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The action of acetylcholine and other drugs on the efflux of potassium and rubidium from smooth muscle of the guinea-pig intestine

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1. A method is described for measuring continuously the efflux of potassium or rubidium from smooth muscle of the guinea-pig.

2. Muscarinic drugs cause at maximum a 100-fold increase in the efflux rate, due to a direct increase in permeability and only to a minor extent secondary to depolarization. With acetylcholine the dose response curve for producing efflux is displaced to 1,000 times higher concentrations than that for contraction.

3. The shift varies with different agonists. The efflux and contractile responses to agonists are antagonized to an equivalent extent by atropine and several other reversible antagonists but benzhexol has a relatively greater effect on efflux. An estimate of spare receptors was obtained with benzilylcholine mustard and was similar for both responses. Dibenamine and local anaesthetics led to a parallel shift of the contraction dose response curve but a depression without shift in the efflux response.

4. The most satisfactory explanation of these results is that there are two types of the muscarinic receptor in the smooth muscle of the guinea-pig intestine.

Nearly all our knowledge of the quantitative pharmacology of smooth muscle has been derived from measurements of the contraction of the muscle in response to agonist drugs. In contrast there have been fewer attempts to study the quantitative features of drug action by measuring other changes produced by drugs. It is now well established that acetylcholine and other agonists that produce muscle contraction increase ionic movements (Born & Bülbring, 1956 ; Weiss, Coalson & Hurwitz, 1961 ; Durbin & Jenkinson, 1961 ; Bass, Hurwitz & Smith, 1964).

In this paper we describe an investigation of the quantitative nature of the effects of agonists on potassium and rubidium efflux from intestinal muscle of guinea-pigs, studied in parallel with measurement of contractions.

Methods

Experiments were carried out on strips of longitudinal muscle from the small intestine of the guinea-pig prepared by the method of Rang (1964) and on carefully cleaned strips of taenia coli.

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Fine silk threads were tied on either end of strips about 2 cm long (weight 5–10 mg) which were then mounted at their resting length on 3 mm stainless steel rods for subsequent handling. The preparations were first equilibrated in bicarbonate Krebs solution gassed with a 95% oxygen and 5% carbon dioxide mixture and maintained at 33° C. After 1–2 hr they were transferred to a small bath in which the solution was continually mixed by a gas lift. The bath contained the loading solution which included either ^{86}Rb (specific activity 3–17 mc/mmole) or ^{42}K (carrier free admixed with ^{43}K) at a concentration of 30–200 μc/ml. Bathing for 60–75 min sufficed to produce a high degree of labelling.

The preparations were then transferred to the experimental flow chamber illustrated in Fig. 1.

The chamber consisted of a hole 1.5 mm diameter and 4 cm in length drilled in a Perspex block. Entry and exit tubes entered the sides of the chamber at the top and bottom respectively. One of the threads attached to the muscle was tied to the actuating hook of a transducer fixed into the top of the bath and the other thread was anchored to the bottom of the bath.

Krebs bicarbonate solution (Na 143 mM ; K 5.5 mM ; Ca 2.5 mM ; Mg 2.4 mM ; Cl 139 mM ; HCO_3 25 mM ; glucose 11 mM) equilibrated with 95% oxygen and 5% carbon dioxide was drawn through the bath and then through a β-counter at a constant rate (6 ml./min) by a roller pump. The Perspex block was immersed in a thermostatted water bath held at 31.5° C. The volume of the bath was ~0.07 ml.,

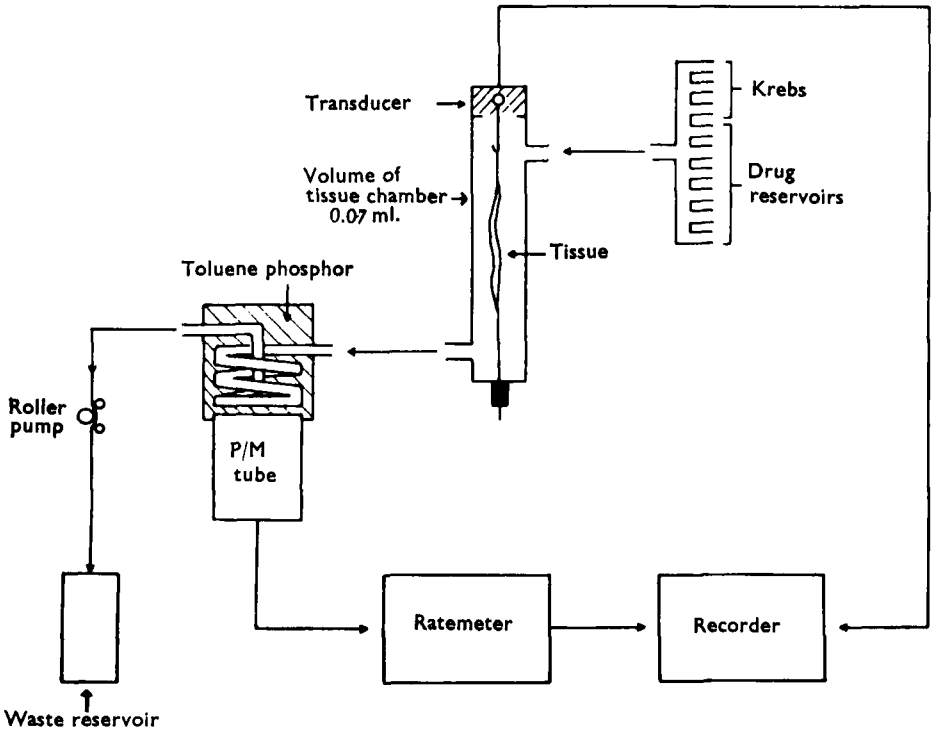

FIG. 1. Diagrammatic representation of experimental arrangement. For details see text.

so the turnover time of the fluid in the empty bath was ~0.7 sec. An electromagnetic valve controlled by a recycling timer replaced the control Krebs solution by solution containing drugs for predetermined periods.

The β-counter was made from a flat spiral of very thin-walled glass tubing (internal diameter 2 mm, volume 1 ml.) mounted in a well machined out of a duralumin block. A clear glass faceplate was cemented on to the front of the duralumin well. The space between the spiral and the outer chamber was filled with a liquid scintillation medium (0.4% DPO ; 0.01% POPOP in toluene). The face plate was optically coupled to the end window of an EMI photomultiplier with silicone oil and the whole apparatus enclosed in a light tight lead castle.

The photomultiplier output was measured on a scaler-rate meter (EKCO N610A) the output of which was recorded on one channel of a Rikadenki potentiometric recorder. The background counting rate was 10 c.p.m. and the counting efficiency was 35–45% for ^{86}Rb and 60–70% for ^{42}K. The integration time imposed by the whole system was approximately 12 sec.

In most experiments the length of the muscle was recorded by a simple photoelectric isotonic transducer the output of which was linear with change of length. In a few experiments isometric tension recordings were made with a semiconductor strain gauge.

In testing the response to drugs the usual procedure was to use an exposure time of 15 sec and an interval between doses of 4 min. In the text this schedule has been used unless otherwise indicated.

Drugs

The following gifts of drugs are acknowledged: oxotremorine, oxotremorine M and furmethide (May and Baker); benzilyldimethylbutanol (Brocades, Amerstam); muscarine (Ciba); dibenamine (Smith, Kline and French); benzilycholine mustard and methylfurmethide (Dr. E. W. Gill).

Results

Immediately after setting up, the preparation rapidly lost some isotope, but within a few minutes this settled down to a steady basal efflux which remained unchanged over several hours.

We have expressed effluxes in terms of the efflux rate constant which is the fractional rate of loss per unit time.

Thus the rate constant

$$f = \frac{\triangle A}{\triangle t . A_t}$$

Where $\triangle A$ represents the counts lost in the time interval $\triangle t$ and A_t is the amount of isotope in the tissue at the mid point of the interval $\triangle t$.

In order to determine A_t, the counts released from the tissue over the whole course of the experiment were integrated and extrapolated to infinity. This gives the value of A_∞. A_t is the difference between A_∞ and the total counts lost up to the middle of $\triangle t$.

In the ileum the basal efflux rate of ^{42}K was 0.011 ± 0.001 min^{-1} ($n = 10$) and of ^{86}Rb 0.013 ± 0.002 min^{-1} ($n = 20$). These are not significantly different. In the

taenia the basal ^{86}Rb efflux rate was 0.008 ± 0.001 min^{-1} ($n = 15$). This is significantly lower than in the ileum. No experiments with ^{42}K were carried out on the taenia.

Change in efflux produced by carbachol

When the ileum was exposed to carbachol at a concentration of 3×10^{-7} g/ml. or greater for 15 sec a large increase in efflux resulted (Fig. 2). The rise in counting rate started after a latency of about 10 sec, rose rapidly and reached a peak in about 10–20 sec and then returned to the basal level over the succeeding 1–3 min. The lag in the response is accounted for by the time taken for the drug to reach the muscle from the changeover tap and then for the released radioactivity to reach the sensitive volume of the β-counter. The latency of the tissue response must be very small, certainly less than 2 sec. The efflux produced by repeated carbachol doses of the same magnitude was constant and reproducible usually within $\pm 10\%$ and always within $\pm 20\%$ of the mean value when tested over periods of several hours. The response was graded with dose as can be seen in Fig. 2 and was very large compared with the resting flux. Concentrations of carbachol of 10^{-7} g/ml. and lower gave relatively small increases in flux which bore a less constant relationship to the dose level.

A complete dose response curve to carbachol is shown in Fig. 3 in which it can be seen that the maximum efflux rate constant was about 1.1 min^{-1} and a half maximum response was obtained at 6.5×10^{-6} g/ml. (3.6×10^{-5}M). Experimental

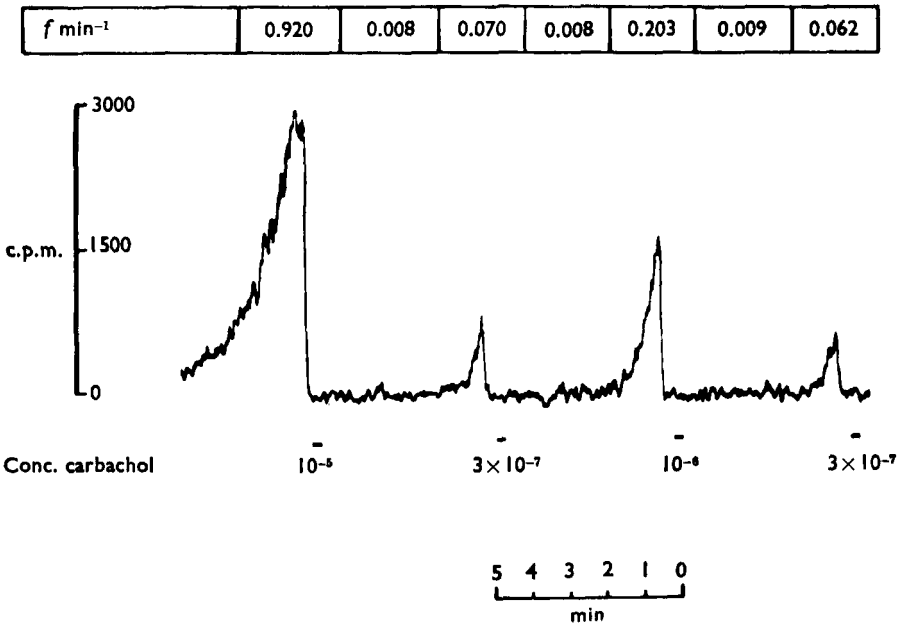

FIG. 2. Record of radioactivity leaving the longitudinal muscle of the ileum following loading with ^{86}Rb. Record reads from the right. Exposures to carbachol for 15 sec are indicated by the bars (doses in g/ml.). Calculated values of the efflux rate constant f are shown in the box above the trace. The values 0.009, 0.008, 0.008 refer to the resting flux in the interdose periods, the other figures to the corresponding drug periods.

points at the top of the curve were difficult to obtain because the preparation readily became refractory after a large dose and took up to an hour to recover ; in some circumstances complete recovery was not obtained. The reason for this deterioration at high concentrations of agonist is not known and is unlikely to be solely the result of large losses of intracellular potassium. This effect has been noticed by other authors in studying contractile responses in the ileum (Paton, 1961).

Because we frequently needed to make many comparisons of efflux on the same muscle preparation it was desirable to avoid this effect by not using concentrations of carbachol greater than 10^{-5} g/ml., but on the other hand it was highly desirable to be able to assess both the maximum response and the apparent affinity constant under different conditions.

We circumvented this difficulty by fitting the results to theoretical curves of the mass action type which were defined by two parameters, namely, the maximum efflux rate ($f$max) and an apparent affinity constant (K_f). Thus

$$\frac{f}{f\text{max}} = \frac{K_f C}{1 + K_f C}$$

Both $f$max and K_f were to be calculated, so fitting was achieved by choosing arbitrary values of $f$max, calculating K_f from the experimental points and testing the goodness of fit by the χ^2 test. By successive trials and interpolation, the values of $f$max and K_f giving the best fit were found. Values at the tail end of the curve usually corresponding to concentrations of carbachol less than 10^{-6} g/ml. were not included for reasons pointed out earlier. Excellent fits were usually obtained as may be seen from the continuous line in Fig. 3 which was fitted to the experimental data shown there. In tests on other complete curves it was shown that accurate prediction of the parameters could be achieved by calculation from data up to

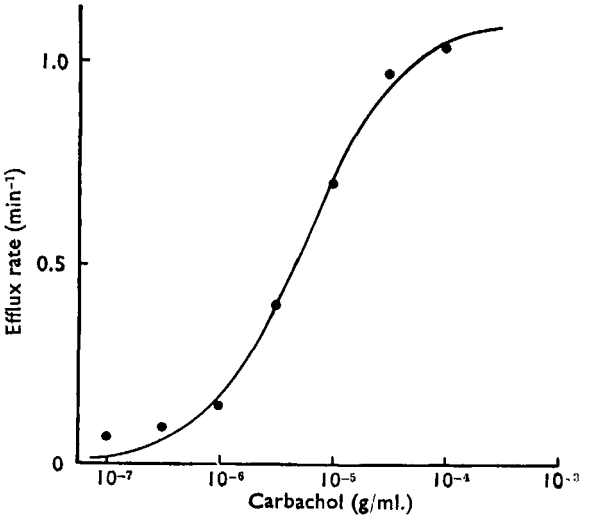

FIG. 3. Efflux rates obtained on ileum loaded with ^{86}Rb in response to carbachol. The points are experimentally determined values. The curve is a mass action curve fitted as described in the text.

10^{-5} g/ml. or 50% of the *f*max. This method of calculation was therefore adopted for the remainder of the experiments to be described.

In forty-eight preparations of ileum studied with Rb the value for *f*max for carbachol was 1.00 ± 0.062 min^{-1} and for five preparations studied with K, *f*max for carbachol was 1.07 ± 0.22 min^{-1}; the difference is not significant. The value of the apparent affinity constant of carbachol was $K_f = 6.60 \pm 0.34 \times 10^4$ $moles^{-1}$ for Rb and again the value for K was not significantly different.

The spread of values among the preparations examined over a period of a year was not very great as can be seen from Fig. 4.

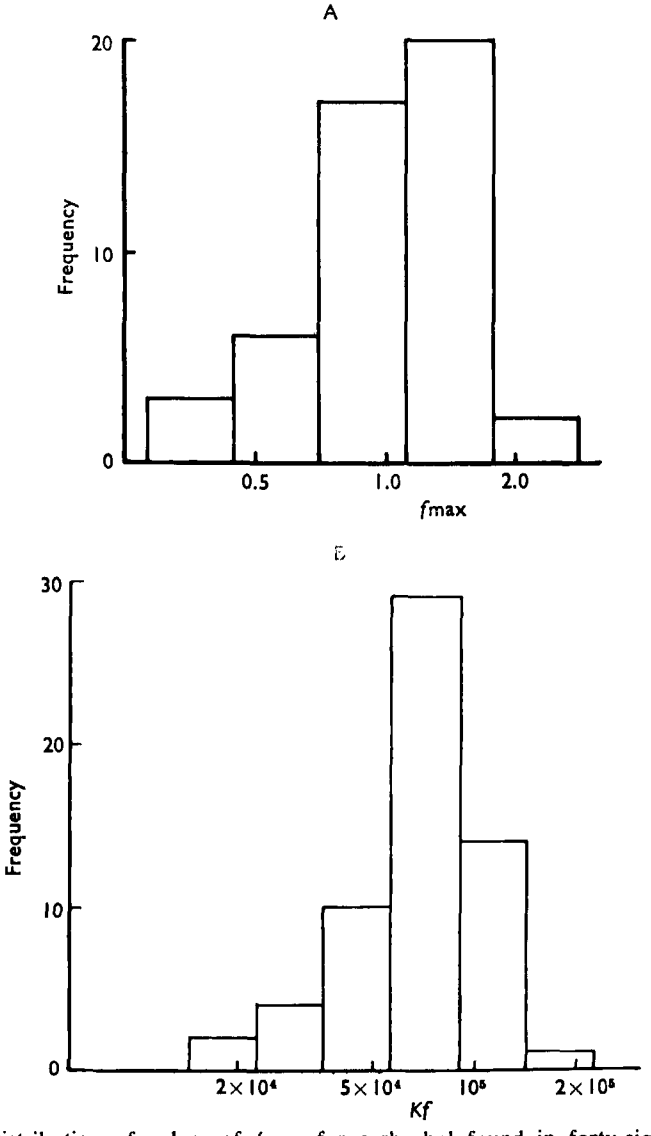

FIG. 4. (A) Distribution of values of *f*max for carbachol found in forty-eight preparations of ileum loaded with ^{86}Rb and examined over a period of a year. (B) Distribution of values of the apparent affinity constant of carbachol for efflux K_f in the same group.

Results obtained with Rb on taenia were quite similar giving for carbachol $f\max = 1.10 \pm 0.16$ min^{-1} and $K_f = 6.75 \pm 0.33 \times 10^4$ moles^{-1}.

Effect of duration of exposure to agonists

In the experiments so far described, the preparation was exposed to the drug for 15 sec and a 4 min interval allowed between doses. The efflux rate constant was, however, substantially independent of the time of exposure to the drug in the range 5–120 sec as may be seen in Fig. 5. There was no sign of fade with longer contact time. Exposures of less than 5 sec gave variable results, probably because the exposure time was then close to the exchange time for the fluid in the organ-bath.

Contribution of depolarization to the increased efflux in response to carbachol

Carbachol and other parasympathomimetic agonists are known to depolarize intestinal smooth muscle (Bülbring & Kuriyama, 1963 ; Burgen & Spero, in preparation). Depolarization will increase the electrochemical potential of potassium inside the muscle fibre and hence increase its tendency to leave the fibre and thus at least part of the increased efflux caused by carbachol might be secondary to depolarization. That this was not likely to account entirely for the increased efflux was evident from the experiments of Durbin & Jenkinson (1961) who found that carbachol could still produce an increase in potassium efflux from taenia depolarized by immersion in a solution in which all the sodium had been replaced by potassium. We have found that an increase of external potassium produced reproducible increases in efflux rate in the ileum reaching a maximum of 0.10–0.15 min^{-1} when the external potassium was made isotonic. This parallels very well the depolarization produced by isotonic potassium in the taenia (Kuriyama, 1963). This efflux rate, however, falls far short of the maximum that can be produced by carbachol. Furthermore, raised potassium does not seriously interfere with the increased efflux produced by carbachol (Table 1).

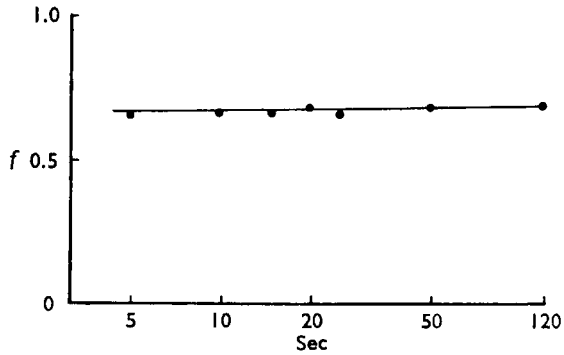

FIG. 5. Values of efflux rate of ileum loaded with ^{86}Rb when exposed to carbachol 3×10^{-6} g/ml. for periods ranging from 5 to 120 sec.

TABLE 1. *Increase in rubidium efflux rate (min^{-1}) caused by carbachol*

Concentration of carbachol (g./ml.)	Ileal muscle bathed in	
	4·7 mM K	148 mM K
10^{-6}	0·151	0·124
3×10^{-6}	0·423	0·371

Responses to other parasympathomimetic drugs

The responses to carbachol were clearly of a muscarinic type for they were readily antagonized by atropine and other antagonists of muscarinic actions and were unaffected by hexamethonium (4×10^{-5} g/ml.). Other muşcarinic agonists produced increases in efflux in the ileum and with the exception of drugs already known to be partial agonists on the contractile response, produced curves of the same form and with the same maximum efflux rate constant as carbachol. The actual values for *f*max for rubidium ranged from 0.78 to 1.67 min^{-1} (Table 2). No correlation existed between *f*max and the *Kf* values which ranged from 1.60×10^3 to 7.68×10^5 M^{-1}. The most active substance examined was an oxotremorine analogue (called here oxotremorine - M) in which the pyrrolidine group was related by a trimethylammonium group.

TABLE 2. *Parameters of agonist action in guinea-pig ileum*

	*Kf** (M^{-1})	K_c (M^{-1})	*f*max* (min^{-1})	K_c/Kf	No.
Acetylcholine	$3{\cdot}60 \pm 0{\cdot}34\dagger \times 10^3$	$3{\cdot}67 \pm 1{\cdot}42\dagger \times 10^6$	$0{\cdot}910 \pm 0{\cdot}076\dagger$	1020	3
(±)-Methacholine	$5{\cdot}53 \pm 0{\cdot}74 \times 10^3$	$3{\cdot}79 \pm 0{\cdot}08 \times 10^6$	$1{\cdot}150 \pm 0{\cdot}030$	683	3
Carbachol	$7{\cdot}71 \pm 0{\cdot}93 \times 10^4$	$2{\cdot}56 \pm 1{\cdot}34 \times 10^7$	$0{\cdot}913 \pm 0{\cdot}150$	332	10
(±)-Bethanechol	$7{\cdot}52 \pm 2{\cdot}76 \times 10^3$	$4{\cdot}74 \pm 1{\cdot}20 \times 10^5$	$1{\cdot}210 \pm 0{\cdot}390$	63	3
Dilvasene	$1{\cdot}05 \pm 0{\cdot}04 \times 10^4$	$5{\cdot}29 \pm 0{\cdot}97 \times 10^5$	$0{\cdot}780 \pm 0{\cdot}055$	51	3
(±)-Muscarine	$5{\cdot}53 \pm 0{\cdot}46 \times 10^4$	$8{\cdot}03 \pm 1{\cdot}42 \times 10^5$	$1{\cdot}000 \pm 0{\cdot}100$	14	4
Oxotremorine-M	$7{\cdot}68 \pm 0{\cdot}77 \times 10^5$	$9{\cdot}81 \pm 2{\cdot}14 \times 10^6$	$1{\cdot}470 \pm 0{\cdot}210$	12	4
Oxotremorine	$6{\cdot}91 \pm 2{\cdot}50 \times 10^4$	$6{\cdot}07 \pm 1{\cdot}24 \times 10^6$	$1{\cdot}410 \pm 0{\cdot}220$	9	3
Methylfurmethide	$1{\cdot}63 \pm 0{\cdot}27 \times 10^5$	$8{\cdot}01 \pm 2{\cdot}56 \times 10^5$	$1{\cdot}670 \pm 0{\cdot}290$	5	4
Furmethide	$7{\cdot}17 \pm 1{\cdot}79 \times 10^3$	$1{\cdot}70 \pm 0{\cdot}08 \times 10^4$	$1{\cdot}570 \pm 0{\cdot}880$	2·5	4
Pentyl TMA	$2{\cdot}39 \pm 0{\cdot}35 \times 10^3$	$7{\cdot}10 \pm 0{\cdot}48 \times 10^3$	$1{\cdot}370 \pm 0{\cdot}120$	3·0	4
TMA	$1{\cdot}60 \pm 0{\cdot}27 \times 10^3$	$5{\cdot}06 \pm 0{\cdot}32 \times 10^3$	$0{\cdot}800 \pm 0{\cdot}250$	3·2	3
Hexyl TMA	$3{\cdot}73 \pm 1{\cdot}47 \times 10^2$	$5{\cdot}44 \pm 1{\cdot}49 \times 10^2$	$0{\cdot}530 \pm 0{\cdot}023$	1·5	4
Heptyl TMA	$3{\cdot}50 \pm 1{\cdot}32 \times 10^2$	$7{\cdot}19 \pm 0{\cdot}34 \times 10^2$	$0{\cdot}233 \pm 0{\cdot}049$	2·0	3
Octyl TMA	$1{\cdot}49 \times 10^2$	—	0·038	—	1
Pentylethyl, DMA	$5{\cdot}53 \pm 0{\cdot}13 \times 10^2$	$2{\cdot}83 \pm 0{\cdot}12 \times 10^3$	$0{\cdot}052 \pm 0{\cdot}003$	5·1	3
Pilocarpine	$2{\cdot}95 \pm 0{\cdot}08 \times 10^3$	$6{\cdot}42 \pm 0{\cdot}26 \times 10^3$	$0{\cdot}184 \pm 0{\cdot}03$	2·2	2

* Efflux of rubidium. † S.E. of mean.

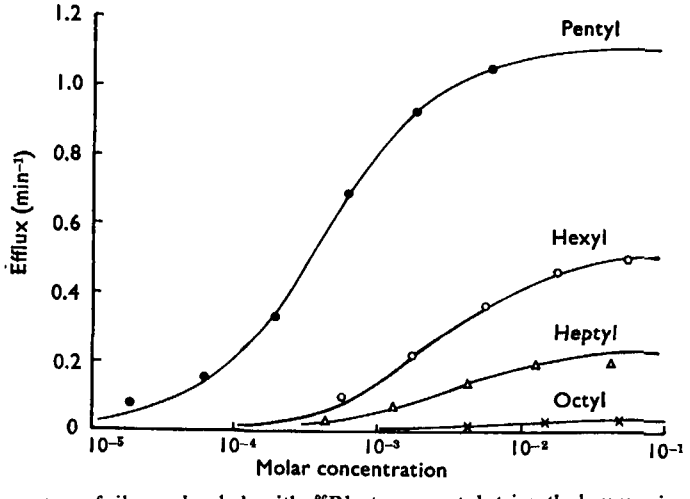

FIG. 6. Efflux rates of ileum loaded with ^{86}Rb to *n*-pentyl trimethylammonium (●——●); *n*-hexyl trimethylammonium (○——○); *n*-heptyl trimethylammonium (△——△); *n*-octyl trimethylammonium (×—— ×). Exposure to drug for 15 sec.

It is interesting that acetylcholine and methacholine, the only two agonists susceptible to hydrolysis by cholinesterase gave curves similar to those of the stable drugs and these curves were not appreciably altered by the anticholinesterases, neostigmine (10^{-6} g/ml.) or tetraethyl pyrophosphate (10^{-7} g/ml.).

Some drugs were classifiable as partial agonists in that the *f*max was reduced below that seen for the agonists just considered. The development of partial agonist behaviour is nicely seen in the alkyltrimethylammonium series in which the C_5 member is a full agonist, the C_6 and C_7 members are partial agonists and the C_8 member is a very weak agonist (Fig. 6). Pentyl ethyldimethylammonium and pilocarpine were also partial agonists.

In the case of partial agonists the magnitude of the efflux rate constant was not independent of contact time, but steadily decreased with time. The curves in Fig. 6 were obtained from experiments in which a 15 sec exposure was used and thus underestimate the efflux rates that would be found if very short exposures were experimentally practical. The values for these agonists in Table 2 are subject to the same limitations.

Relationship between efflux and contraction

Fig. 7 shows the result of a typical experiment in which both the efflux and contractile responses to carbachol were measured simultaneously. Both curves fit mass action curves satisfactorily but they are not coincident for the efflux curve lies far to the right of the contraction curve. In ten such experiments the apparent affinity constant for the contraction response was $K_c = 2.56 \pm 1.34 \times 10^7$ M^{-1} whereas the efflux affinity constant was $K_f = 7.71 \pm 0.93 \times 10^4$ M^{-1} giving a ratio between these parameters of 332. With acetylcholine and methacholine the displacement was even larger (Table 2). Bethanechol and dilvasene showed smaller differences whereas the compounds listed below oxotremorine in the table showed ratios of 5 or less. In no case was the apparent affinity for the efflux response greater than

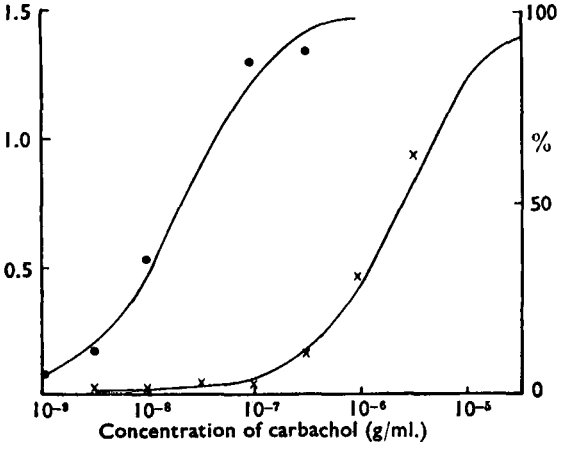

FIG. 7. Comparison of the dose-response curves for contraction and Rb efflux in the ileum. Carbachol was the agonist. ●——●, Contraction (as % of maximum response); ×——×, efflux (min^{-1}).

that for contraction. No clearcut relationship exists between the absolute values of either K_c or K_f and their ratio. All that can be said is that the weakest agonists tend to have a low value of K_c/K_f.

It is clear from Fig. 7 that the small increase in efflux occurring with low doses of carbachol is associated with the contractile response and probably with the generation of action potentials. This foot on the efflux curve was especially evident when acetylcholine was the agonist.

Effects of muscarine antagonists

The efflux response to carbachol was readily antagonized by atropine. In the experiment illustrated in Fig. 8 the dose ratio against contraction and efflux are seen to be identical. In a total of six preparations the affinity constant of atropine measured against an efflux response produced by carbachol was $7.53 \pm 1.08 \times 10^8$ M^{-1} and against the contractile response was $6.54 \pm 0.98 \times 10^8$ M^{-1}. The difference is not significant ($P > 0.5$). The association and dissociation kinetic rate constants were also determined and were not significantly different for the two responses.

A similar lack of selective antagonism was also found with four other antagonists (Table 3). Benzhexol was exceptional in that it was 8.5 times as active against the efflux response as against contraction.

None of the antagonists reduced *f*max even though concentrations giving dose ratios of up to 1,000 were used.

Benzilycholine mustard

When the alkylating antagonist benzilycholine mustard (Gill & Rang, 1966) was used a similar pattern was seen for both responses. With a short exposure to

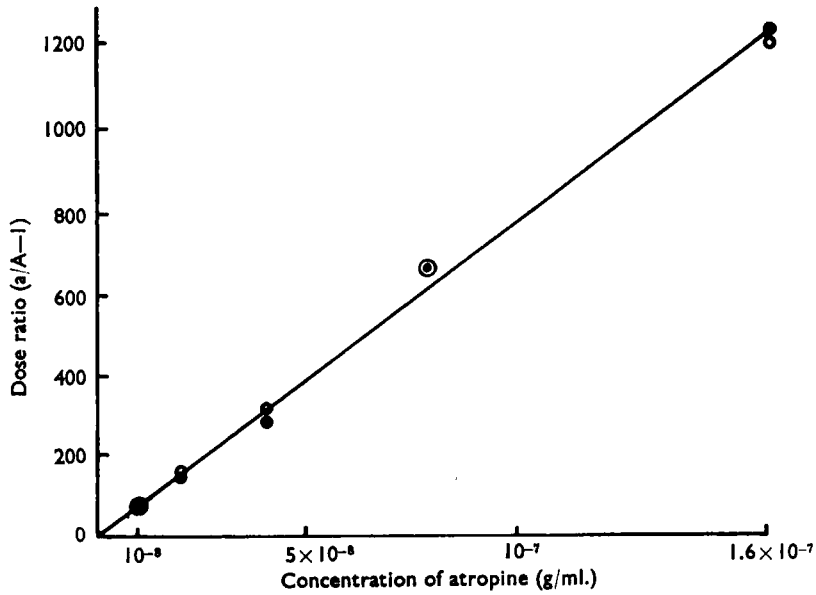

FIG. 8. Antagonism of carbachol action by atropine. Dose ratios (a) for efflux (●) and contraction (○) responses in the ileum as a function of atropine concentration (A).

2×10^{-8} g/ml. the dose response curves for carbachol were displaced to the right without depression of the maximum responses and with longer times of exposure further displacement occurred with depression of the maxima (Figure 9). Taking the ratio of apparent affinity before treatment with the alkylating agent to that at which the maximum response begins to be depressed as a measure of spare receptor availability gave in the example in Figure 9 a ratio of 83 for contraction and 150 for efflux. These ratios were lower than found in most experiments in which the average ratio was 273 ± 47 for contraction and 384 ± 46 for efflux.

TABLE 3. *Affinity constants of reversible antagonists*

	For efflux* K_A M^{-1}	For contraction* K_A M^{-1}	Significance of difference (P)
Atropine	$7{\cdot}53 \pm 1{\cdot}08\dagger \times 10^8$	$6{\cdot}54 \pm 0{\cdot}98\dagger \times 10^8$	$>0{\cdot}5$
Lachesine	$3{\cdot}90 \pm 0{\cdot}43 \times 10^8$	$4{\cdot}09 \pm 0{\cdot}07 \times 10^8$	$>0{\cdot}6$
Tricyclamol	$1{\cdot}32 \pm 0{\cdot}27 \times 10^9$	$1{\cdot}64 \pm 0{\cdot}36 \times 10^9$	$>0{\cdot}5$
Methyl-scopolamine	$1{\cdot}14 \pm 0{\cdot}13 \times 10^9$	$1{\cdot}15 \pm 0{\cdot}06 \times 10^9$	$>0{\cdot}9$
Benzilyl dimethyl butanol	$2{\cdot}10 \pm 0{\cdot}12 \times 10^6$	$4{\cdot}14 \pm 0{\cdot}95 \times 10^6$	$>0{\cdot}1$
Benzhexol	$6{\cdot}60 \pm 0{\cdot}02 \times 10^{10}$	$7{\cdot}70 \pm 0{\cdot}32 \times 10^9$	$<0{\cdot}001$

* Agonist carbachol; isotope ^{86}Rb. † S.E. of mean.

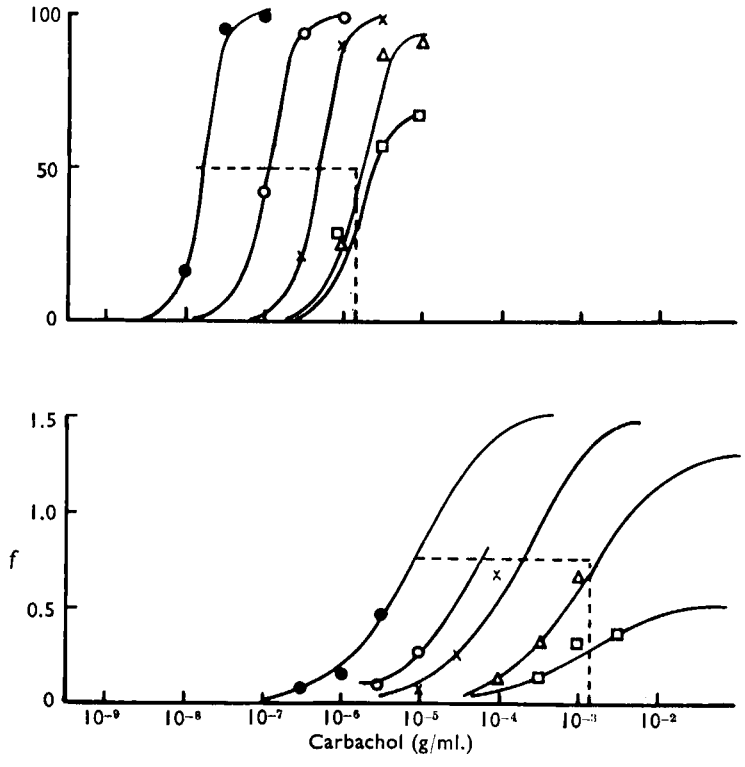

FIG. 9. Effect of exposure to benzilyl choline mustard on contraction and efflux responses produced by carbachol. ●——●, Control curves; benzilyl choline mustard 2×10^{-8} g/ml.; ○——○, 4 min; ×——×, 9 min; △——△, 15 min; □——□, 25 min. Notice that both curves show a displacement to the right before the maximum is depressed.

Dibenamine

The results obtained in similar experiments using dibenamine as the alkylating agent were rather different. While the contraction response showed displacement followed by depression of the maximum, the response curve for efflux was immediately depressed without significant shift of the apparent affinity constant for carbachol (Fig. 10).

Local anaesthetics

Both lignocaine and cinchocaine produced a reduction in the resting potassium efflux of the ileal muscle at concentrations as low as 2×10^{-7} g/ml. Both agents caused a reduction in *f*max without any shift in the efflux dose response curve and also caused a shift in the contractile dose response curve to the right. In these actions cinchocaine was about 5–15 times as active as lignocaine.

Other agonists

A few experiments were carried out with histamine. There was a K_c/K_f ratio of approximately 60, but the maximum efflux was only 0.106 min^{-1}. In the presence of mepyramine the contraction response was displaced to the right whereas the efflux response was depressed without displacement.

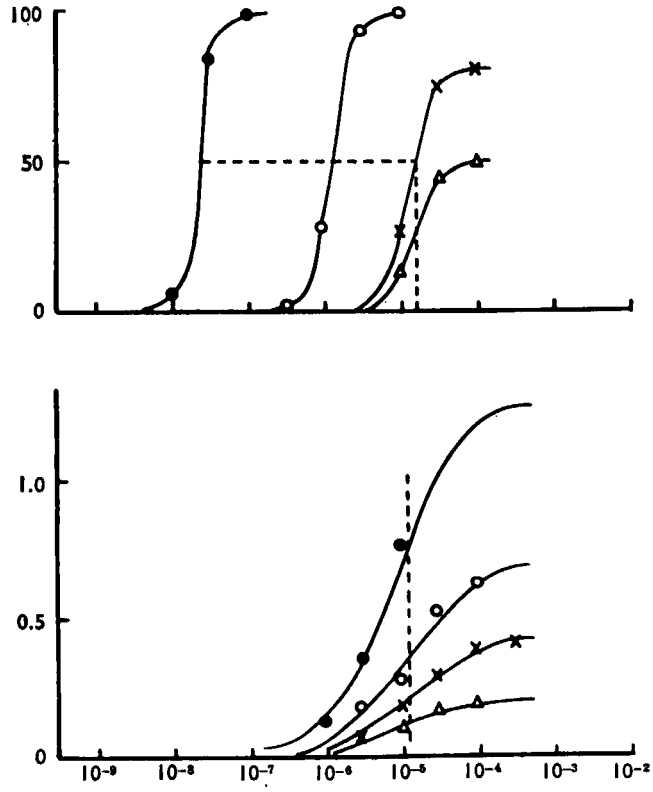

FIG. 10. Effect of exposure to dibenamine on contraction and efflux responses produced by carbachol. ●——●, Control response ; exposure to dibenamine 10^{-6} g/ml. for: ○——○, 4 min ; ×——×, 10 min ; △——△, 18 min.

Angiotensin and caffeine also produced maximum effluxes of ~0.1 min^{-1} and a K_c/K_f of the order of 20. The efflux response to catecholamines was very small.

Discussion

The results obtained in the experiments described in this paper show that parasympathomimetic drugs can produce a very large increase in the efflux of potassium and rubidium from the longitudinal muscle of the ileum and taenia coli of the guinea-pig. This effect is presumably accompanied by correspondingly large changes in influx of sodium and perhaps calcium because the overall effect of the drugs is to produce a depolarization of the fibres which is accountable as a result of an excess entry of cations over those leaving. The magnitude of these changes is comparable with those occurring at the motor endplate in skeletal muscle as deduced from potential and conductance changes (Ginsborg, 1967).

The receptors responsible for efflux response in smooth muscle clearly belong to the muscarinic class as shown by the lack of antagonism by hexamethonium and the ready antagonism by atropine and its congeners.

One of the most interesting features of the action of some of the agonists is that the dose range needed to activate the efflux response is much higher than that needed to produce contraction. The possibility must be considered that at these high doses release of acetylcholine from the intestinal parasympathetic fibres is occurring and it is this that leads to the efflux response. This is made highly implausable by the fact that acetylcholine itself displays the largest ratio in activity (K_c/K_f) as regards the two responses. Furthermore, there are several facts that argue against an effect on nerve endings. The sensitivity of the efflux response is altered only to a minor extent by isotonic potassium in the bath fluid although this would be expected to produce both maximal activation of acetylcholine release and rapid depletion of the stores, nor is it significantly altered by changes in calcium concentration in the bath fluid which would produce considerable changes in both acetylcholine release by nerve stimulation, or the release mechanism described by McKinsky & Koelle (1967). Furthermore it has been shown (Bennett & Rogers, 1967) that the cholinergic fibres in the taenia synapse on only certain fibres in a bundle and that the response in the remaining fibres occurs as a result of the spread of electronically propagated junctional potentials through intercellular contact zones, or by the propagation of action potentials through these junctions. These findings suggest that the effects of released acetylcholine is confined to a local response in the immediate vicinity of the nerve fibres. We have shown that the major part of the efflux response is not secondary to depolarization of the membrane but is a more direct consequence of drug interaction with the cellular receptors. Exposure to an agonist in high concentration can lead within a minute to the loss of a large fraction of the total radioactivity in the tissue ; this is not compatible with a focal action on only a few fibres of a muscle bundle. This seems to argue very strongly against an indirect action of agonists through acetylcholine release from nerve fibres.

The most straightforward explanation why a lower concentration of agonist is needed to produce contraction than efflux is that contraction is normally initiated by depolarization of rather sparsely distributed pacemaker zones at which action potentials are generated which then spreading through the remainder of the smooth muscle syncytium cause a general increase in tone. This mechanism is entirely

comparable with that in skeletal muscle where agonist drugs produce depolarization at the motor end plate and this initiates propagated action potentials which result in the contraction of the fibre. If these pacemaker zones occupy only a small fraction of the total membrane surface it is easy to see why their activation should not lead to any large increase in ion efflux. Indeed it might be expected that such local efflux as did occur might be inconsiderable compared with that due to the resulting increase in the action potential firing rate.

As pointed out earlier, however, the massive nature of the efflux response points quite clearly to a distribution of receptors responsible for this response which must be relatively general.

We can reconcile these two aspects of the problem if we regard receptors as being widely distributed over the cell surface but being more densely congregated in the pacemaker zones; it does not seem unreasonable to suggest that the latter may be mainly or perhaps wholly in the vicinity of the cholinergic motor nerve fibres. The parallel with the organization of skeletal muscle and its motor nerve is then apparent. The theory as proposed so far explains the displacement of the dose-response curves for efflux and contraction with respect to each other on the basis of a built-in amplifier due to local concentration of receptors in focal zones and propagation by action potentials which results in a greater sensitivity of the tissue as regards the contractile response.

This will not, however, account for the finding that the K_c/K_f ratio is not constant but varies from 2.5 to 1,020 for the full agonists examined. It should be noticed also that there is no simple relationship between the ratio K_c/K_f and the magnitude of either parameter. For example, acetylcholine has a K_c of 3.67×10^6 M^{-1} and $K_c/K_f = 1{,}020$ yet oxotremorine-M which is a little more active in producing contraction ($K_c = 9.81 \times 10^6$ M^{-1}) has a $K_c/K_f = 12$. If the top nine agonists in Table 2 are arranged in order of potency it is seen that the order is strikingly different for the two responses (Table 4). These substances demonstrate a classical difference in structure-activity relationship for the two responses and the most direct pharmacological interpretation of such an occurrence is that we are dealing with distinct receptor types concerned respectively with generating action potentials and with producing major changes in the ion flux.

It is to be expected, however, that if two distinct receptors are present they will also display differences in their behaviour to antagonists.

As far as atropine was concerned, no discrimination was found between the responses; neither the antagonist affinity constant nor the kinetic constants were different. Nor were there significant differences with lachesine, tricyclamol, methyl-

TABLE 4. *Order of potency*

	Contraction	Efflux
Highest	Carbachol	Oxotremorine M
	Oxotremorine M	Methylfurmethide
	Methacholine	Carbachol
	Acetylcholine	Oxotremorine
	Muscarine	Muscarine
	Methylfurmethide	Dilvasene
	Oxotremorine	Bethanechol
	Dilvasene	Methacholine
Lowest	Bethanechol	Acetylcholine

scopolamine, or benzilyl-dimethylbutanol. Benzhexol was, however, 8.5 times as active on efflux as on contraction. This discrepancy is interesting especially in view of the finding by Paton & Rang (1965) that the activity of benzhexol in competing with the uptake of tritiated atropine by ileum did not accord with its activity as an antagonist of the contractile response to methylfurmethide. In this case, however, the competitive " affinity " of benzhexol was lower than its direct antagonistic affinity.

It seems unwise to rely on this exception, however, and we would rather come to the conclusion that the experiments with reversible antagonists do not support the idea of distinct receptor types, although they do not exclude it because it is quite possible to suppose, as has been suggested by Ariens (1964), that the main specificity of antagonists is concerned with combination with an area of the cell membrane adjoining a more restricted site concerned with combination with agonists. It is possible therefore that while there are distinct receptor variants in the agonist " areas," the antagonist " areas " are identical.

The experiments with the alkylating antagonist benzilylcholine mustard take us some way further in this analysis because not only was the change of dose ratio produced by different periods of exposure quite similar for both responses, but so was the estimate of a receptor reserve of some 300 400 fold. This estimate of receptor reserve has the effect of reducing the putative affinity constant of carbachol for efflux to 2.83×10^2 M^{-1}—a singularly low value and that for contraction to 2.68×10^4 M^{-1}.

The particular interest of these values is that they are still in a ratio of nearly 100. We cannot therefore explain the differences between affinities on the assumption that the spare receptor availability for the contractile response is sufficiently greater than that for the efflux response to account for the lower sensitivity of the latter.

The experiments in which dibenamine was used as alkylating antagonist are of very considerable interest. On the contractile response low doses produced a parallel shift in the dose response curve leading to a similar estimate of the apparent receptor reserve (420 ± 19) to that found with benzilylcholine mustard but at the same time the dose response curve for efflux showed no shift along the concentration axis, but only a depression of the whole curve including the estimated *f*max. The end result is that with sufficiently long exposures to dibenamine the dose response curves for contraction and efflux are superimposable. If we were to take these results at their face value, it would imply just what the results with benzilylcholine mustard seem to have disposed of, namely that the difference in the dose response curves for contraction and efflux is due to a difference in spare receptor ratios.

We also found, however, that no depression of *f*max occurred with concentrations of atropine sufficient to give large dose ratios and this cannot occur with a slowly dissociating antagonist except where a considerable receptor reserve is present (Rang, 1966). We would therefore propose an alternative explanation of the results with dibenamine—namely, that this agent either directly interferes with ionic permeability sites or with the coupling between the drug receptor and these sites. Thus it may be that as the flux mechanism becomes inactivated so the depolarization produced by a given receptor utilization declines and hence the action potential generation is decreased. More receptors therefore need to be utilized to produce

the same depolarization and rate of firing and hence the dose response curve is shifted to the right. This interpretation draws attention to an inherent ambiguity in the determination of spare receptor ratios with this type of agent.

A rather similar kind of behaviour was seen with the local anaesthetics lignocaine and cinchocaine. Here again there was a shift to the right in the contraction dose-response curve and a depression without shift in the efflux dose response curve. There was one interesting point of difference ; the local anaesthetics diminished the resting efflux whereas dibenamine did not. This suggests that the local anaesthetics may be acting directly on the ionic permeabilty mechanism.

The alkyltrimethylammonium series give us further information. Pentyl trimethylammonium was a full agonist for both responses, but hexyl trimethylammonium was a partial agonist for both responses and to the same extent. In heptyltrimethylammonium the maximum responses were reduced further and again in parallel. This parellelism of reduction of *f*max and *C*max was also evident with pilocarpine and pentyl ethyl dimethylammonium. This is consistent with the interpretation that the affinity of partial agonists for the two responses is similar and that the efficacy is also similar. However, another explanation seems as likely. Suppose that the partial agonists have only very low affinities for the receptors in the foci generating the action potentials, then a generalized increase in permeability and depolarization of the cells will be the initial event and with its accompanying influx of Ca ions might lead to a contraction by a wholly local non-propagated response. The measurement of an affinity constant for the contractile response will then be quite spurious because all it will measure is the activation of the more general permeability response, and it will then follow that the contractile response will have to follow rather closely the efflux response. The same state of affairs may indeed pertain also to those full agonists that show low K_c/K_f ratios. If this is the case then it suggests that the differences in structure activity relationships seen in the agents with large K_c/K_f ratios may also apply to the agents with low K_c/K_f but are concealed by the experimental methods in the work reported here. It should be possible to examine this question further by the intracellular electrode technique where one can examine whether in the case of the partial agonists and agonists with the low K_c/K_f ratios, the contraction response is associated with an increase in the firing rate of action potentials or whether the correlation is more with depolarization and calcium entry. Work of this type is now in progress. If this thesis is sustained it suggests caution in the interpretation of structure activity relationships for agonists based on contraction in smooth muscle.

Finally, we have considered the possibility that the differences between the two responses might be due to diffusional barriers around the receptors, but in this case one would need to endow these barriers with such unusually selective properties towards different agonists and antagonists as to make it highly implausible.

We believe that the most probable explanation of our experiments is that there are two subtypes of the muscarinic receptor on smooth muscle, one possibly located in the vicinity of the neural endings and the other elsewhere, and that these receptors are distinguishable in their responsiveness to muscarinic agonists but are not discriminated by the antagonists studied with the possible exception of benzhexol. We are conscious that the evidence presented here does not provide unequivocal support for this thesis.

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