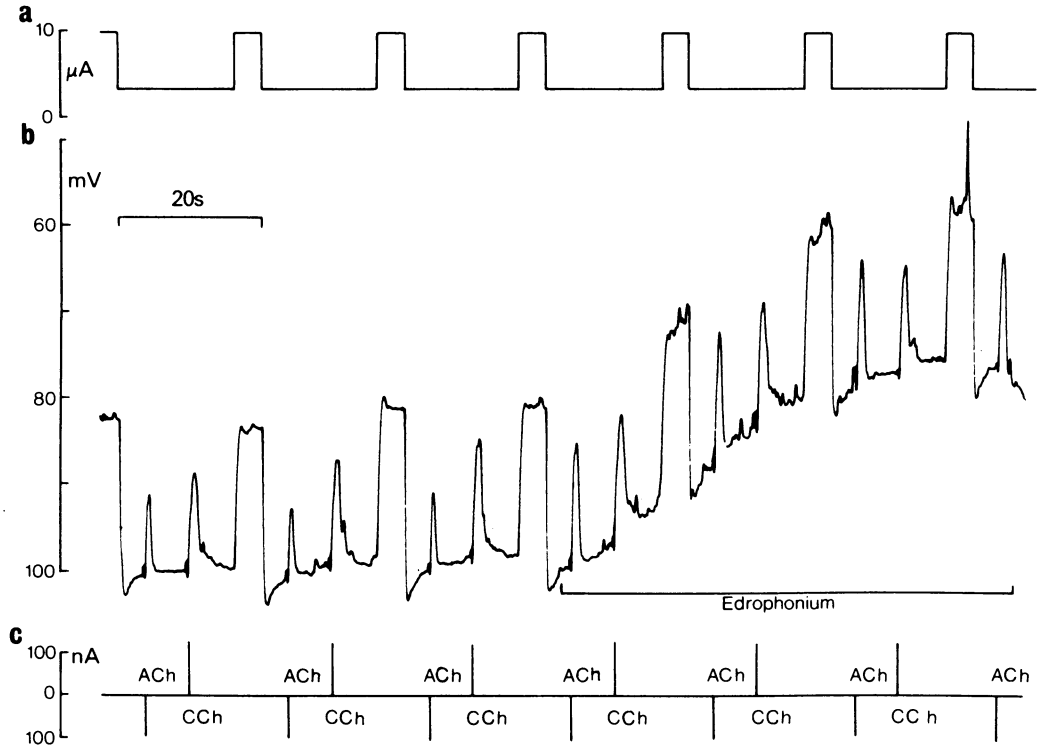

Figure 4 Effect of the anticholinesterase, edrophonium, on the responses to the iontophoretic application of acetylcholine (ACh) or carbachol (CCh). A hyperpolarizing electrotonic potential was evoked every 20 s during which the agonists were applied. Edrophonium (4×10^{-6} M) was applied in the bathing solution for the period indicated by the bracket. (a) Sucrose-gap current monitor; (b) membrane potential recorded intracellularly at 36°C; (c) iontophoretic current monitor, pulses 150 ms duration. Tetrodotoxin (2×10^{-7} M) was present throughout.

that to carbachol was reduced, or unchanged (Figure 4). The potentiation of the acetylcholine response occurred before edrophonium depolarization started and persisted after the membrane potential had recovered to its previous value. In many experiments in the presence of an anticholinesterase, the simple monophasic shape of the acetylcholine response was converted to a complex multi-peaked shape, very similar to that observed when a stable agonist was applied.

If the differences in latency and time to peak between acetylcholine and carbachol are caused by the hydrolytic activity of cholinesterase in the tissue, then anticholinesterases should reduce or remove these differences. This was found to be so, although it was not possible to use concentrations of anticholinesterase which completely inhibit cholinesterase activity because of their substantial depolarizing action. Table 2 contains the results of an experiment in which some 13 applications of 5×10^{-6} M edrophonium were made to a tissue at various times and the effects on the latency and time to peak of responses to acetylcholine and carbachol measured.

In the presence of edrophonium, the size of the acetylcholine response was increased by some 80%, while the response to carbachol was reduced, although not significantly. Time to peak was increased in the case of acetylcholine, although latency was reduced (Table 2A). It has previously been observed that increasing the amount of acetylcholine released by increasing the iontophoretic pulse, reduces latency (Bolton, 1976) so this effect is expected. The latency and time to peak of carbachol responses were unaffected. If the response to acetylcholine was matched in size to that of carbachol before application of edrophonium, the increase in size of the acetylcholine response made direct comparison of responses to the two agonists invalid in the presence of edrophonium. For this reason, the iontophoretic current releasing acetylcholine was reduced in the presence of edrophonium, in order to match sizes of responses to the two agonists.

In these conditions, the time to peak of the acetylcholine responses approached that of carbachol responses, the difference declining from 196 to 99 ms. This decline was significant ($P < 0.05$) on a one tail test. It seems likely that, if cholinesterase could have been completely inhibited, this difference would have disappeared. The effect on latency was smaller, the difference declining from 57 to 43 ms, but this decline was not significant (Table 2B).

These results suggest strongly that it is the hydrolytic activity of cholinesterase in the tissue which is responsible for the differences existing between the responses to acetylcholine and stable agonists, and not differences in the kinetics of binding to muscarinic receptors.

Efflux experiments

Our failure to detect effects which could be ascribed to the heterogeneous binding characteristics of muscarinic agonists made it of some interest to investigate the differential effects of these agonists on ^{86}Rb or ^{42}K efflux and on contraction, originally described by Burgen & Spero (1968; 1970). To avoid as far as possible the complicating effect of stimulating nicotinic receptors of nerve cells of Auerbach's plexus, and the release of acetylcholine from nerve endings, tetrodotoxin (2×10^{-7} M) was routinely added to the bathing solution in these experiments.

After perfusion for an initial 15 min, the ^{42}K efflux rate remained virtually constant for periods in excess of 2 h (Figures 6 and 7) in the absence of drug application. This could imply a single rate limiting process is operating within the tissue and it is likely that this process is the passage of ^{42}K across the plasma membranes of the smooth muscle cells. The basal efflux rate was found to be $1.36 \pm 0.06 \times 10^{-2}$ min^{-1} (14 animals, 33 strips).

Application of a muscarinic agonist, such as carbachol, caused an increase in the rate of loss of ^{42}K. When the duration of application was varied from 5 to 40 s the peak tensions generated by the muscle were very similar. However, increasing the duration of carbachol application greatly increased the peak effect on the ^{42}K efflux rate (Figure 5). It was found that increasing the duration of application beyond 3 min (for low concentrations) or 1 min (for high concentrations) had no further effect on the increase in the efflux rate. Periods in excess of these times were, therefore, used in subsequent experiments (e.g. see

Table 2 Effect of inhibiting the activity of cholinesterase in the tissue with edrophonium (4×10^{-6} M) in one experiment

A *Iontophoretic releasing pulse not changed*

	Acetylcholine		*Carbachol*	
	Control	In presence edrophonium	Control	In presence edrophonium
Lat (ms)	208 ± 11 (18)	167 ± 11 (22)	246 ± 16 (20)	236 ± 15 (23)
	$P < 0.025$		NS	
TTP (ms)	615 ± 32 (19)	816 ± 28 (23)	928 ± 25 (20)	881 ± 32 (23)
	$P < 0.001$		NS	
Size (mV)	5.0 ± 0.3 (19)	9.0 ± 0.4 (23)	8.1 ± 0.5 (20)	7.4 ± 0.3 (23)
	$P \ll 0.001$		NS	

B *Peak sizes of responses matched*

	In normal solution ($n = 39$)			
	Acetylcholine	*Carbachol*	*Difference*	*Significance*
Lat (ms)	151 ± 6	209 ± 12	57 ± 11	$P < 0.001$
TTP (ms)	585 ± 24	781 ± 29	196 ± 28	$P \ll 0.001$
Size (mV)	5.7 ± 0.3	6.1 ± 0.2	0.4 ± 0.2	NS
	In edrophonium ($n = 18$)			
	Acetylcholine	*Carbachol*	*Difference*	*Significance*
Lat (ms)	162 ± 10	205 ± 18	43 ± 14	$P < 0.01$
TTP (ms)	782 ± 39	882 ± 51	99 ± 41	$P < 0.05$
Size (mV)	8.1 ± 0.6	7.8 ± 0.5	0.3 ± 0.5	NS

In A, responses were compared before and after changing to a solution containing edrophonium. The iontophoretic releasing pulses were not changed. The response to acetylcholine was increased in size while that to carbachol was slightly reduced. Latency (Lat) and time to peak (TTP) of the acetylcholine responses were affected while these measures of the carbachol responses were not. The number of responses measured is given in parentheses. In B, pairs of similar-sized responses to acetylcholine and carbachol were chosen. In the presence of edrophonium the differences between the latency and time to peak of acetylcholine and carbachol responses were reduced, although the effect on latency is not significant.

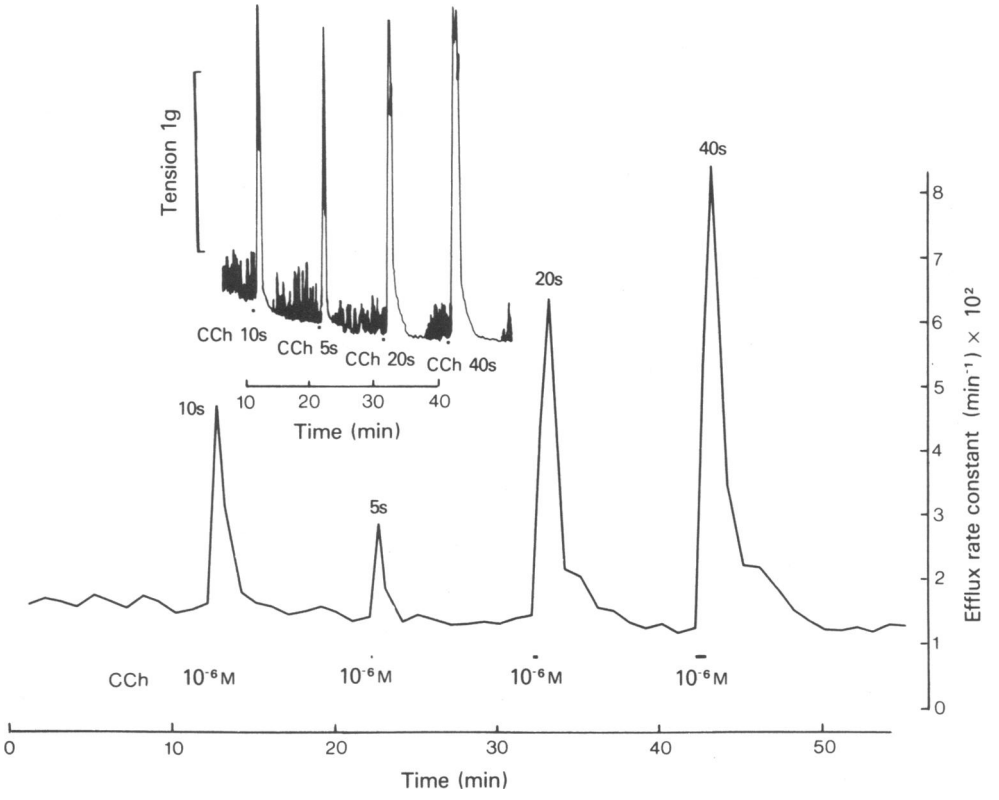

Figure 5 Effect of varying the duration of carbachol (10^{-6} M) application on ^{42}K efflux from longitudinal muscle of guinea-pig ileum. In this experiment, tetrodotoxin was omitted from the bathing solution and tension measured semi-isometrically. The inset shows the contractions of the muscle to the drug.

Figures 6 and 7). Thus, low concentrations of carbachol were applied for 4 min while high concentrations (10^{-5} and 10^{-4} M) were applied for 90 s. Upon application of high concentrations, the effect on efflux rate reached a peak within 30 to 60 s and then declined despite the presence of carbachol in the bathing solution, suggesting that a process of desensitization was occurring. The interval between drug applications was 10 to 15 min and concentrations of drugs were given in ascending order.

It was found with all muscarinic agonists tested that maximum shortening of the muscle occurred at a concentration lower than that required to produce a maximum increase in ^{42}K efflux rate. Results of typical experiments using methylfurmethide and TMA are illustrated in Figures 6 and 7.

The maximum increase in ^{42}K efflux rate could be very different for different muscarinic agonists. The averaged results of five experiments of the type described for each of the four drugs acetylcholine, carbachol, methylfurmethide and TMA, are shown in Figure 8. The maximum increases in ^{42}K efflux rate produced by carbachol and methylfurmethide were very similar, about 6 to 7 times the basal efflux rate. In contrast, TMA appears to show the characteristics of a partial agonist; a concentration of 10^{-2} M produced a smaller increase in efflux rate than that obtained with carbachol, or methylfurmethide. Also, the TMA log dose-response curve was less steep. However, TMA was found to produce a maximum contraction no less than the maximum contraction produced by carbachol.

The maximum increase in ^{42}K efflux rate produced by acetylcholine was much less than that produced by the three stable agonists. To find out whether this could be attributed to hydrolysis of acetylcholine by cholinesterase in the tissue, the following experimental design was used. The maximum increase in ^{42}K efflux rate of which the tissue was capable was estimated by giving carbachol (10^{-5} M). A close-to maximally effective concentration (10^{-5} M) of acetylcholine was applied and the efflux response to this was matched by applying carbachol (2×10^{-7} M). Acetylcholine (10^{-5} M) and carbachol (2×10^{-7} M) were then applied in the presence of neostigmine (2×10^{-7} M). The results of a typical experiment are shown in

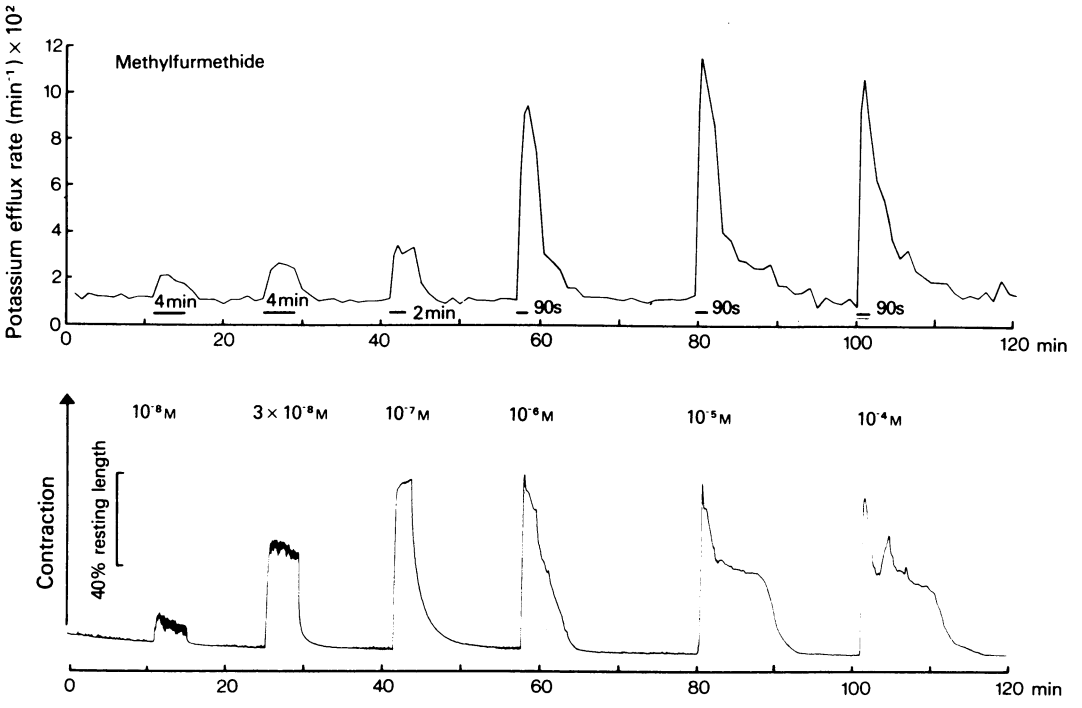

Figure 6 Effect of methylfurmethide on ^{42}K efflux (upper trace) and muscle length measured in the same tissue. Methylfurmethide was applied for the periods indicated by the short horizontal bars.

Figure 9 (b,c) which also summarizes results from four experiments of this type (Figure 9a). In order to avoid bias due to desensitization, the order in which the drugs were given was varied in different experiments, but neostigmine was always given in the latter part of each experiment. In neostigmine (2×10^{-7} M) the response to a previously maximally effective concentration of acetylcholine (10^{-5} M) was substantially increased, although the response was still less than that obtained with carbachol (10^{-5} M). This may be due to a decline in the responsiveness of the tissue with time (see responses to carbachol (2×10^{-7} M), Figure 9). These experiments suggest that the smaller maximum increase in ^{42}K efflux rate attained with acetylcholine when cholinesterase is not inhibited, is caused by poor penetration of acetylcholine into the tissue.

Inhibition of cholinesterase also increased the maximum contraction attainable with acetylcholine. In the absence of anticholinesterase, carbachol produced a maximum contraction larger than that obtained with acetylcholine on the same tissues. Neostigmine (2×10^{-7} M) shifted the acetylcholine log dose-response curve to the left and increased its maximum. It had a similar but much smaller effect on the carbachol log dose-response curve possibly due to its direct excitant action (Figure 4). In the presence of neostigmine, the acetylcholine and carbachol log dose-response curves were about parallel in their midranges, and had similar maxima (Figure 10).

A comparison was made of the abilities of four muscarinic agonists to increase ^{42}K efflux rate and to produce contraction using these results. On each preparation the concentration producing 50% of maximum contraction (EC_{50} contraction) and that producing 50% of the maximum increase in ^{42}K efflux rate (EC_{50} efflux) were obtained by interpolation on the log dose-response curves. The mean EC_{50} efflux/EC_{50} contraction ratios are shown in Table 3. For acetylcholine, carbachol and methylfurmethide, this ratio was about 20. The ratios for carbachol and methylfurmethide were close, 18.2 and 17.0 respectively. This was a surprising finding, as Burgen & Spero (1968) found ratios of 330 (carbachol) and 5 (methylfurmethide) and on this basis, and on the basis of varying ratios for other muscarinic agonists, suggested that some muscarinic agonists could preferentially activate pathways leading to an increase in membrane permeability. However, no difference was evident in our experiments between the relative effects of carbachol and methyl furmethide on contraction and on efflux (Figure 11).

In an attempt to resolve the discrepancy, a further series of experiments was done under conditions similar to those used by Burgen & Spero (1968; 1970).

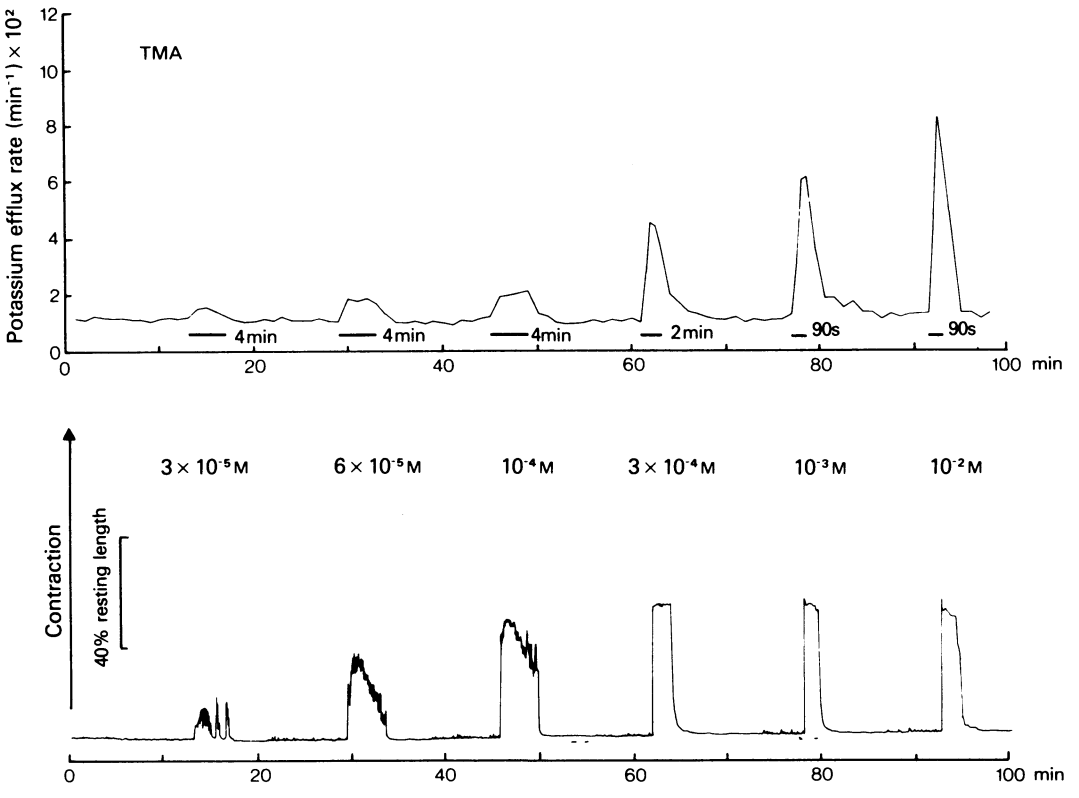

Figure 7 Effect of tetramethylammonium (TMA) on ^{42}K efflux (upper trace) and muscle length in the same tissue. TMA was applied for the periods indicated by the horizontal bars.

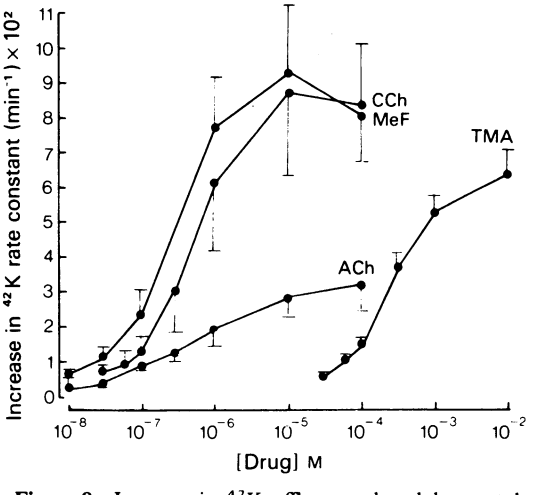

Figure 8 Increase in ^{42}K efflux produced by acetylcholine (ACh), carbachol (CCh), methylfurmethide (MeF) and tetramethylammonium (TMA). The results (mean and one s.e. mean) for each drug are the average values obtained on five preparations from different animals.

The loss of intracellular potassium was estimated using $^{86}R6$ as a marker, tetrodotoxin was omitted and and the magnesium concentration in the bathing solution raised from 1.2 to 2.4 mM. The basal ^{86}Rb efflux rate after 15 min perfusion with Rb-free solution was $1.38 \pm 0.04 \times 10^{-2}$ min^{-1} (6 animals, 11 strips). The EC_{50} efflux/EC_{50} contraction ratios for carbachol and methylfurmethide were 10.1 ± 1.6 and 30.2 ± 11.4 and, again, these were not significantly different (Table 2).

In all the above comparisons, carbachol and methyl furmethide were applied to different preparations. Experiments were therefore carried out in which comparisons were made on the same tissue. Equipotent molar ratios were estimated for contraction and for increase in ^{42}K efflux rate relative to carbachol, for methylfurmethide and butyltrimethylammonium (butyl TMA). Butyl TMA had a very similar potency relative to carbachol on ^{42}K efflux rate and on contraction. Methylfurmethide seemed slightly more potent relative to carbachol in causing contraction than in producing loss of ^{42}K (Table 3). This difference was in the opposite direction to that found by Burgen & Spero (1968) and was statistically signifi-

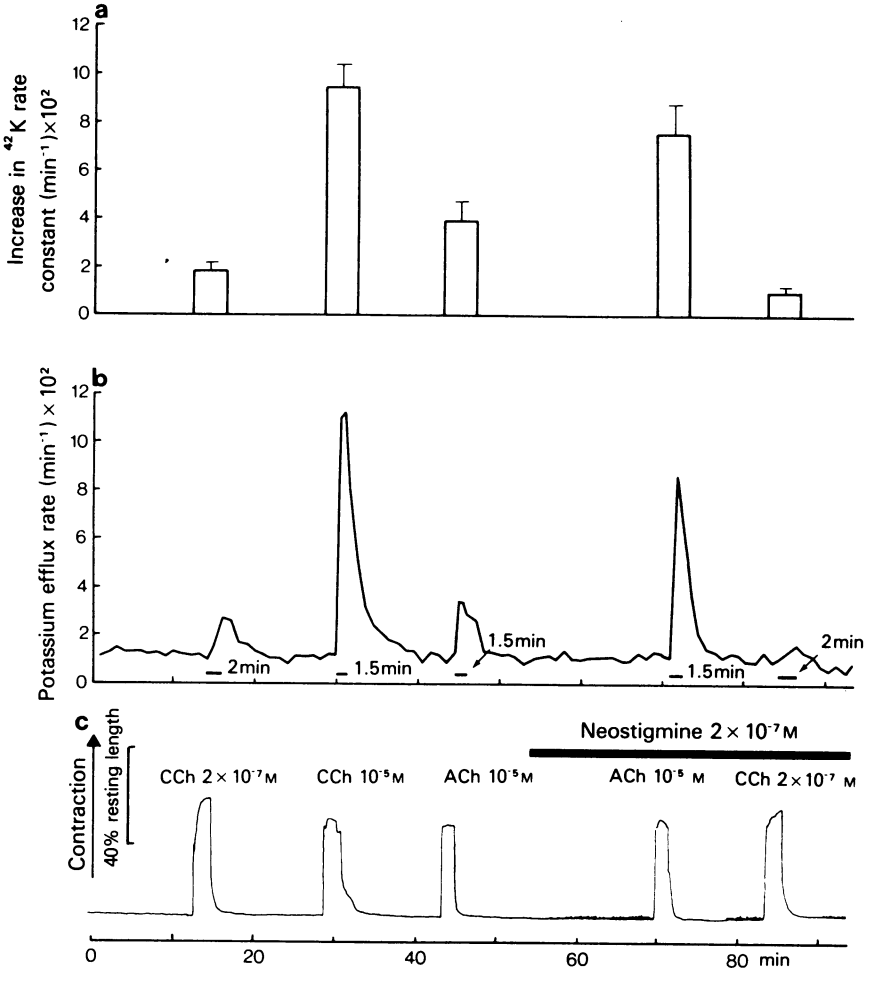

Figure 9 The effect of inhibiting cholinesterase with neostigmine (2×10^{-7} M) on the ^{42}K efflux responses to acetylcholine and to carbachol. The data from one experiment are shown in (b) (^{42}K efflux) and (c) (muscle shortening). Also shown (a) are the average increases in ^{42}K efflux rate constant for 4 strips from different animals to the concentrations of the drugs shown in (c).

cant ($P < 0.05$). The reason for this small difference was not pursued, but probably arises because the chosen concentrations of drugs applied lay in slightly different regions of the dose-response curves.

The smaller EC_{50} efflux/EC_{50} contraction ratio seen with TMA may arise partly because it was assumed that 10 mM TMA produced a maximum efflux response. However, this was not the whole explanation because if it was assumed that concentrations of TMA greater than 10 mM produced an increase in ^{42}K efflux rate comparable to that produced by high concentrations of carbachol, or methylfurmethide, this ratio was only increased to about 9. At concentrations in the millimolar range, TMA may well be having other effects.

Burgen & Spero (1970) also reported that the increase in ^{86}Rb efflux produced by carbachol was very insensitive to varying the calcium concentration in the range 1.25 to 10 mM, whereas the contractile response was sensitive to such variations. We found only a small effect on the EC_{50} contraction for carbachol, in two series of experiments, of raising the calcium concentration of the bathing solution from 2.5 to 5.0 mM (the magnesium concentration was 2.4 mM in these experiments, as in Burgen & Spero's) (Figure 12).

Discussion

Both the electrophysiological and efflux results we have obtained are consistent with the view that all the

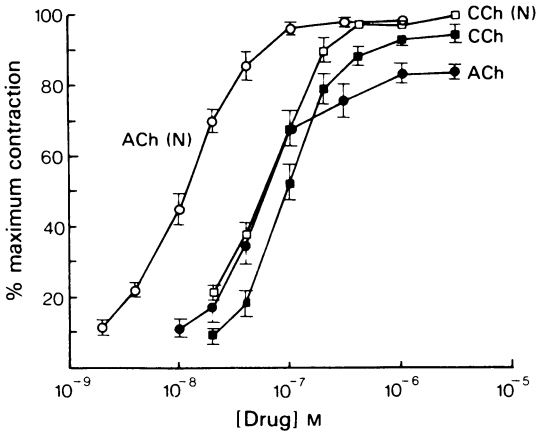

Figure 10 Effect of inhibiting cholinesterase with 2×10^{-7} M neostigmine (N) on contractions in response to acetylcholine (ACh) and carbachol (CCh).

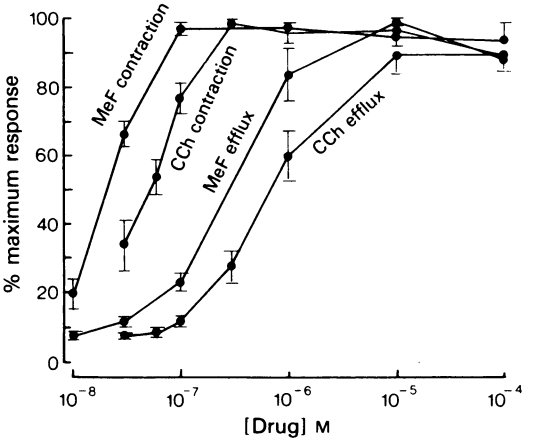

Figure 11 Contraction and increase in ^{42}K efflux in response to methylfurmethide (MeF) and carbachol (CCh). Contractions and ^{42}K efflux responses on individual strips were expressed as a percentage of the maximum responses. The results shown for each drug are the mean values obtained from 5 strips from different animals; vertical lines show s.e. mean.

muscarinic agonists tested interact in an essentially similar way with muscarinic receptors to produce their effects on membrane potential, conductance and permeability, and to elicit contraction of smooth muscle. This conclusion is at variance with that reached by others (Burgen & Spero, 1968; 1970) but no constructive suggestions can be made to explain the discrepancies between our results and theirs, as we have been unable to reproduce the results of their crucial experiments. Our findings provide support for the concept (Bolton, 1979) that activation of muscarinic receptors open ion channels in the smooth muscle membrane associated with these receptors and these channels allow inorganic ions, including potassium, to cross the membrane with increased facility. The resulting increases in membrane permeability and conductance produces depolarization, action potential discharge, and contraction.

However, in the present study, we have not attempted to dissect components which contribute to the increase in ^{42}K efflux rate. Depolarization itself opens channels which probably allow an increased loss of potassium from the cell. Muscarinic agonists at

Table 3 Relative potencies of muscarinic agonists in producing contraction and increase in ^{42}K or ^{86}Rb efflux

A, *EC_{50} efflux/EC_{50} contraction ratios*

	^{42}K efflux			*^{86}Rb efflux*		
	Arithmetic mean ± s.e. mean	Geometric mean and 95% confidence limits		Arithmetic mean ± s.e. mean	Geometric mean and 95% confidence limits	
Acetylcholine	29 ± 18	14 (2–78)	$n = 5$			
Carbachol	18 ± 5.2	16 (3–32)	$n = 5$	10 ± 1.6	9.5 (6.6–14)	$n = 6$
Methylfurmethide	17 ± 3.2	16 (6–44)	$n = 5$	30 ± 11	22 (7–68)	$n = 5$
Tetramethylammonium	4.1 ± 0.7	3.9 (2.4–6.3)	$n = 5$			

B *Equipotent molar ratios relative to carbachol*

	^{42}K efflux			*Contraction*		
Methylfurmethide	0.69 ± 0.11	0.66 (0.43–1.0)	$n = 5$	0.43 ± 0.03	0.42 (0.34–0.53)	$n = 5$
Butyltrimethyl ammonium	32 ± 5.5	30 (16–58)	$n = 4$	35 ± 2.3	35 (28–43)	$n = 4$

The arithmetic mean ± s.e. mean and geometric mean with 95% confidence interval are given for the indicated number (n) of experiments done (A) to compare the effect on ^{42}K efflux or ^{86}Rb efflux and on contraction, and (B) to estimate the equipotent molar ratios relative to carbachol on ^{42}K efflux and on contraction.

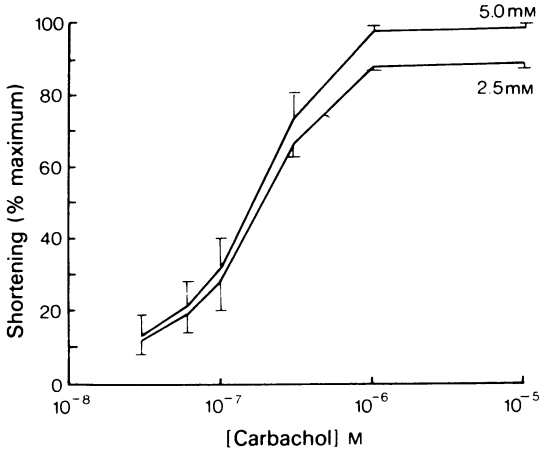

Figure 12 Effects of different calcium concentrations (5 and 2.5 mM) on carbachol-induced contraction. The magnesium concentration was 2.4 mM and tetrodotoxin was omitted from the bathing solution in these experiments. Carbachol was applied for 10 s periods.

lower concentrations increase action potential discharge and at higher concentrations produce a substantial steady depolarization, abolishing action potential discharge (Bolton, 1972). Both action potential discharge and steady depolarization themselves, by opening potential-sensitive potassium channels, may contribute to the increased ^{42}K efflux rate, and depolarization alone would be expected to increase ^{42}K efflux. A third contribution may come from the opening of channels associated with the muscarinic receptor itself, as ^{42}K efflux is known to increase upon application of carbachol in smooth muscle depolarized in high potassium solution (Durbin & Jenkinson, 1961).

The time course of iontophoretic responses do not seem to give much indication of whether the high or low affinity binding sites for muscarinic agonists are the receptors involved in the responses. If a forward rate constant (k_1) of about 10^7 M^{-1} s^{-1} is assumed, the high affinity site ($K = 7 \times 10^{-7}$ M for carbachol) and the low affinity site ($K = 10^{-4}$ M approximately) give backward rate constants (k_2) of 7 s^{-1} and 10^3 s^{-1}. If the high affinity sites were the receptors involved, then the rate constant of the declining phase of the iontophoretic response might be about 0.1 s, if dissociation from combination with the receptor determines its rate of decay (cf. Paton, 1961; Rang, 1966). As carbachol would dissociate much more quickly from the low affinity sites, some other process, such as buffered diffusion, would have to be supposed as determining the declining phase of the iontophoretic response. In any case, the complex nature of the declining phase, consisting of several peaks, suggests that perhaps patches of receptors may be involved. The peaks may represent incipient action potentials, created by intense polarization of small areas of the three-dimensional electrical syncytium, which is the smooth muscle tissue (George, 1961; Tomita, 1970). Presumably the more transient lifetime of acetylcholine (when cholinesterase is active) limits its spread, so that incipient action potentials generally do not develop on the descending phase.

The picture which emerges from the results described here is that concentrations of the natural transmitter, acetylcholine, reaching the receptors are reduced by the activity of cholinesterase in the tissue. However, it would appear that acetylcholine, or its stable analogues, interact in an essentially similar way with muscarinic receptors which presumably represent binding sites for muscarinic agonists detected by others.

Longitudinal smooth muscle of ileum binds 100 to 150 pM propylbenzilyl choline mustard per g wet wt. of tissue (Fewtrell & Rang, 1973; Burgen, Hiley & Young, 1974a) which indicates some 100 binding sites per μm^2 if these are distributed uniformly. (In this calculation an extracellular space of 35% was assumed and that 1.4 μm^2 plasma membrane was associated with 1 μm^3 of intracellular volume, Gabella, 1975.) Birdsall, Burgen & Hulme (1978b) have suggested from thermodynamic considerations that the low affinity sites for specific binding of muscarinic agonists represent the receptors by which the physiological responses are mediated. The present results do not throw much additional light on the problem except in so much as the various muscarinic agonists known to have very different selectivity for the two types of binding site produced rather similar patterns of effects. This suggests that either the different affinities for the two types of binding site are insufficient to be reflected in the physiological responses, or that either the low or the high affinity site alone represent the receptors proper. The evidence from the use of irreversible alkylating antagonists at the muscarinic receptor (Burgen & Spero, 1968; Taylor *et al.*, 1975) is that, following presumed occlusion of most of the receptors, responses to carbachol continue to increase substantially beyond 2×10^{-5} M, a concentration at which 97% of available high affinity sites ($K = 7 \times 10^{-7}$ M) would be occupied by carbachol. This implies that the low affinity site ($K \approx 10^{-4}$ M) may be involved. Nevertheless, responses to carbachol do not increase beyond about 10^{-4} M after alkylation of receptors, even though substantial increases in occupancy of low affinity sites are presumably possible. Experiments which we have done, but have not described here, support these findings and underline the difficulty of positively indentifying receptors as either high or low affinity binding sites. It is possible, as Birdsall *et al.* (1978b) suggest,

that the low affinity sites are the receptors—but that responses are reduced above about 10^{-4} M carbachol, despite the increase in occupancy, for some other reason e.g. 'channel blocking'. The phenomena of 'autoinhibition' and bell-shaped log dose-response curves are well known. It would appear that the availability of receptors normally does not limit the ^{42}K efflux response, but that this is limited in some other way, possibly by the availability of ion channels, implying that there is not a one-to-one ratio of receptors and ion channels which they operate, and possibly indicating that there is an enzymatic rather than a direct coupling between them.

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References

BIRDSALL, N.M.J., BURGEN, A.S.V., HILEY, C.R. & HULME, E.C. (1976). Binding of agonists and antagonists to muscarinic receptors. *J. Supramol. Structure*, **4**, 367–371.

BIRDSALL, N.J.M., BURGEN, A.S.V. & HULME, E.C. (1978a). The binding of agonists to brain muscarinic receptors. *Mol. Pharmac.*, **14**, 723–736.

BIRDSALL, N.J.M., BURGEN, A.S.V. & HULME, E.C. (1978b). Correlation between binding properties and pharmacological responses of muscarinic receptors. In *Cholinergic Mechanisms and Psychopharmacology*. ed. Jendon, D.J. New York: Plenum Press.

BIRDSALL, N.J.M. & HULME, E.C. (1976). Biochemical studies on muscarinic acetylcholine receptors. *J. Neurochem.*, **27**, 7–16.

BELD, A.J. & ARIENS, E.J. (1974). Stereospecific binding as a tool in attempts to localize and isolate muscarinic receptors. *Eur. J. Pharmac.*, **25**, 203–209.

BOLTON, T.B. (1972). The depolarizing action of acetylcholine or carbachol in intestinal smooth muscle. *J. Physiol.*, **220**, 647–671.

BOLTON, T.B. (1975). Effects of stimulating the acetylcholine receptors on the current voltage relationships of the smooth muscle membrane studied by voltage clamp of potential recorded by microelectrode. *J. Physiol.*, **250**, 175–202.

BOLTON, T.B. (1976). On the latency and form of the membrane responses of smooth muscle to the iontophoretic application of acetylcholine or carbachol. *Proc. R. Soc. B.*, **194**, 99–119.

BOLTON, T.B. (1979). Mechanisms of action of transmitters and other substances on smooth muscle. *Physiol. Rev.* **59**, 606–718.

BOLTON, T.B., TOMITA, T. & VASSORT, G. (1981). Voltage-clamp and the measurement of ionic conductances in smooth muscle. In: *Smooth Muscle: an Assessment of Current Knowledge*. ed. Bülbring, E., Brading, A.F., Jones, A.W. & Tomita, T. London: Edward Arnold.

BURGEN, A.S.V., HILEY, C.R. & YOUNG, J.M. (1974a). The binding of [^{3}H]-propylbenzilylcholine mustard by longitudinal muscle strips from guinea-pig intestine. *Br. J. Pharmac.*, **50**, 145–151.

BURGEN, A.S.V., HILEY, C.R. & YOUNG, J.M. (1974b). The properties of muscarinic receptors in mammalian cerebral cortex. *Br. J. Pharmac.*, **51**, 279–285.

BURGEN, A.S.V. & SPERO, L. (1968). The action of acetylcholine and other drugs on the efflux of potassium and rubidium from smooth muscle of the guinea-pig intestine. *Br. J. Pharmac.*, **34**, 99–115.

BURGEN, A.S.V. & SPERO, L. (1970). The effects of calcium and magnesium on the response of intestinal smooth muscle to drugs. *Br. J. Pharmac.*, **40**, 492–500.

CASTEELS, R. (1969). Calculation of the membrane potential in smooth muscle cells of the guinea-pig's taenia coli by the Goldman Equation. *J. Physiol.*, **205**, 193–208.

COX, B. & LOMAS, D.M. (1972). The effects of eserine and neostigmine on the guinea-pig ileum and on ileal longitudinal muscle strips. *J. Pharm. Pharmac.*, **24**, 541–546.

DEL CASTILLO, J. & KATZ, B. (1957). A study of curare action with an electrical micro-method. *Proc. R. Soc. B.*, **146**, 339–356.

DURBIN, R.P. & JENKINSON, D.H. (1961). The effect of carbachol on the permeability of depolarized smooth muscle to inorganic ions. *J. Physiol.*, **157**, 74–89.

FEWTRELL, C.M.S. & RANG, H.P. (1973). The labelling of cholinergic receptors in smooth muscle. In *Drug Receptors*. ed. Rang, H.P. pp. 211–224. Baltimore: University Park Press.

GABELLA, G. (1975). Hypertrophy of intestinal smooth muscle. *Cell Tiss. Res.*, **163**, 199–214.

GEORGE, E.P. (1961). Resistance values in a syncytium. *Aust. J. exp. Biol. med. Sci.*, **39**, 267–274.

HULME, E.C., BIRDSALL, N.J.M., BURGEN, A.S.V. & MEHTA, P. (1978). The binding of antagonists to brain muscarinic receptors. *Mol. Pharmac.*, **14**, 737–750.

HULME, E.C., BURGEN, A.S.V. & BIRDSALL, N.J.M. (1975). Interactions of agonists and antagonists with the muscarinic receptor. *Colloques d'INSERM*, **50**, 49–70.

KLOOG, Y., SACHS, D.I., KORCZYN, A.D., HERON, D.S. & SOKOLOVSKY, M. (1979). Muscarinic acetylcholine receptors in cat iris. *Biochem. Pharmac.*, **28**, 1505–1511.

KLOOG, Y. & SOKOLOVSKY, M. (1977). Muscarinic acetylcholine receptor interactions: competition binding studies with agonists and antagonists. *Brain Res.*, **134**, 167–172.

KURIYAMA, H. & TOMITA, T. (1970). The action potential in the smooth muscle of the guinea-pig taenia coli and ureter studied by the double sucrose gap method. *J. gen. Physiol.*, **55**, 147–162.

PATON, W.D.M. (1961). A theory of drug action based on the rate of drug-receptor combination. *Proc. R. Soc. B.*, **154**, 21–69.

PURVES, R.D. (1974). Muscarinic excitation: a microelectrophoretic study on cultured smooth muscle cells. *Br. J. Pharmac.*, **52,** 77–86.

RANG, H.P. (1966). The kinetics of action of acetylcholine antagonists in smooth muscle. *Proc. R. Soc. B.*, **164,** 488–510.

SNEDECOR, G.W. & COCHRAN, W.G. (1967) *Statistical Methods.* 6th ed. Ames, Iowa: Iowa State University Press.

SNYDER, S.H., CHANG, K.J., KUHAR, M.J. & YAMAMURA, H.I. (1975). Biochemical identification of the mammalian muscarinic receptor. *Fedn Proc.*, **34,** 1915–1921.

TAYLOR, I.K., CUTHBERT, A.W. & YOUNG, M. (1975). Muscarinic receptors in rat intestinal muscle: comparison with the guinea-pig. *Eur. J. Pharmac.*, **31,** 319–326.

TOMITA, T. (1970). Electrical properties of mammalian smooth muscle. In *Smooth Muscle.* ed. Bülbring, E., Brading, A.F., Jones, A.W. & Tomita, T. pp. 197–243. London: Edward Arnold.

WARD, D. & YOUNG, J.M. (1977). Ligand binding to muscarinic receptors in intact longitudinal muscle strips from guinea-pig intestine. *Br. J. Pharmac.*, **61,** 189–197.

YAMAMURA, H.I. & SNYDER, S.H. (1974). Muscarinic cholinergic receptor binding in the longitudinal muscle of the guinea-pig ileum with [^{3}H]-quinuclidinyl benzilate. *Mol. Pharmac.*, **10,** 861–867.

YOUNG, J.M. (1974). Desensitisation and agonist binding to cholinergic receptors in intestinal smooth muscle. *FEBS Letters*, **46,** 354–356.

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