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MUSCARINIC RECEPTORS IN RAT SYMPATHETIC GANGLIA

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1 Potential changes in isolated superior cervical ganglia of the rat produced by muscarinic-receptor agonists were recorded by an extracellular 'air-gap' method.

2 Muscarinic agonists produced a delayed low-amplitude ganglion depolarization, frequently preceded by a hyperpolarization. Potentials were enhanced by reducing $[K^+]_o$ or $[Ca^{2+}]_o$.

3 Mean ED_{50} values (μM) for depolarization at 25°C were: oxotremorine 0.004, methylfurmethide 0.11, (±)-muscarine 0.24, furmethide 1.56, pilocarpine 4.81 and AHR-602 (*N*-benzylpyrrolidylacetate methobromide) 10.8. Responses produced by oxotremorine, pilocarpine and AHR-602 showed some characteristics of 'partial agonism'. ED_{50} values (μM) for choline esters (measured in the presence of 2.5 mM hexamethonium) were: acetylcholine 3.2, methacholine 59 and bethanechol 78.

4 Responses to muscarine were antagonized by hyoscine (K_I 0.49 nM) atropine (K_I 0.24 nM) methylscopolamine (K_I 0.09 nM) lachesine (K_I 0.15 nM) and (weakly) by hexamethonium (K_I 0.2 mM). Propylbenzilylcholine mustard produced irreversible antagonism with an apparent onset rate constant of 2×10^5 $M^{-1}s^{-1}$.

5 Depolarization was accompanied by facilitation of submaximal ganglionic transmission.

6 Muscarine (1 to 100 μM) initially reduced, then increased, the rate of $^{86}Rb^+$-efflux from isolated ganglia at both 6 and 120 mM $[K^+]_o$. These effects were reduced by 1 μM hyoscine.

7 No consistent change in the amounts of cyclic 3',5'-guanosine monophosphate in isolated ganglia accompanying muscarinic depolarization could be detected.

8 Mean against ED_{50} values (μM) for contracting the rat isolated ileum were: oxotremorine 0.012, methylfurmethide 0.29, (±)-muscarine 0.48, pilocarpine 7.8 and AHR-602 9.9. Mean antagonist K_I values (nM) were: hyoscine 0.17, atropine 0.34 and lachesine 0.27.

9 It is concluded that ganglionic muscarinic receptors are quite similar to ileal receptors in terms of agonist ED_{50} and antagonist K_I values, and that the major difference between them lies in the greater 'efficacy' of certain agonists (pilocarpine, AHR-602 and McN-A-343) on the ganglion.

Introduction

The ability of muscarinic-receptor agonists to excite sympathetic ganglion cells has been recognized for some considerable time (Dale & Laidlaw, 1912; Marrazzi, 1939; Ambache, 1949a and b; Trendelenburg, 1954; Ambache, Perry & Robertson, 1956; Konzett & Waser, 1956; Volle, 1966a and b). However, pharmacological characterization of the receptors involved has so far been rather limited. In part this may reflect the inconsistency of the excitation (see for example Trendelenburg, 1954; Ambache *et al.*, 1956) and the low amplitude of the underlying depolarization (Volle, 1966a and b).

The primary aim of the present experiments was to obtain more detailed information about the pharmacological nature of the receptors responsible for 'muscarinic' depolarization of rat sympathetic ganglia. For this purpose, the depolarization of rat isolated superior cervical ganglia has been recorded by a suitably-sensitive and stable extracellular-recording method (Brown & Marsh, 1975). This type of recording does not readily permit conclusions to be drawn about the mechanism of the potential changes. Although the latter was not the primary objective of the present study, the results of a few measurements of $^{86}Rb^+$ efflux and cyclic guanosine 3',5'-monophosphate changes in the ganglion are described which may be pertinent to the interpretation of the potential changes. Effects of muscarine and some other agonists recorded with intra-

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cellular electrodes are described in the subsequent paper (Brown & Constanti, 1980).

Methods

Superior cervical ganglia were isolated from Wistar rats (200 to 300 g, either sex) anaesthetized with urethane. The outer connective tissue sheaths of the ganglia were removed, and the ganglia maintained in Krebs solution bubbled with 95% O_2 and 5% CO_2 (pH 7.3 to 7.4) at (usually) 25°C.

Surface-depolarization

Ganglion depolarization was measured by the extracellular air-gap method described by Brown & Marsh (1975), with a reference electrode on the postganglionic nerve trunk. D.c. potentials were monitored continuously on a potentiometric chart-recorder at a gain of 1 or 2.5 mV full-scale deflection, filtered to give a minimum time-constant of about 1 s. The ganglion was superfused continuously at 1 ml/min. Access of drug in known concentration to the ganglion was monitored by the deliberate introduction of an air bubble, so that the precise time of drug application (less perfusion dead-time) was known: bars on the records show this contact-time. Most ganglia were left overnight at 4°C before starting experiments, to allow demarcation potentials to subside. This did not affect the general form of the electrical responses.

Transmission

Ganglionic action potentials produced by single preganglionic stimuli (0.6 ms duration, 0.2 Hz) were recorded by use of a three-chambered bath similar to that described by Brown & Marsh (1978). Action potentials were displayed on a chart-recorder using a peak-height detector (Courtice, 1977) to 'hold' the response for sufficient time for a full pen-deflection.

$^{86}Rb^+$-efflux

Ganglia were 'loaded' with $^{86}Rb^+$ (40 μCi/ml, 1 mM RbCl) for 3 h to near-saturation as described by Scholfield (1977a and b). Subsequent efflux of $^{86}Rb^+$ was measured by transferring the ganglia through a series of vials containing Krebs solution at 25°C at 2 min intervals. Radioactivity in the vials and that remaining in the ganglion at the end of the washout period was measured in a liquid scintillation counter by Cerenkov emission and the efflux rate coefficient calculated as described by Brown & Scholfield (1974). In a few experiments efflux was monitored continuously by inserting the ganglion in a small chamber, perfusing the chamber with Krebs solution, and passing the effluent solution through a flow cell inserted in the well of a radiation detector (see Scholfield, 1977a and b). The output from the detector (a Tracerlab 'Coruflow') was coupled via a ratemeter to a chart recorder, using a ratemeter time-constant of (usually) 5 or 15 s. Results obtained by the transference and continuous flow recording were essentially similar.

Cyclic guanosine 3',5'-monophosphate (cyclic GMP)

After the overnight storage at 4°C (as used for electrical recording) the ganglia were pre-equilibrated in Krebs solution for 60 min at 25°C, then exposed to agonist for the desired time. Thereafter, they were transferred to 300 μl buffer solution (50 mM Tris plus 4 mM disodium edetate (EDTA): pH 7.5) at 100°C for 3 min, homogenized and centrifuged for 2 min at 14,000 *g*. The cyclic GMP content of the supernatant was measured by radioimmunoassay (Radiochemical Centre, Amersham), and protein measured by the method of Lowry, Rosebrough, Farr & Randall (1951). In some experiments the ganglia were pretreated for 10 min with 0.5 mM isobutylmethylxanthine (IBMX) before adding agonist, to inhibit phosphodiesterase.

Rat ileum

Contractions of the isolated terminal ileum of the rat bathed in Krebs solution at 25°C were recorded on a smoked drum with a frontal writing lever.

Data analysis

Dose-response data was analyzed assuming a hyperbolic relationship between response (y) and drug concentration (x), such that $y = y_{max}.x/(K + x)$. Least-squares estimates for the kinetic constants y_{max} and K were sought directly using Powell's (1968) method for minimizing the sums of squares of deviations of observed and predicted values for y. Antagonist potencies (K_I values) were estimated from the shift in the value of K in the presence of a known concentration of antagonist (I), assuming the competitive relationship $K' = K(1 + I/K_I)$: in this case responses with and without antagonist (I = I and I = 0 respectively) were processed simultaneously to find a common value for y_{max} and values for K and K_I. Curves were constrained to go through the origin. All responses were normalized against those to a standard concentration of muscarine applied in the same experiment, to reduce variations in absolute response amplitude in different experiments. Data points were not normally weighted since (i) insufficient replicates (usually 4) were obtained at each concentration with most agonists to justify selected weightings and (b) tests on the more extensive muscarine raw data

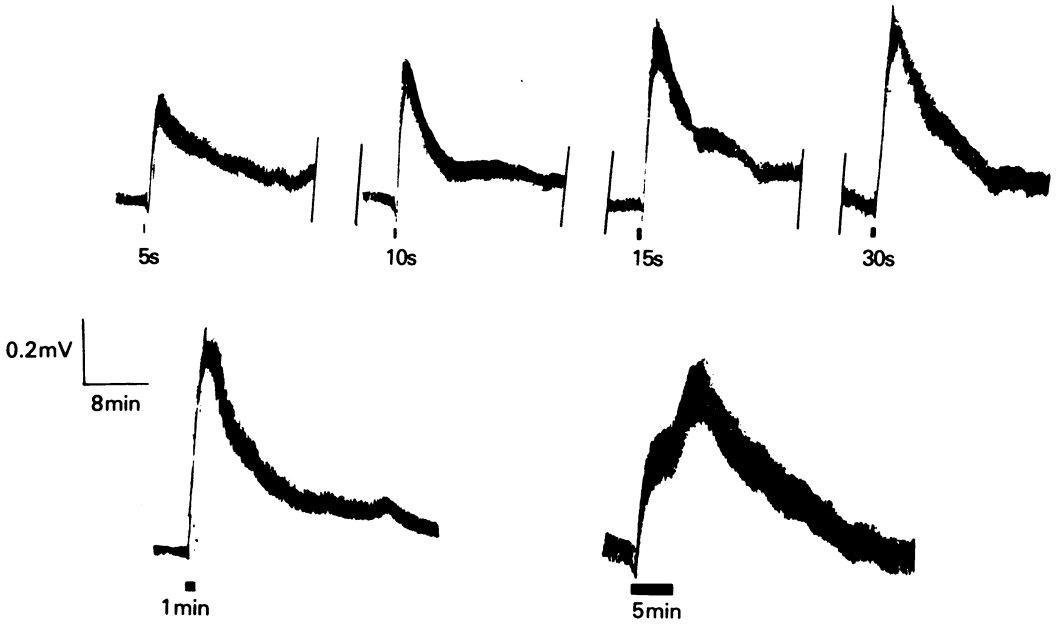

Figure 1 Depolarization of a single rat superior cervical ganglion produced by (±)-muscarine (1 μM) applied (at dots) for different durations. Vertical calibration: change in d.c. potential (bar = 0.2 mV; depolarization upwards). Horizontal calibration: time scale (bar = 8 min). Forty min was allowed for recovery between applications.

revealed no appreciable difference in the estimates of the kinetic parameters on weighting data points according to standard errors of means or sample standard deviations when compared with unweighted estimates.

Estimates for kinetic parameters for normalised muscarine data were cross-checked against non-normalized responses. The accuracy of estimates for y_{max}, K and K_1 were expressed as 95% confidence limits (CL), assuming a normal distribution of errors. The coefficient of serial correlation was normally $< +0.5$, > -0.5, suggesting that any departures from the assumed hyperbolic relationships were insufficiently consistent to warrant the search for alternative expressions.

Solutions

The Krebs solution used had the following composition (mequiv/l): Na^+ 143, K^+ 5.9, Ca^{2+} 2.52, Mg^{2+} 1.3, Cl^- 127, HCO_3^- 25, SO_4^{2-} 1.3, $H_2PO_4^-$ 1.2 and D-glucose 11. The K^+ concentration was altered by adding or omitting KCl (normally 4.7 mM). A Tris-buffered solution (in which 10 mM Tris base, buffered to pH 7.4 with HCl, was substituted for 25 mM $NaHCO_3$) was used to test elevated Ca^{2+} concentrations (added as $CaCl_2$); adjustment of osmolarity was not found necessary.

Drugs

The following drugs were used (with sources): acetylcholine chloride, atropine sulphate, hyoscine hydrobromide, nicotine hydrogen tartrate (all from BDH), (±)-muscarine chloride, bethanechol chloride (carbamyl-β-methylcholine), carbachol (carbamyl choline), methacholine (acetyl-β-methylcholine), physostigmine salicylate, (−)-noradrenaline bitartrate, pilocarpine hydrochloride, hexamethonium bromide, yohimbine (all from Sigma) and lachesine hydrochloride (Evans Medical Ltd.). Furmethide, AHR-602 (*N*-benzyl-3-pyrrolidyl-acetate methobromide) and McN-A-343 ((3-*m*-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium) were gifts from Smith, Kline and French Laboratories, Robins Research Laboratories and McNeil Laboratories respectively. Dr A. Ungar and Dr L.L. Iversen and Dr J.M. Young, provided samples of methyl furmethide, 8-bromocyclic GMP and propylbenzilylcholine mustard (PrBCM), respectively. $^{86}Rb^+$ was purchased from the Radiochemical Centre, Amersham, as $^{86}RbCl$ in 0.9% w/v NaCl solution (saline).

Results

Surface-depolarization

Figure 1 shows responses of the rat superior cervical

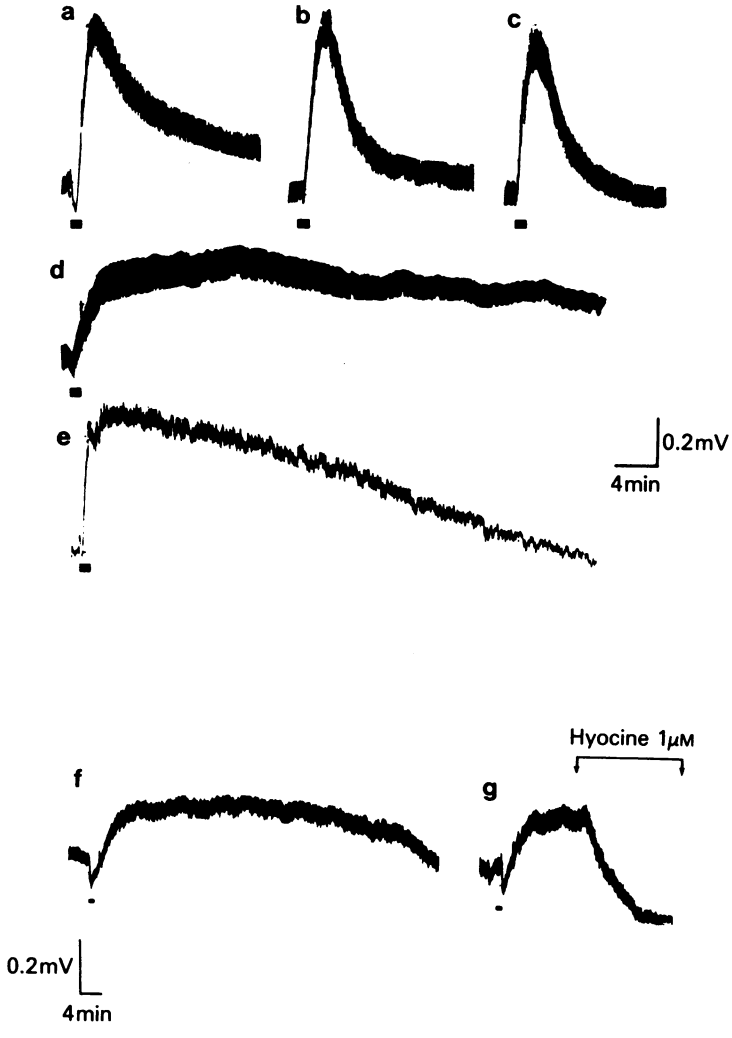

Figure 2 Representative effects of some different muscarinic agonists on rat isolated ganglia, each applied for 1 min: (a) (±)-muscarine 1 μM; (b) methylfurmethide 1 μM; (c) AHR-602 (*N*-benzyl-3-pyrrolidyl acetate) 100 μM; (d) oxotremorine 10 nM; (e) pilocarpine 100 μM; (f and g) oxotremorine 100 μM, repeated twice, with hyoscine 1 μM added after 10 min in (g). Calibrations: vertical, 0.2 mV depolarization (upwards); horizontal, 4 min.

ganglion to 1 μM (±)-muscarine applied for various lengths of time. The initial, and immediate, response was usually a small hyperpolarization of the ganglion, followed (after about 30 s) by a slow depolarization. The depolarization did not reach its peak value until about 3 min after starting the drug application, that is, after the drug had been washed off; indeed, with prolonged exposure (5 min: sixth response in Figure 1), the peak response was delayed until the drug was removed. Recovery to baseline was slow, requiring about 30 to 40 min. Essentially similar effects were produced by several other 'muscarinic' agonists, differing primarily in (i) their tendency or otherwise to produce an immediate hyperpolarization, and (ii) the speed of offset (Figure 2). Responses to pilocarpine and oxotremorine were particularly prolonged. This appeared to result from prolonged receptor-activation, since hyoscine (see below) produced a rapid reversal of the depolarization (Figure 2g).

Temperature

The amplitude of the depolarization produced by (±)-muscarine was essentially independent of temperature over the range of 5° to 37°C, but offset became slower as the temperature was reduced. In

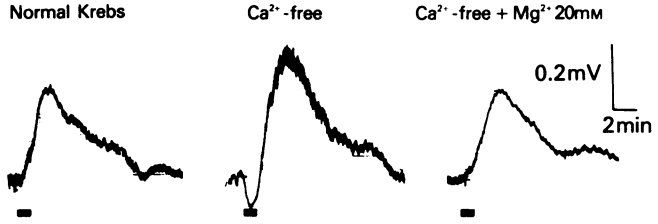

Figure 3 Effects of varying external $[Ca^{2+}]$ on the depolarizing action of muscarine. Responses of a single ganglion to (±)-muscarine 1 μM (1 min application) in normal Krebs solution (2.5 mM $[Ca^{2+}]$), 30 min after removing Ca^{2+}-free solution, and then after adding 20 mM $[Mg^{2+}]$ to the Ca^{2+}-free solution.

other experiments described below a temperature of 25°C was used.

Calcium

Both the immediate hyperpolarization and the subsequent depolarization were increased when Ca^{2+} ions were omitted from the superfusion fluid. This effect was reversed by raising $[Mg^{2+}]$ to 20 mM (Figure 3). The effect of removing Ca^{2+} was independent of the concentration of muscarine used. Elevation of Ca^{2+} from 2.5 to 5 or 10 mM (in Tris-buffered solution, to preclude precipitation of $CaCO_3$) reduced the response to (±)-muscarine.

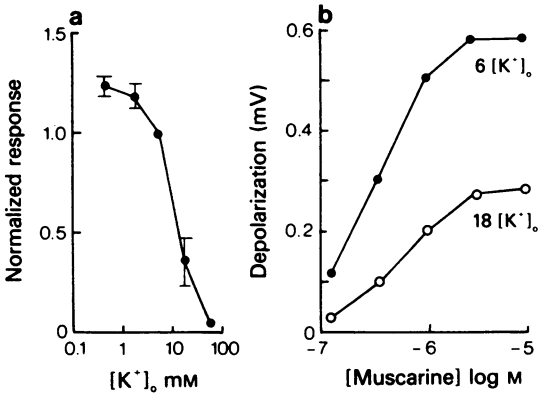

Figure 4 Effects of changing external K^+ concentration ($[K^+]_o$) on the depolarization produced by (±)-muscarine: (a) peak amplitude of the responses to a standard concentration of muscarine (1 μM) plotted against $[K^+]_o$. Results from 3 experiments in which responses were measured after 15 min equilibration in 0.5, 2, 18 and 60 mM $[K^+]_o$ and normalized against bracketed responses in normal Krebs solution (6 mM $[K^+]_o$). Each point shows mean value; vertical lines indicate s.e. mean. $[K^+]_o$ was varied by adding or subtracting KCl to or from normal Krebs solution (KH_2PO_4 being replaced by NaH_2PO_4 in 0.5 mM $[K^+]_o$ solution). (b) Muscarine dose-depolarization curves (see Figure 5) obtained in 6 mM $[K^+]_o$ (●) and 18 mM $[K^+]_o$ (○) (single experiment).

Potassium

The response to muscarine was increased on reducing $[K^+]_o$ from 6 to 2 or 0.5 mM and was suppressed on raising $[K^+]_o$ to 60 mM (Figure 4a). This resulted primarily from a change in maximum response amplitude, rather than from a change in agonist potency (Figure 4b).

Concentration-dependence of muscarinic agonists

The slow recovery from single agonist applications limited the number of dose-response curves obtainable in any one preparation. To overcome this, a form of 'semi-cumulative' dosing was tested (Figure 5). This method makes use of the fact that the maximum depolarization occurred *after* the agonist is removed. Thus, the agonist was applied for a short period (1 min), then washed off and peak amplitude of the ensuing depolarization measured. At the height of the depolarization, a higher concentration of agonist was applied, again for 1 min. The immediate response to this was a large *hyper*polarization (the nature of which is discussed further below), which on washing, reverted to a further depolarization exceeding the preceding maximum. This further depolarization was measured as the response to the second dose, and the drug procedure repeated. The peak amplitudes of the depolarizations recorded in this manner were identical to those recorded after individual applications of muscarine (compare (a) and (b) in Figure 5) and the dose-response curves overlapped. (A corollary to this is that there was no appreciable 'desensitization' to muscarine over this time-scale, notwithstanding the slow recovery). 'Semi-cumulative' dose-response relations could be established in this manner at intervals of about an hour.

Figure 5c shows the mean depolarization produced by (±)-muscarine in 12 ganglia. Unweighted least-squares estimates (see Methods) for the kinetic constants K and y_{max} (with 95% CL) were: K, 0.16 μM (0.05 to 0.26 μM); y_{max}, 0.45 mV (0.39 to 0.51 mV). Comparative responses of the ganglion to other agonists (normalized with respect to the depolarization

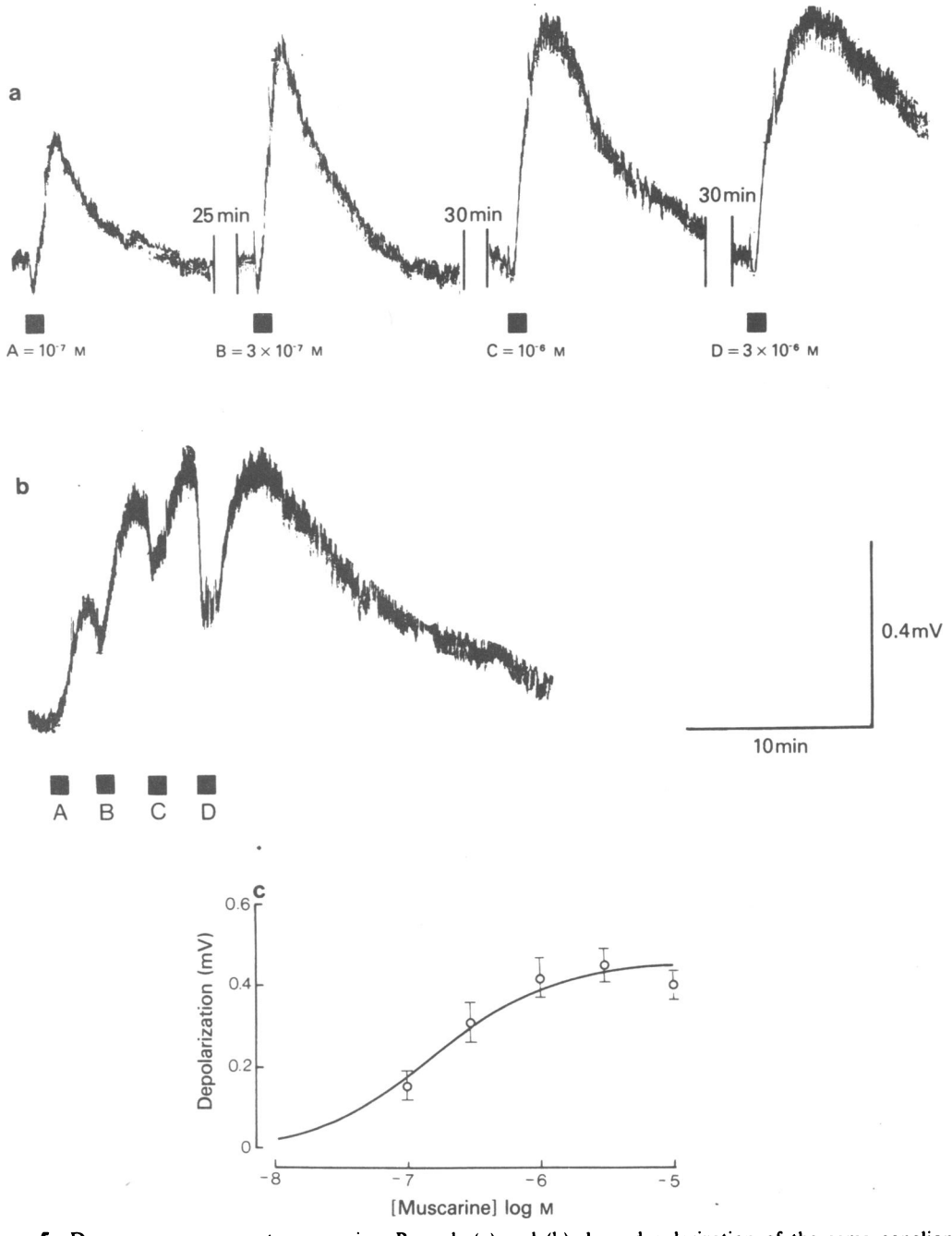

Figure 5 Dose-response curves to muscarine. Records (a) and (b) show depolarization of the same ganglion produced by 1 min application of increasing muscarine concentration applied at 35 to 40 min intervals with recovery in-between (a) or at much shorter intervals, without recovery (b: 'semi-cumulative' dosing, see text). The graph in (c) shows mean depolarizations in 12 ganglia; vertical lines indicate s.e. mean. The curve is an unweighted least-squares fit to the hyperbolic expression $y = y_{max} \cdot x/(K + x)$ where y = depolarization (mV, ordinate), x = muscarine concentrations (M, abscissa on a logarithmic scale) and y_{max} and K are constants (see Methods).

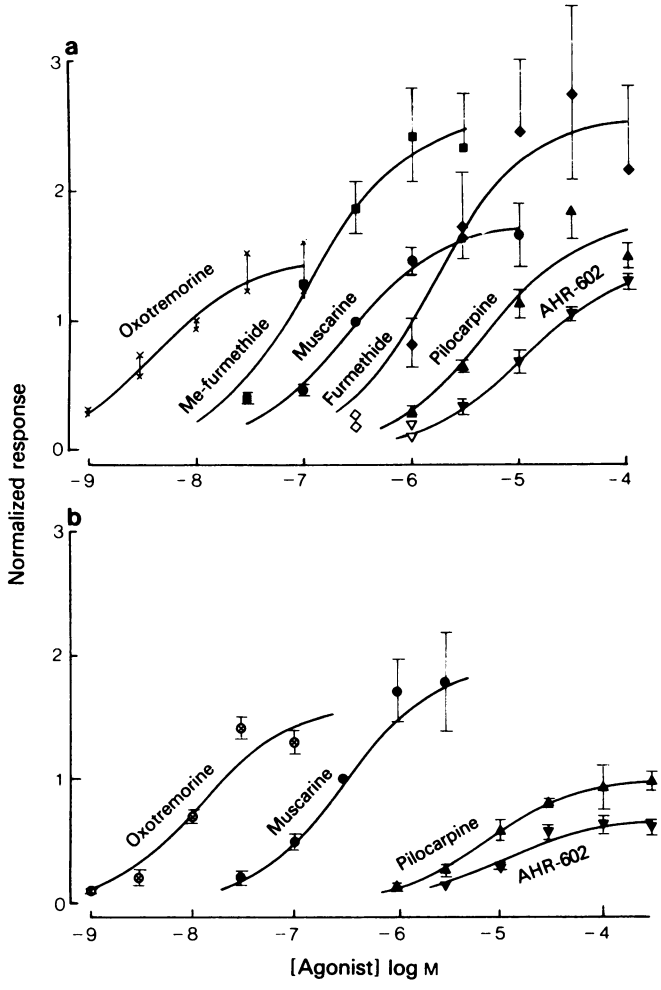

Figure 6 Dose-response curves for (a) the depolarization of rat isolated ganglia and (b) the contraction of the rat isolated ileum by several muscarinic agonists. Filled points show means; vertical lines indicate s.e. ($n \geq 4$); open points are individual measurements. All responses were normalized about those to a standard concentration of muscarine (0.3 μM) applied in the same experiment. Curves are least-squares fits to the hyperbolic expression $y = y_{max}.x/(K + x)$ where y = normalized response and x = agonist concentration (see Methods).

produced by 0.3 μM muscarine, see Methods) are shown in Figure 6. Most showed a reasonable fit to the simple hyperbolic expression $y = y_{max}.x/(K + x)$ (smooth curves), the principal deviation being a tendency to fade at high agonist concentrations. Estimates of the kinetic constants K and y_{max} are listed in Table 1. The potency of the agonists diminished in the order oxotremorine > methylfurmethide > muscarine > furmethide > pilocarpine > AHR-602. Both observed and extrapolated maxima for oxotremorine, pilocarpine and AHR-602 were significantly lower than those to the 'strongest' agonist, methylfurmethide. This, together with the persistent responses to pilocarpine and oxotremorine suggested characteristics of 'partial agonism'. In broad agreement with this, the response to muscarine was reduced in the presence of low concentrations of oxotremorine and suppressed at 1 μM oxotremorine (Figure 7).

'Mixed' agonists

The choline esters acetylcholine (in the presence of physostigmine, 30 μM), methacholine and bethanechol produced a more rapid and larger depolarization than muscarine. The initial, rapid phase of the response was presumably a 'nicotinic' effect since it was suppressed by a high concentration (2.5 mM) of hexa-

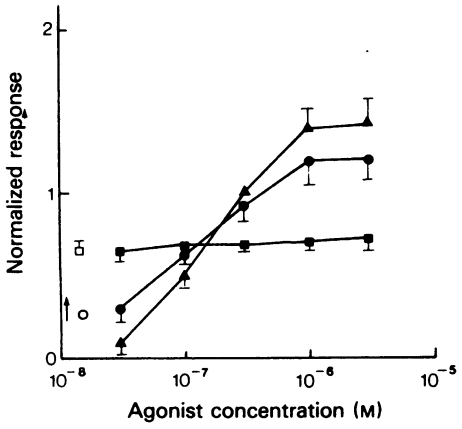

Figure 7 Dose-response curves for muscarine in normal solution (▲) and superimposed upon the depolarization produced by 1 nM (●) and 1 μM (■) oxotremorine. Open symbols show responses to oxotremorine without added muscarine. Each point is the mean of 4 experiments; all responses are normalized to 0.3 μM muscarine applied in the absence of oxotremorine.

methonium: the residual response then resembled in time-course that to muscarine and was abolished by 1 μM hyoscine. Dose-response curves for the agonists (again standardized against muscarine) in the presence of hexamethonium yielded the estimated kinetic constants for their activation of muscarinic receptors listed in Table 1. The order of potency was acetylcholine > methacholine > bethanechol. It may be noted that hexamethonium reduced the sensitivity to muscarine itself about 16 times. Since there was a corresponding reduction in the antagonistic effect of hyoscine under these conditions (see below), this may reflect a side-effect of hexamethonium on the muscarinic receptors. If so, the apparent ED_{50} values for the choline esters in hexamethonium solution should be reduced appropriately to permit comparison with the other agonists applied in the absence of hexamethonium.

McN-A-343 (*4-m-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium*

This compound was introduced as a muscarinic agonist selective for ganglia (Roszkowski, 1961). On the rat ganglion, McN-A-343 (30 to 100 μM) produced a rapid but sustained depolarization which was incompletely antagonized by either hyoscine (1 μM) or hexamethonium (2.5 mM). Attempts to obtain a further resolution of these apparent 'nicotinic' and 'muscarinic' components to the dose-response curve for McN-A-343 were unsuccessful, probably because the two effects overlapped too much (see also Jaramillo & Volle, 1967a; Flacke & Fleisch, 1970).

Reversible antagonists

Responses to muscarine were readily antagonized by hyoscine. A Schild plot (Arunlakshana & Schild, 1959) accorded with a competitive interaction with a pA_2 of 9.5 (K_I = 0.3 nM) (Figure 8). Displacement of dose-response curves by a fixed concentration (3 nM) of hyoscine (see Methods) yielded comparable K_I values

Table 1 Least-square estimates of the constants K and y_{max} (see Methods) for depolarization of isolated ganglia by some muscarinic agonists

Agonist	K(μM)	y_{max}[1]	*Coefficient of serial correlation*	*d.f.*[2]
(±)-Muscarine	0.24 (0.12–0.36)	1.76 (1.56–1.96)	−0.32	55
Methylfurmethide	0.11 (0.04–0.19)	2.54 (2.08–3.00)	−0.67	18
Furmethide	1.56 (−0.51–3.63)	2.60 (1.90–3.30)	−0.32	20
Pilocarpine	4.81 (1.68–7.93)	1.79 (1.50–2.08)	+0.21	18
Oxotremorine	0.0041 (0.0017–0.0066)	1.49 (1.28–1.70)	−0.56	8
*AHR-602	10.86 (6.62–15.0)	1.44 (1.28–1.60)	+0.013	16
Plus hexamethonium (2.5 mM)				
(±)-Muscarine	3.99 (1.74–6.23)	2.48 (2.14–2.82)	−0.14	21
Acetylcholine	3.24 (0.61–5.87)	1.01 (0.82–1.19)	−0.14	18
Methacholine	58.7 (28.3–89.1)	1.68 (1.49–1.86)	−0.31	22
Bethanechol	78.7 (31.1–126.4)	2.11 (1.80–2.43)	+0.65	20

Values were derived from normalized data (see Figure 6a); numbers in parentheses are 95% confidence limits of the estimates, assuming a normal distribution of errors.
[1] Normalized value (y for 0.3 μM muscarine = 1); [2]degreees of freedom; *N-benzyl-3-pyrrolydylacetate methobromide (Franko, Ward & Alphin, 1963).

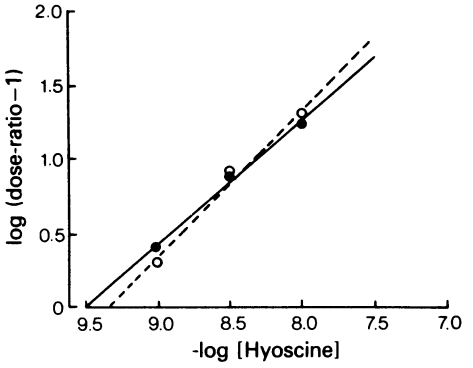

Figure 8 Schild-plots (see Arunlakshana & Schild, 1959) for the antagonism of muscarine by hyoscine in rat isolated ganglion (●) and rat isolated ileum (○), one experiment on each. Lines are calculated least-squares regressions; slopes were 0.85 and 1.0 for the ganglion and ileum respectively; extrapolated pA_2 values were 9.5 and 9.34.

(between 0.1 and 0.9 nM) against other 'pure' agonists. When tested against muscarine and choline esters in the presence of hexamethonium, rather erratic K_I values about ten times greater than those obtained in the absence of hexamethonium were derived. Atropine, lachesine and methylscopolamine were also potent antagonists to muscarine (K_I < 1 nM, Table 2).

Propylbenzilylcholine mustard (PrBCM)

The effect of this irreversible inhibitor (Young, Hiley & Burgen, 1972) was assessed in the manner described by Taylor, Cuthbert & Young (1975). The mustard was cyclized in 10 mM phosphate buffer (pH 7.5, 60 min at 20°C) and diluted to 1 μM in ice-cold Krebs solution for storage.

Exposure of the ganglion to the cyclized PrBCM produced a *parellel* shift of the muscarine dose-response curve. On washing there was a rapid partial reversal within 30 min, followed by a very slow phase

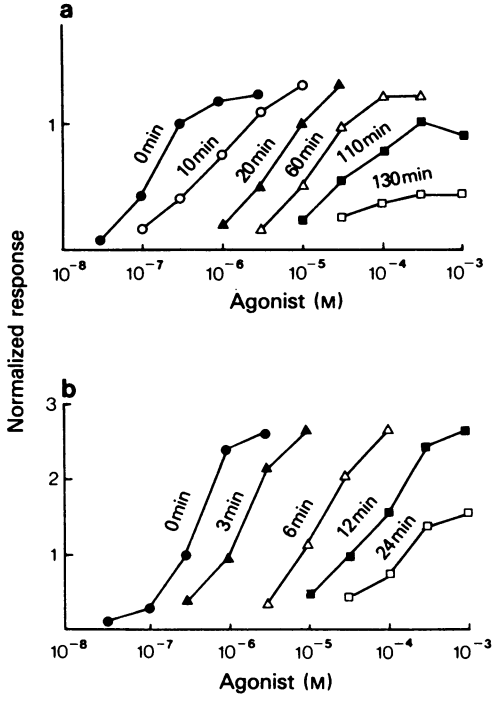

Figure 9 Two experiments showing the shifts of muscarine dose-response curves in (a) the rat isolated ganglion and (b) rat isolated ileum produced by applying propylbenzilylcholine mustard (PrBCM) for increasing lengths of time. After each exposure period, the PrBCM was removed and the preparations thoroughly washed until the agonist gave a stable dose-response curve, to eliminate reversible effects of PrBCM. Times show *total* exposure period to PrBCM. The PrBCM concentrations used were 3 nM in (a) and 10 nM in (b).

of reversal with a half-time of several hours. The residual shift of the dose-response curve after the initial washing remained parallel up to dose-ratios of at least 100, indicating an appreciable number of spare receptors for muscarine-depolarization (Fig. 9). Residual

Table 2 Estimates of K_I for some muscarinic antagonists against (±)-muscarine on the rat superior cervical ganglion and rat ileum, determined from dose-response curve shifts (see Methods)

Antagonist	K_I(nM) *Ganglion*	*Ileum*
Hyoscine	0.49 (0.24–0.73)	0.17 (0.04–0.31)
Atropine	0.24 (0.09–0.40)	0.34 (0–0.68)
Lachesine	0.15 (0.06–0.24)	0.27 (0.01–0.54)
Methylscopolamine	0.09 (0–0.18)	—
Hexamethonium	0.22 (0.07–0.37) × 10^6	—

Numbers in parentheses are 95% confidence limits of the estimates.

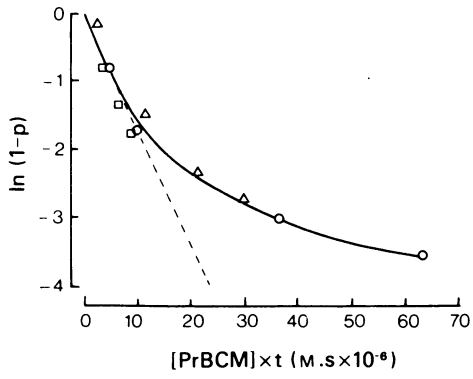

Figure 10 Antagonism of the ganglionic depolarization by propylbenzilylcholine mustard (PrBCM). Ordinate scale: ln (1 − p) where p = PrBCM receptor-occupancy; abscissa scale: product of PrBCM concentration and time(s) of exposure (M.s × 10^{-6}). PrBCM receptor-occupancy (p) was calculated from the irreversible shifts in the dose-response curve obtained as indicated in Figure 9, but restricted to smaller dose-ratios. Three concentrations of PrBCN (□, 3 nM; △, 10 nM; ○, 30 nM) were applied for 5, 20, 35 and 50 min.

antagonist receptor-occupancy after washing for 60 min was measured from the relationship p = (DR − 1)/DR (Paton, 1961) following application of PrBCM for various times at different concentrations. Assuming a reaction scheme (Gill & Rang, 1966)

$$A + R \underset{k_2}{\overset{k_1}{\rightleftarrows}} (AR) \xrightarrow{k_3} (AR') \xrightarrow{k_4} A' + R$$

where (AR) is the reversible PrBCM-receptor complex, (AR') is the alkylated receptor and k_4 is negligibly small, a plot of ln (1 − p) against [A].t should be linear if either (i) $k_3 \gg k_2$ (when k_{app} from the slope = k_1: Gill & Rang, 1966; Taylor *et al.*, 1975), or (ii) $k_3 \ll k_2$ (when $k_{app} = k_3/(1 + K/A)$: Kitz & Wilson, 1962). The plot obtained (Figure 10) was not linear: this may be because k_3 and k_1 are insufficiently different or (perhaps more likely) because agonist receptor-occupancy at large dose-ratios was too great. Assuming the latter, and that $k_3 \gg k_2$ (as previously deduced for BCM and PrBCM) the initial slope suggests a minimum value for k_1 of about 2×10^5 $M^{-1}s^{-1}$; this accords well with previous measurements of pharmacological antagonism in guinea-pig and rat ileum (Young *et al.*, 1972; Taylor *et al.*, 1975).

Ganglion hyperpolarization

The initial hyperpolarization produced by muscarinic agonists is small but becomes larger when superimposed on an ongoing muscarinic depolarization (see Figure 5). Since the hyperpolarizing effect of noradrenaline is also exaggerated under these conditions (Brown & Caulfield, 1978), it seemed possible, as suggested by Libet (1970), that the hyperpolarization might be generated by the release of endogenous catecholamines. A noradrenaline-receptor blocking agent (yohimbine: see Brown & Caulfield, 1978) was used to test this (Figure 11). At 1 μM, yohimbine nearly abolished the response to noradrenaline, reduced the initial hyperpolarization and increased the amplitude of the depolarization, but did not suppress the hyperpolarization superimposed on the on-going depolarization.

Transmission

In agreement with previous observations (Marrazzi, 1939; Trendelenberg, 1954; 1955; Konzett & Waser, 1956; Volle, 1966a and b; Schulman & Weight, 1976), agonists such as muscarine (1 μM) or methylfurmethide (1 μM) augmented the ganglionic response to submaximal preganglionic stimulation. This effect was sometimes extremely prolonged, greatly outlasting the depolarization.

$^{86}Rb^+$-efflux

The resting rate coefficient for $^{86}Rb^+$-efflux into normal Krebs solution attained a 'basal' value of between 0.01 and 0.02 min^{-1} after 60 min or so, in agreement with previous observations on this tissue using both $^{86}Rb^+$ and $^{42}K^+$ (Scholfield, 1977a). Reduction or elevation of external [K^+] over the range 2 to 60 mM reduced or increased the efflux rate coefficient respectively (Figure 12a). The changes in rate coefficient were somewhat less than those expected from the 'constant-field' expression for ionic flux were the membrane potential governed solely by the K^+ gradient (Figure 12b). This, of itself, is not surprising, since the latter assumption is unlikely to hold, particularly at low values for $[K^+]_o$. The cut-off at high values of $[K^+]_o$ may partly reflect delays in extracellular clearance at high efflux rates (see Brown & Scholfield, 1974). Addition of muscarine (1 to 100 μM) in the presence of 120 mM [K^+] (to prevent effects secondary to membrane potential changes, cf. Durbin & Jenkinson, 1960) reduced the rate-coefficient for ^{86}Rb-efflux (Figure 13). This effect was replicated by carbachol (in the presence of hexamethonium) but not by nicotine, and was antagonized by 1 μM hyoscine. In some ganglia, the fall in ^{86}Rb-efflux was sustained for 10 to 20 min; in others it was succeeded by a secondary increase in the flux rate. The maximum fall in efflux rate-coefficient, at 100 μM muscarine, was between 20 and 30% of the resting rate. Although the efflux rate coefficient in high-K^+ solution was large, it fell gradually towards that in normal Krebs solution;

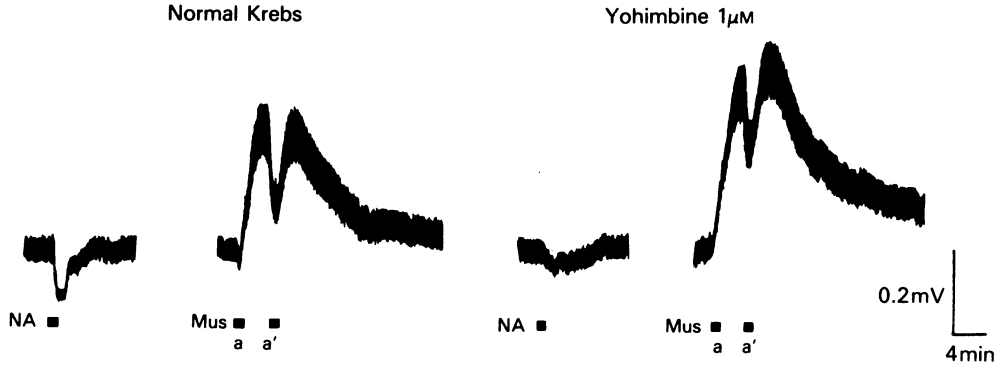

Figure 11 Effects of yohimbine 1 μM on ganglionic responses to noradrenaline (NA) 1 μM and to successive applications, at (a) and (a') of (±)-muscarine (Mus) 1 μM.

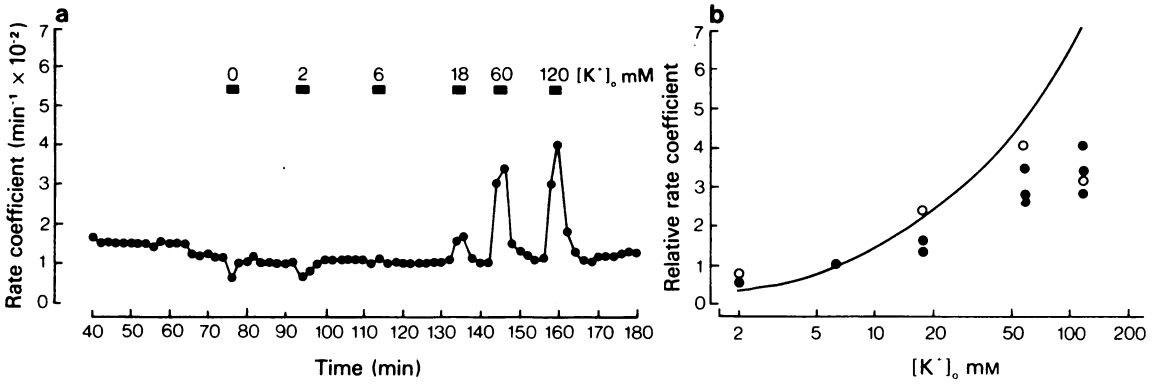

Figure 12 Effects of altering $[K^+]_o$ (by adding or subtracting KCl) on the rate-coefficient for efflux of $^{86}Rb^+$ from a rat isolated superior cervical ganglion into a stream of normal Krebs solution (containing 5.9 mM $[K^+]_o$). The upper record (a) shows a plot of rate-coefficient ($min^{-1} \times 10^{-2}$) against time transcribed from a ratemeter output of effluent count rate from a superfused ganglion. In (b) the results from 4 experiments are summarized, with peak rate coefficients expressed as a multiple of that into normal Krebs solution. Efflux into normal Krebs solution (●) and into Krebs solution containing ouabain 1 mM (○, to inhibit active re-accumulation of $^{86}Rb^+$) was measured. The curve shows the expected change if the membrane potential V were a Nernst function of $[K^+]_o$, $V = (RT/F) \cdot \ln([K^+]_o/[K^+]_i)$, and $^{86}Rb^+$-efflux (M_o) varied with V as determined by the 'constant field' equation:

$$M_o = \frac{VF}{RT} \cdot P_{Rb^+} \cdot [^{86}Rb^+]_i \cdot \frac{\exp(-VF/RT)}{1 - \exp(-VF/RT)}$$

where P_{Rb^+} is the permeability of the cell membrane to Rb^+.

the fractional decrease produced by muscarine did not appear to depend on the magnitude of the resting rate-coefficient.

Cyclic guanosine 3',5'-monophosphate

It has been suggested (Weight, Petzold & Greengard, 1974; Kebabian, Steiner & Greengard, 1975) that muscarinic depolarization of sympathetic ganglia is mediated through an increase in intracellular cyclic GMP. Two tests related to this hypothesis were made: the action of cyclic GMP and its 8-bromo-derivative on surface potentials was tested; and total cyclic GMP levels in the tissue measured.

GMP-depolarization

In 8 out of 10 ganglia, cyclic GMP or 8-bromo-cyclic GMP (1 mM) had no discernible effect on surface potentials. In two tests a very small depolarization occurred which (unlike that to muscarine, see above) was not increased in a Ca^{2+}-free solution.

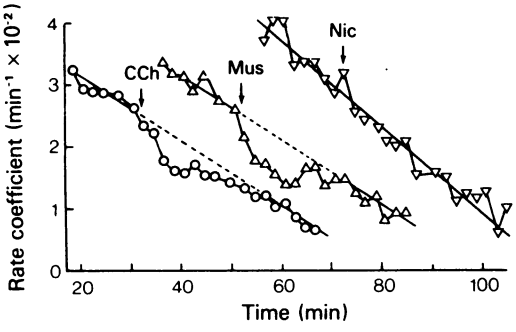

Figure 13 Effects of carbachol (10 μM: CCh), muscarine (10 μM: Mus) and nicotine (10 μM: Nic) on the rate-coefficient for ^{86}Rb efflux observed simultaneously in 3 ganglia. Each ganglion was 'loaded' with $^{86}RbCl$ (0.17 mM in normal Krebs solution) and subsequent efflux into a solution containing 59 mM K_2SO_4 in replacement for 118 mM NaCl (=124 mequiv. K^+/l) plus 1 mM hexamethonium monitored at 2 min intervals by the transference method (see Methods). Drugs were applied for 4 min at the arrows. The time-scale (abscissa scale) reads from the end of the loading period for the first ganglion (carbachol added); other curves are displaced by 20 min intervals for convenience of display (all drugs being added after 32 min washing).

Cyclic GMP levels

Muscarine (1 μM or 100 μM, applied for 2 to 5 min) did not consistently raise tissue cyclic GMP levels, irrespective of whether or not the tissue had been pretreated with the phosphodiesterase inhibitor, IBMX (0.5 mM; Table 3). In contrast, addition of 50 mM K^+ for 3 min raised levels about three fold.

Experiments on the rat ileum

The object of these experiments was to see how the sensitivity of a smooth muscle from the same species and strain of animal to muscarinic agonists and antagonist matched that of the superior cervical ganglion. Dose-contraction curves to muscarinic agonists were constructed by cumulative drug addition, again using muscarine itself as an internal standard for normalization. Normalized curves for oxotremorine, muscarine, pilocarpine and AHR-602 are illustrated in Figure 6b. The sensitivities of the ileum and ganglion (cf. Figure 6a) to these agonists are clearly similar, but the maximum ileal responses to pilocarpine and AHR-602 are lower. Mean kinetic constants for agonist-induced ileal contractions, estimated in the same way as those for ganglion depolarization (see Methods) are listed in Table 4. Hyoscine antagonized both ileal and ganglionic responses to muscarine equally, the pA_2 on the ileum being about 9.34 (K_I 0.5 nM; Figure 8). Comparable K_I values (<1 nM) were deduced for atropine and lachesine (Table 2).

In agreement with van Rossum (1962), McN-A-343 did not produce any appreciable contraction of the ileum but instead antagonized the effect of other agonists at concentrations around 0.1 to 1 mM.

Discussion

The principal conclusion we draw from these experiments is that the muscarinic receptors responsible for depolarization of rat ganglia are essentially similar to those mediating contractions of the rat ileum, with one main exception: that certain 'partial agonists' (pilocarpine, AHR-602 and McN-A-343) are more efficacious on the ganglion than the ileum.

Antagonists

The sensitivities of the rat ganglion and ileum to the reversible antagonists atropine, hyoscine and lache-

Table 3 Effects of muscarine and K^+ on cyclic guanosine 3',5'-monophosphate (cyclic GMP) levels in isolated superior cervical ganglia of rat

Agonist	*Cyclic GMP concentration* (pmol/mg protein, mean ± s.e.) *control*	*test*	*test/control*	n
[1]Muscarine 1 μM	2.16 ± 0.18	2.68 ± 0.33	1.32 ± 0.24	5
[1]Muscarine 1 μM [2]+IBMX 0.5 mM	2.56 ± 0.52	2.92 ± 0.54	1.23 ± 0.21	5
[1]Muscarine 100 μM [2]+IBMX 0.5 mM	2.53 ± 0.66	3.41 ± 0.98	1.93 ± 0.76	3
KCl 50 mM +IBMX 0.5 mM	4.02 ± 0.56	13.07 ± 2.73	3.31 ± 0.67	7

[1]Muscarine was added for 2.5 min, KCl for 3 min. [2]Isobutylmethylxanthine (IBMX) was added 10 min before the other agonist.

sine (Table 2) were indistinguishable. All three were potent antagonists of ganglion depolarization, with apparent K_I values <1 nM. This contrasts with dog adrenal gland receptors, which appear strikingly insensitive to atropine (K_I 0.15 μM; Henderson & Ungar, 1976). An appreciable similarity between ganglionic and ileal receptors in the rat is also indicated by the effect of the irreversible antagonist PrBCM, which shows an apparent onset rate constant at the ganglion (2×10^5 $M^{-1}s^{-1}$) comparable to that reported for both rat (Taylor *et al.*, 1975) and guinea-pig (Young *et al.*, 1972) intestines. It would, however, be premature to conclude an exact identity of rat ganglionic and ileal antagonist-sites in view of recent evidence for subclasses of muscarinic receptors as defined by less traditional antagonists than those which we have used in the present work (Barlow, Berry, Glenton, Nikolau & Soh, 1976; Hammer, Berrie, Birdsall, Burgen & Hulme, 1980). One curiosity is that the potency of the tertiary antagonists observed in the present experiments exceeded by some ten times that usually reported in either pharmacological (Paton & Rang, 1965; Burgen & Spero, 1968) or ligand-binding (Yamamura & Snyder, 1974a,b; Ward & Young, 1974; Hulme, Birdsall, Burgen & Mehta, 1978) experiments. In some instances this might, in part, reflect temperature differences (see Barlow *et al.*, 1976), but this is not the entire explanation since the K_I for atropine, on the rat ganglion at 34°C (4.6×10^{-10}M; M.P. Caulfield, personal communication) appears to differ relatively little from those we have observed. Further, the present values are compatible with the IC_{50} values reported against radiolabelled QNB binding in rat ganglia (Burt, 1978).

Agonists

Agonist potencies on the ganglion and ileum were also strikingly similar as measured by their ED_{50} values (K in Tables 1 and 4), both individually and in rank order.

Interpretation of this is, of course, limited by consideration of efficacy. However, in the case of muscarine itself, the number of 'spare receptors' in the two tissues was similar as judged from the effect of the irreversible antagonist PrBCM (see Figure 9), so the comparable potencies of muscarine do appear to reflect similar receptor-affinities.

The ganglion and ileum differ most noticeably in their responsiveness to the 'weak' agonists, pilocarpine and AHR-602. Although these compounds are equipotent on the two tissues in terms of ED_{50} measurements, the maximum responses of the ileum to pilocarpine and AHR-602 were clearly less (as a fraction of that to a 'strong' agonist such as muscarine or methylfurmethide) than those of the ganglion. This difference is not an artifact of the normalization procedure since the maximum responses to muscarine, normalised in the same way, showed no such differences. McN-A-343 showed an even more striking difference, in that it behaved as an agonist on the ganglion but an antagonist on the ileum. This accords with the observations of van Rossum (1962); McN-A-343 contracted the guinea-pig ileum (via parasympathetic ganglion stimulation?) with a pD_2 of 4.8 but acted as an antagonist on the rat ileum with a pA_2 of 4.8. Thus, the selective ganglion-stimulating actions of McN-A-343 and AHR-602 described by Roszkowski (1961) and Franko *et al.* (1963) respectively may be attributed to a greater efficacy on the ganglion than on smooth muscle. (Pilocarpine also shows a relatively strong ganglion-stimulant action compared with its other peripheral muscarinic effects: Dale, 1912; Ambache, 1949; Trendelenburg, 1954; 1955; Levy & Ahlquist, 1962).

The other striking feature of the ganglionic response to muscarinic agonists is its long duration. This was most noticeable with pilocarpine, AHR-602 and especially oxotremorine. (The long lasting effect of oxotremorine has also been noted after close-arterial injection to cat ganglia *in vivo*: Jaramillo & Volle, 1967b). In part this might result from cellular uptake of these lipid-soluble tertiary bases: evidence has previously been adduced that cellular uptake provides a reservoir for prolonging the action of nicotine on the ganglion (Brown & Scholfield, 1972). However, it may also reflect partial agonist characteristics, in keeping with binding characteristics to homogenized brain tissue (Birdsall, Burgen & Hulme, 1978).

Table 4 Estimates of kinetic constants for contraction of the rat isolated ileum by some muscarinic agonists (see Table 1)

Agonist	K (μM)	y_{max} (*normalized*)
(±)-Muscarine	0.30 (0.18–0.43)	1.93 (1.51–2.35)
Methylfurmethide	0.29 (0.03–0.54)	1.71 (1.28–2.14)
Pilocarpine	7.80 (4.46–11.13)	1.00 (0.91–1.10)
Oxotremorine	0.012 (0.004–0.019)	1.60 (1.29–1.91)
AHR-602	9.91 (3.61–16.21)	0.67 (0.57–0.77)

Mechanism of the potential change

As pointed out in the Introduction, the present experiments were not designed with the primary purpose of elucidating the mechanism of muscarinic depolarization, but certain observations warrant comment.

(a) *The role of* K^+ The K^+-dependence of the depolarization, and the initial reduction in ^{86}Rb-efflux, accord with previous electrophysiological evidence in amphibian ganglia that the depolarization is driven by a reduced K^+-conductance (Weight & Votava, 1970; Kuba & Koketsu, 1974; 1976). Unfortunately, the relatively small fall in ^{86}Rb-efflux (-30%), superimposed on a rather fluctuating background flux-rate, has so far limited our attempts to analyze the receptive-mechanism in detail.

(b) *Cyclic GMP* In contrast to previous reports (Weight *et al.*, 1974; Kebabian *et al.*, 1975), muscarinic depolarization of rat isolated ganglia recorded under the present experimental conditions was not associated with a demonstrable increase in cyclic GMP, even when measured in the presence of a phosphodiesterase-inhibitor. Further, 8-bromo-cyclic GMP itself had negligible depolarizing action. Although depolarization by cyclic GMP derivatives has been detected in other ganglia (McAfee & Greengard, 1972; Hashiguchi, Ushiyama, Kobayashi & Libet, 1978; but see Busis, Weight & Smith, 1978), the response differs in nature from that to muscarinic agonists (Dun, Kaibara & Karczmar, 1978). A further doubt regarding an intermediary role for cyclic GMP is that the response to muscarinic agonists was enhanced on removing Ca^{2+}, and was not reduced even when tested in a Ca^{2+}-free/EDTA solution, conditions under which cyclic GMP formation is greatly reduced (see for example, Kebabian *et al.*, 1975). It seems likely, as suggested by Busis *et al.* (1978), that changes in cyclic GMP levels (where they occur) are coincidental with muscarinic depolarization rather than causative.

(c) *Hyperpolarization* The initial response to addition of muscarinic agonists usually consisted of a ganglion hyperpolarization. This was normally very small, but was intensified when superimposed on an on-going muscarinic depolarization. Further, the maximum depolarization was not generated until removal of the agonist, as though during the presence of the agonist there is a component of 'inhibition' which declines at a faster rate than the depolarization.

Hyperpolarizing actions of muscarinic agonists on sympathetic ganglion cells have been frequently described (see Volle, 1966; Brown, 1966; Libet, 1970; Hartzell, Kuffler, Stickgold & Yoshikami, 1977). In rabbit ganglia the hyperpolarization has been attributed to the release of a catecholamine (Libet, 1970); in amphibia, on the other hand, this effect is direct (Weight & Padjen, 1973; Hartzell *et al.*, 1977). In the rat isolated ganglion evidence for an indirect, catecholamine-mediated effect was equivocal. The intensified hyperpolarization observed with cumulative agonist-addition accords with the enhanced hyperpolarization produced by noradrenaline during a muscarinic depolarization (see Brown & Caulfield, 1978). However, this intensified hyperpolarization was not blocked by yohimbine, which readily blocks the effect of exogenous noradrenaline (acting on 'α_2'-receptors in the rat ganglion: Brown & Caulfield, 1979), though the initial hyperpolarization and 'inhibition' during exposure to muscarine was reduced. Further, muscarine hyperpolarization was enhanced, rather than diminished, by removing Ca^{2+}-ions (in contrast to the report of Libet, 1970): so, if a catecholamine is released, an exocytotic process is unlikely to be involved.

Implications

One area in which the present observations may be helpful is the interpretation of studies on the central nervous system. The electrophysiological consequences of muscarinic receptor activation in central neurones show a qualitative similarity to those in ganglia (see, for example, Krnjević & Phillis, 1963; Curtis & Ryall, 1966; Krnjević, 1969; Krnjević, Pumain & Renaud, 1971; Dingledine, Dodd & Kelly, 1977), but are more difficult to quantify. In consequence, central receptors have so far been characterized primarily from measurements of radiolabelled ligand-binding (Yamamura & Snyder, 1974a, Birdsall *et al.*, 1978). Although these are difficult to correlate directly with the pharmacological response of intact central neurones, our experiments on sympathetic ganglia suggest that the ligand-binding properties of the central muscarinic receptor probably reflect their functional characteristics fairly accurately.

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