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TWO COMPONENTS OF MUSCARINE-SENSITIVE MEMBRANE CURRENT IN RAT SYMPATHETIC NEURONES

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SUMMARY

1. Membrane currents induced by muscarine (I_{mus}) were recorded in voltage-clamped neurones in isolated rat superior cervical ganglia.

2. Two components of I_{mus} were regularly recorded: an inward current resulting from inhibition of the outward K^+ current, I_M; and an outward current attributable to the reduction of a steady inward current. The presence of these two components caused a 'cross-over' in the current–voltage curves at -50 ± 3 mV in neurones impaled with KCl-filled micro-electrodes or at -63 ± 4 mV in neurones impaled with K-acetate-filled electrodes.

3. Both components of I_{mus} were prevented by atropine. Both persisted in Krebs solution containing tetrodotoxin (1 μM), Cd^{2+} (200 μM) or 0 Ca^{2+}.

4. When I_M was inhibited by external Ba^{2+} or internal Cs^+ only the outward component of I_{mus} could be detected. This component reversed at $+3 \pm 2$ mV in cells impaled with CsCl-filled electrodes or at -20 ± 3 mV in cells impaled with Cs-acetate-filled electrodes. The reversal potentials agreed with those for the currents induced by γ-aminobutyric acid ($+4 \pm 2$ mV and -16 ± 3 mV with CsCl and Cs acetate electrodes respectively). Replacement of external NaCl with Na acetate (so reducing external Cl^- concentration ($[Cl^-]_o$) from 155 to 22 mM) shifted the reversal potential for I_{mus} by +25 and +14·5 mV in two cells impaled with CsCl-filled electrodes. A tenfold reduction of external $[Na^+]$ (by glucosamine replacement) did not significantly alter the reversal potential for I_{mus} in KCl or CsCl-impaled cells.

5. Under conditions where I_M is already inhibited, the residual outward component of I_{mus} can lead to hyperpolarization and inhibition of neuronal activity in unclamped cells.

6. We conclude that both inward and outward components of I_{mus} result from direct activation of muscarinic receptors on the ganglion cells. The inward component results from I_M inhibition. We suggest that the outward component results from inhibition of another, voltage-independent current I_X which largely comprises a Cl^- current. The inward component induces membrane depolarization and an increased excitability; the outward component can lead to hyperpolarization and reduced excitability.

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INTRODUCTION

Stimulation of cholinergic afferents to sympathetic neurones produces two types of excitatory post-synaptic potential (e.p.s.p.): an initial 'fast' e.p.s.p., mediated through the action of acetylcholine on nicotinic receptors; and a delayed 'slow' e.p.s.p., generated via muscarinic (atropine sensitive) receptors (Kobayashi & Libet, 1968; Nishi, Soeda & Koketsu, 1969; Weight & Votava, 1970). The latter can be selectively replicated by applying a muscarinic receptor stimulant, such as muscarine itself, to the ganglion cells (Brown & Adams, 1980; Brown & Constanti, 1980).

Previous voltage-clamp studies on frog (Brown & Adams, 1980; Adams, Brown & Constanti, 1982*b*; MacDermott & Weight, 1982; Akasu & Koketsu, 1982) rat (Constanti & Brown, 1981) and rabbit (Hashiguchi, Kobayashi, Tosaka & Libet, 1982) sympathetic neurones have shown that the principal common effect of muscarine is to inhibit a voltage-dependent K^+ current termed the 'M-current', I_M (see Adams, Brown & Constanti, 1982*a*). The consequent decline in outward K^+ current is the source of the inward (depolarizing) current at membrane potentials positive to -70 mV. However, some experiments suggest that other membrane currents might also be modified or induced by muscarinic receptor stimulation (see, for example, Kobayashi & Libet, 1970; Kuba & Koketsu, 1976; Ivanov & Skok, 1981; McCort, Nash & Weight, 1982; Akasu & Koketsu, 1982; Hashiguchi *et al.* 1982).

One previous observation at variance with the 'M-current hypothesis', made on unclamped rat sympathetic neurones, was that current–voltage curves constructed from voltage transients induced by current pulses in the presence and absence of muscarine *intersected* some 10–20 mV negative to the resting potential, as though the direction of the potential change reversed when the cell was hyperpolarized (Brown & Constanti, 1980). There is some evidence for a similar reversal of the acetylcholine-induced potential change in rabbit ganglion cells by membrane hyperpolarization (Kobayashi & Libet, 1970; Ivanov & Skok, 1981). This would not be expected to occur if muscarine only inhibited I_M because I_M is normally deactivated at -70 mV, i.e. positive to the normal K^+ equilibrium potential; hence current flow through open M-channels is always outward and inhibition of I_M should always produce an inward (depolarizing) current.

In previous voltage-clamp studies on rat ganglion cells (Constanti & Brown, 1981) muscarine-induced currents at hyperpolarized levels were not explored in sufficient detail to address this anomaly. We have therefore undertaken a further series of experiments on the membrane currents induced by muscarine (I_{mus}) in voltage-clamped rat sympathetic neurones. We confirm the reversal of I_{mus} on membrane hyperpolarization and show that it results from the inhibition of a steady inward current with a relatively positive reversal potential. This current can be readily separated from I_M and is sensitive to the anion in the impaling micro-electrodes and in the bathing fluid: it may therefore be a muscarine-sensitive species of Cl^- current.

METHODS

The techniques employed in this study were essentially the same as those described previously by Brown & Constanti (1980) and Constanti & Brown (1981). In brief, superior cervical ganglia were rapidly excised from rats killed by air injection under anaesthesia, their outer connective tissue

sheaths removed and ganglia mounted *in vitro* under flowing Krebs solution continuously bubbled with 95% oxygen/5% carbon dioxide (pH 7·4) and maintained at 30 °C. Tris-buffered Krebs solution was normally used, of composition (mM): NaCl, 133; KCl, 5·9; $MgCl_2$, 1·2; $CaCl_2$, 2·5; Tris base, 10 (buffered to pH 7·4 with 8·3 mM-HCl); D-glucose, 11. (Ionic concentrations: 133 mM-Na^+, 5·9 mM-K^+, 1·2 mM-Mg^{2+}, 2·5 mM-Ca^{2+}, 155 mM-Cl^-.) In some experiments bicarbonate-buffered Krebs solution, containing 118 mM-NaCl and 25 mM-$NaHCO_3$, 4·7 mM-KCl and 1·2 mM-KH_2PO_4, was used, with qualitatively identical results. For Cl^--deficient solution, NaCl in Tris-buffered solution was replaced with 133 mM-Na^+ acetate ($[Cl^-]$ = 21·6 mM). Na^+-deficient solution contained 11·8 mM-NaCl, 106 mM-D(+)glucosamine hydrochloride and 25 mM-Tris base, in addition to the usual concentrations of $CaCl_2$ and $MgCl_2$ and KCl, giving 11·8 mM-Na^+ and 131 mM-Cl^- (pH 7·02–7·14). For the corresponding control solution the Tris concentration was raised to 25 mM and the NaCl concentration reduced to 93 mM by adding 25 mM-Na acetate. After titrating to pH 7·1 with HCl this yielded 118 mM-Na^+ and 128 mM-Cl^-. In each experiment junction potential changes at the Ag/AgCl reference electrode on transferring to Cl^--deficient and Na^+-deficient solutions were estimated by measuring the d.c. changes between the KCl or CsCl recording micro-electrode and the reference electrode after withdrawing the former from the cell, assuming potential changes at the KCl/Krebs or CsCl/Krebs junctions to be −4 mV (Caldwell, 1968). Junction potential changes with low-Na^+ solution were small (−3 mV) and were neglected. Low-Cl^- solution produced a shift of between +4 and +12 mV with respect to the KCl electrode, for which a correction was made. (In model-cell tests an additional slow (20–30 min) drift up to +20 mV at the Ag/AgCl electrode was recorded. No equivalent slow base-line current drift was apparent during experimental recording. If present this would lead to an *underestimate* of the membrane depolarization and positive reversal potential shift in low-Cl^- solution.)

Individual neurones were impaled with a single micro-electrode (see below for micro-electrode solutions) and voltage clamped through an amplifier switching between current-passing and voltage-recording modes at 1−3 kHz, with a 50% duty cycle. Capacitance was compensated to remove any step in the voltage excursion in voltage-recording mode, prior to imposition of the clamp. Under voltage clamp the gain and phase was adjusted to obtain as 'square' a voltage trajectory during imposed voltage commands as possible. The clamp voltage settling time was $\leqslant$ 2 ms. Normally the recorded voltage attained 90% of the command voltage but clamp-gain had sometimes to be compromised to avoid oscillation. Care was taken to ensure that the voltage drop across the electrode had dissipated within the 0·16–0·5 ms interval between the end of current injection and voltage sampling, primarily by testing whether the recorded voltage was strongly affected by changing the capacity compensation. The electrotonic length (L) of rat sympathetic neurones has been estimated at 0·67 (Henon, Brown & McAfee, 1981) implying that these neurones are electrically quite compact with a voltage attenuation of $\leqslant$ 20% along the dendrites in the steady state (Rall, 1977). However, we would not claim to have achieved a full clamp of soma and dendrites: our saving grace in these experiments is that muscarine reduces membrane conductance and hence may be expected to improve rather than worsen clamp performance, and that we have been solely concerned with very slow currents or steady-state current measurements, rather than kinetic measurements. Currents were filtered at (usually) 300 Hz and recorded using Gould recorder (2200 or 200 series).

Micro-electrodes were filled with one of the following solutions: 3 M-KCl, 4 M-K acetate (brought to pH 7 with acetic acid); 2 M-CsCl; or 3 M-Cs acetate (pH 7). Electrode resistances were: KCl, 40–60 MΩ; K acetate, 70–80 MΩ; CsCl, 30–40 mΩ; Cs acetate, 90–100 MΩ.

Drugs were applied by bath perfusion using a pump. The bath volume was usually adjusted to $\simeq$ 1·2 ml and solution flow rate $\simeq$ 3 ml min^{-1}.

RESULTS

The problem addressed in this paper is illustrated in Figs. 1 and 2. Fig. 1 is compiled from Figs. 1 and 2 of Adams *et al.* (1982*b*): it shows the effect of the muscarinic acetylcholine-receptor agonist, muscarine, on a voltage-clamped bull-frog ganglion cell. Fig. 2 shows an analogous experiment on a rat ganglion cell. Both cells were impaled with KCl-filled electrodes, and the same experimental protocol was used in

each test: the cell was first clamped at a potential (V_H) where sufficient M-channels are open to ensure that a constant outward M-current (I_M) contributes substantially to the holding current; and the M-channels were then induced to close by superimposing short hyperpolarizing commands. The resultant time-dependent decline in I_M is seen

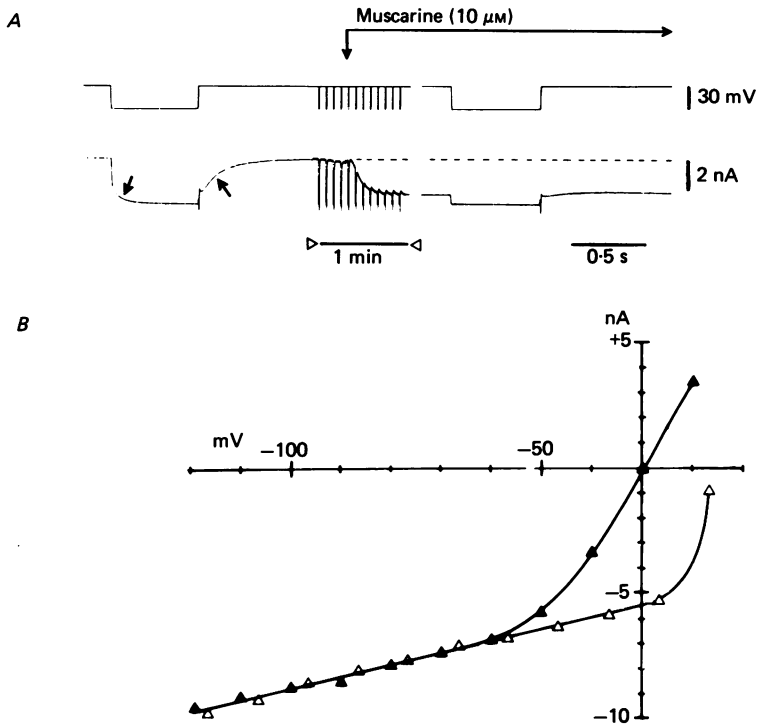

Fig. 1. Effect of muscarine on a voltage-clamped bull-frog sympathetic neurone. (Reproduced from Figs. 1 and 2 of Adams *et al.* 1982*b*.) *A*, voltage-clamp records: upper trace, voltage; lower trace, current (inward current downwards). Holding potential −30 mV. The recorder was slowed 100 times between ▷ and ◁. *B*, Current–voltage curves in the absence (▲) and presence (△) of muscarine. Currents are measured as deflexions from initial holding current at the end of each 0·58 s voltage jump. See Adams *et al.* (1982*b*) and text p. 338 for further details.

as an inward relaxation on the current records (↓); a corresponding outward relaxation at the end of the command (↑), superimposed on the transient outward current I_A (cf. Fig. 2), signifies the redevelopment of I_M on returning to the holding potential.

In the frog ganglion (Fig. 1) muscarine suppressed these time-dependent relaxations, signifying that it inhibits I_M; and there was a consequential inward shift in the holding current, reflecting the loss of the steady outward current that previously flowed through the open M-channels. In contrast there was no change in the steady current level attained at the end of the hyperpolarizing command pulses, where M-channels are already shut. This implies that the inward current generated by muscarine derives entirely from the inhibition of I_M. The current–voltage curve in

Fig. 1*B* confirms this interpretation. The control current–voltage curve shows two zones: a linear zone negative to −70 mV (the 'leak' current) and an outwardly rectifying zone positive to −70 mV, reflecting the increasing contribution of the M-current to the steady-state current. Only the latter component is changed by muscarine; the linear component remains the same and the two curves overlap below −70 mV. Most significantly, the two current–voltage curves do not cross each other. This is because the reversal potential for I_M is −90 mV, some 20 mV negative to the threshold for M-channel opening, and hence, in the steady state, current flow through the M-channels is always outward, never inward.

An analogous test on a rat ganglion cell (Fig. 2) yielded a rather different result. As in the frog cell, muscarine reduced the amplitude of the I_M relaxations induced by voltage jumps (arrowed), and induced the inward current shift at the holding potential to be expected from I_M inhibition. This agrees with previous results on rat neurones (Constanti & Brown, 1981). However, when the current levels attained at the *end* of the hyperpolarizing commands are compared, it is clear that muscarine has induced an *outward* current shift at the more negative potentials. As a result, the current–voltage curves (Fig. 2*B*) *cross over* each other at about −50 mV. This is not due to an unusually positive reversal potential for I_M itself. Thus, I_M relaxations remained inwards at command potentials down to −80 mV or more, as shown in Fig. 2*A*; and the reversal potential estimated from the intersection of the 'instantaneous' and steady-state current–voltage curves in Fig. 2 (V_M) was about −89 mV. This 'cross-over' effect was confirmed in all but two of eighteen cells impaled with a KCl electrode. The average reversal potential for the muscarine-induced current flow (V_{mus}) was $-50{\cdot}4 \pm 2{\cdot}7$ mV (mean ± S.E. of mean, $n = 14$: Table 1), and ranged from −34 to −66 mV. In all cases V_{mus} was appreciably positive to V_M (average, $-84{\cdot}5 \pm 2{\cdot}1$ mV at 5·9 mM-external K^+).

Some minor variations between the experiments illustrated in Figs. 1 and 2 need comment. *First*, the rat neurone was clamped at a more negative holding potential (−45 mV, as against −30 mV for the frog neurone). This was principally to minimize the large transient outward current (I_A, marked ▼ in Fig. 2*A* and other, slower outward currents generated on repolarizing the cell after a hyperpolarizing command. I_A is also present in frog neurones (Adams *et al.* 1982*a*) but seems relatively more pronounced in rat neurones (Constanti & Brown, 1981; Galvan, 1982) and tended to distort the repolarizing current trajectory, obscuring I_M. Even at −45 mV, sufficient I_A remained to obscure the early phase of the outward I_M relaxation, whose apparent amplitude therefore underestimates the total contribution of I_M to the steady-state holding current. (I_A can be inhibited by 4-aminopyridine, but only partially and only at high concentrations; Galvan, 1982.) One consequence of the more negative holding potential is that I_M is less well-developed and makes a smaller contribution to the steady membrane current in the rat cells than in frog cells, but this may be partly offset by a slightly more negative activation threshold for I_M in rat cells (Constanti & Brown, 1981). *Secondly*, muscarine, even at 20 μM, clearly reduced I_M to a lesser extent in Fig. 2 than in Fig. 1. This probably does not represent a consistent difference between the two preparations, since both showed some variability in the precise amount of inhibition produced by muscarine (cf. Fig. 6 in Adams *et al.* 1982*b*). In the present context it is helpful in showing that the cross-over effect is not a

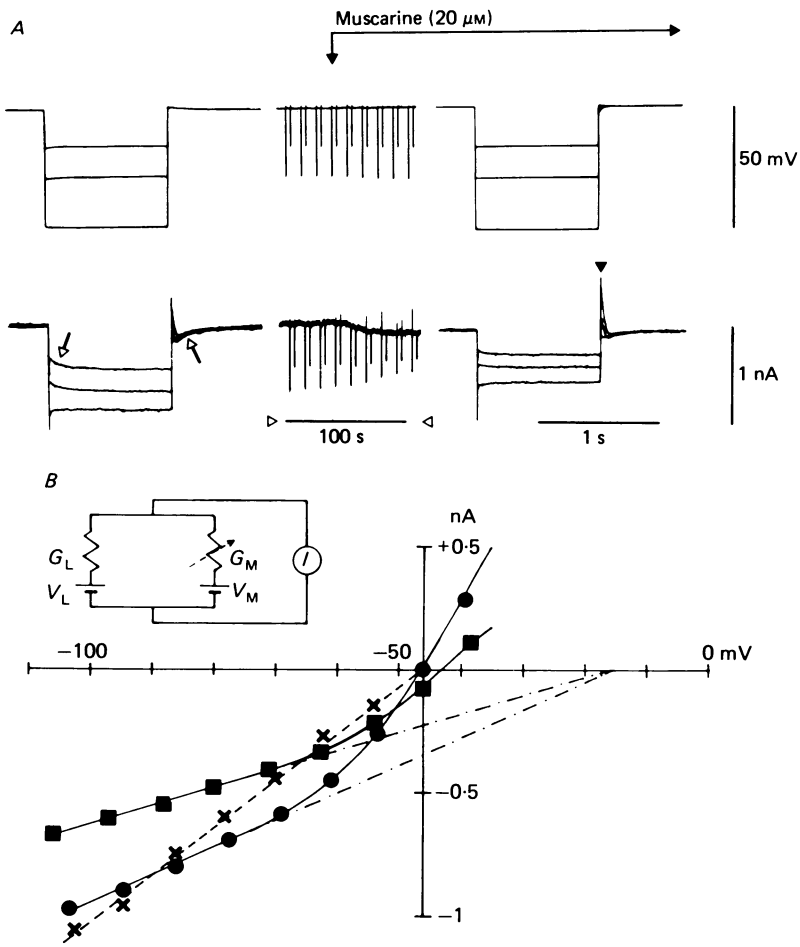

Fig. 2. Response of a rat superior cervical ganglion neurone to 20 μM-*dl*-muscarine added to the perfusing Krebs solution for the period indicated. The Krebs solution contained 0·2 mM-$CdCl_2$ (see text). The neurone was impaled with a KCl-filled micro-electrode, clamped at a holding potential (V_H) equal to the original resting potential of −46 mV, and subjected to a series of 1 s voltage commands (upper records in *A*). Superimposed tracings of currents evoked by three such commands (to −62, −78 and −96 mV are shown in the lower records of *A*. The smaller commands produced inward (↓) and outward (↑) relaxations associated with the decline and redevelopment of the M-current (I_M); the largest command carries the potential beyond the reversal potential for I_M, so that the initial I_M relaxation during the command is reversed to an outward relaxation. The transient outward current I_A (▼) is activated following the larger commands. Just before and during addition of muscarine the recorder was slowed 100 times (between ▷ and ◁) while alternating commands to −62 and −78 mV were applied. The graph in *B* shows the current attained at the end of each 1 s voltage command plotted against command potential (filled symbols) before (●) and during (■) muscarine perfusion. Crosses (×) show 'instantaneous' currents before muscarine measured at the onset of the voltage jumps after subtracting capacity transients (i.e. by extrapolating the slow currents to zero time). (Voltages for instantaneous and steady currents sometimes differ by 1–2 mV because of limited clamp gain.) The intersection point of the steady-state and instantaneous curves gives the reversal potential of I_M (V_M = −89 mV). The interrupted lines are extrapolated 'leak' currents I_L, intersecting at V_L = −15 mV. Curves were constructed using the circuit diagram indicated. G_L, leak conductance; G_M, M-conductance; V_L, leak current reversal potential; V_M, M-current reversal potential; *I*, membrane current. (See text p. 344 for further explanation and numerical values.)

consequence of excessive muscarinic activation. *Thirdly*, a low concentration of $CdCl_2$ (200 μM) was added to the Krebs solution bathing the rat ganglion. This was to inhibit slow time-dependent Cl^- currents activated by hyperpolarizing pulses (see Selyanko, 1984). These are very pronounced in KCl-filled cells, and seriously interfere with the

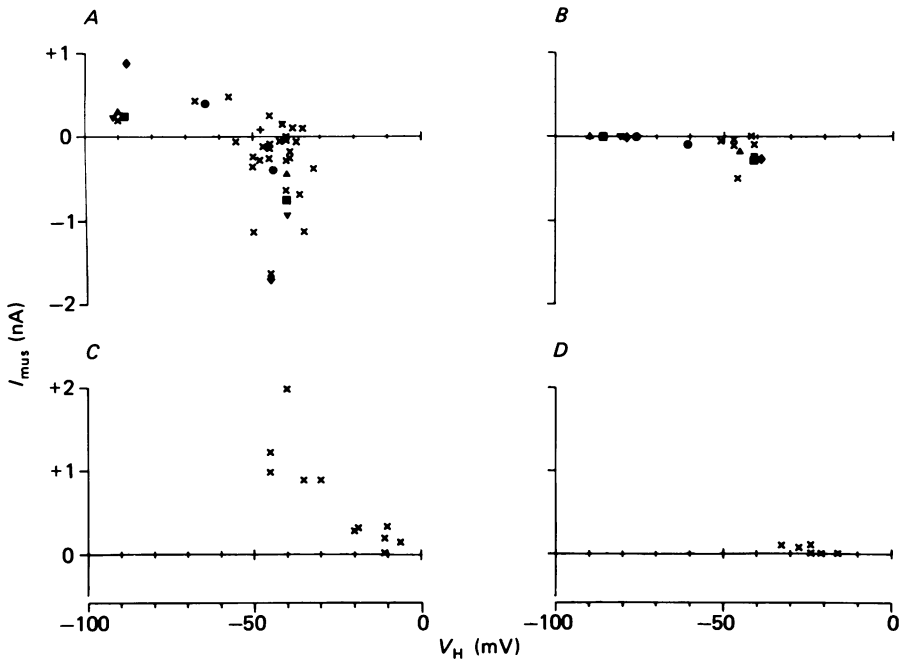

Fig. 3. Amplitudes of steady currents induced by 10 μM-*dl*-muscarine at different clamp holding potentials in cells impaled with electrodes filled with KCl (*A*), K acetate (*B*), CsCl (*C*) and Cs acetate (*D*). Outward current is positive. Filled symbols (●, ▲, ▼, ■) refer to experiments in which muscarine was applied twice in the same neurone at different holding potentials; crosses (×) refer to single tests in separate neurones.

I_M relaxations to such an extent that the reversal of the I_M tail current is difficult to detect. Cd^{2+} did not materially affect the actions of muscarine depicted in Fig. 2; indeed the data in Table 1 refer to cells without added Cd^{2+}.

A corollary to the cross-over effect shown in Fig. 2 is that application of muscarine at steady hyperpolarized holding potentials should produce a clear *outward* current. This was true for cells impaled with KCl electrodes, as shown in Fig. 3*A*: all cells held negative to −55 mV responded with an outward current whereas all but five cells held positive to −55 mV gave an inward current. The cell depicted in Fig. 2 was therefore fully representative of the sample tested.

The amplitudes of the outward steady current at negative holding potentials depicted in Fig. 3*A* are generally rather less than those predicted from the displacements of the current–voltage curves measured from more positive holding potentials, such as that illustrated in Fig. 2. There are two reasons for this. First, in the absence of Cd^{2+}, the current–voltage curves usually showed strong inward rectification at hyperpolarized potentials, which appeared to be enhanced in

muscarine solution. Since this rectification is time dependent (see Selyanko, 1984), the current–voltage curves would tend to converge more when the potential is held at a hyperpolarized level for several minutes than after a brief command of a second or so: in other words, the latter had not reached a true steady state. Secondly, the responses may be affected by slow changes in intracellular ionic concentrations at hyperpolarized clamp potentials: this effect was particularly pronounced using acetate-filled cells, and is described further below.

Conductance measurements

A second manifestation of I_M inhibition by muscarine is a fall in conductance as measured by the instantaneous current steps accompanying a voltage step (Adams *et al.* 1982*b*).

To assess the effect of muscarine on membrane conductance in rat ganglia, the 'instantaneous input conductance' (G_{in}) at different membrane potentials was estimated by measuring the currents induced by short (30 ms), small ($\leqslant 10$ mV) voltage pulses superimposed on the longer voltage commands. These pulses were long enough for the capacity transient to subside but too short and small to materially alter the state of the M-system. The M-conductance itself (G_M) shows minimal instantaneous rectification (Adams *et al.* 1982*a*). Assuming that other conductances at hyperpolarized potentials also show no fast time-dependent voltage sensitivity or instantaneous rectification (see below), G_{in} provides a measure of the sum total of membrane chord conductances at the selected membrane potential.

Fig. 4 shows an example of these instantaneous input conductance tests, taken from the same experiment as that illustrated in Fig. 2. Several points emerge. *First*, the instantaneous conductance G_{in} measured at the holding potential of -46 mV (20 nS) agrees with that given by the slope of the 'instantaneous' current–voltage curve at -46 mV (the interrupted line) in Fig. 2 rather than with that given by the 'steady-state' current–voltage curve. This confirms that the pulses do not perturb the resting state of G_M. *Secondly*, G_{in} is reduced when the voltage pulses are superimposed on hyperpolarizing commands, in a manner approximating to that expected for the voltage dependence of G_M, and then remains constant at its reduced level of about 11 nS at -80 mV downwards. This accords with previous measurements of G_{in} in frog neurones (cf. Adams *et al.* 1982*b*). *Thirdly*, addition of muscarine reduced G_{in} *throughout* the voltage range, including that at membrane potentials > -75 mV where G_M appeared not to contribute to the measured input conductance. The latter was reduced by about 38%, from 11·2 to 7·9 nS approximately. (By subtracting the voltage-insensitive component of conductance, it appeared that G_M was reduced by about 50%.) This contrasts strongly with the effect of muscarine on the conductance of frog neurones, where negligible change in the voltage-insensitive component of G_{in} was recorded at hyperpolarized levels by concentrations of muscarine reducing G_M by $\geqslant 80\%$ (cf. Fig. 4 in Adams *et al.* 1982*b*).

Interpretation of current–voltage curves: two components of conductance change

Current–voltage curves in frog ganglion cells over the range -100 to -30 were previously interpreted as the resultant of two separate current flows; an outward

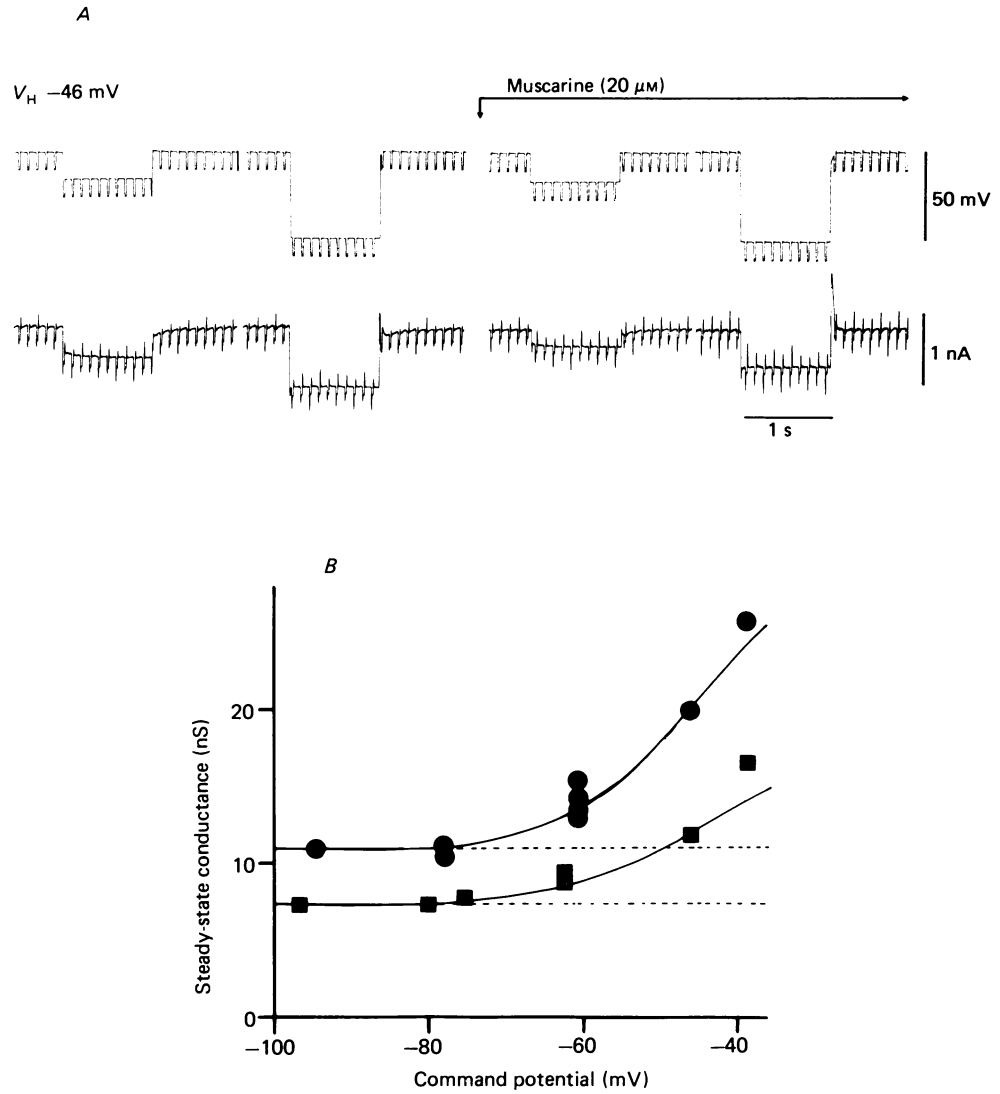

Fig. 4. Effect of muscarine on membrane conductance (same experiment as in Fig. 2). Instantaneous input conductance was estimated by applying 10 mV hyperpolarizing commands of 30 ms duration at 10 Hz (*A*, upper records) and measuring the resultant current displacement (*A*, lower records). The graph in *B* shows 'steady-state' conductance measurements plotted against command potential before (●) and during (■) muscarine perfusion. ('Steady-state' conductances are those achieved by the end of the 1 s voltage steps shown in *A*.) The curves are drawn assuming two parallel conductances G_L and G_M (see inset to Fig. 2*B*). G_L is a linear (voltage independent) conductance and is assigned values of 11·2 and 7·9 nS in the absence and presence of muscarine (dashed lines). G_M varies with potential according to text eqn. (4), with voltage-dependent parameters for rat neurones given on p. 344 (see Constanti & Brown, 1981); the maximum value for G_M ($\bar{G}_M$) is set at 18 and 8 nS before and during muscarine respectively.

component of voltage-dependent M-current (I_M) at potentials positive to -70 mV superimposed on an inward 'leak' current I_L (Adams *et al.* 1982*a*).

The net current at any voltage V is then

$$I(V) = I_L(V) + I_M(V), \tag{1}$$

with

$$I_L(V) = G_L[V - V_L], \tag{2}$$

and

$$I_M(V) = G_M(V)[V - V_M], \tag{3}$$

where G_L and $G_M(V)$ are the conductances and V_L and V_M the reversal potentials respectively. G_L was assumed to be invariant with voltage whereas $G_M(V)$ varied with voltage according to the general expression:

$$G_M(V) = \bar{G}_M \left\{1 + \exp\left(\frac{V_0 - V}{A}\right)\right\}^{-1}, \tag{4}$$

where $\bar{G}_M$ is the maximum M-conductance, V_0 is the potential where $G_M(V)/\bar{G}_M = 0{\cdot}5$ and A is a constant expressing the voltage sensitivity of G_M (6·5 mV for frog cells). The effect of muscarine illustrated in Fig. 1 could then be quite simply interpreted as resulting from a selective reduction in G_M without any alteration in the other constants G_L, V_L, V_M, V_0 or A; indeed the curves in Fig. 1 were constructed on this basis (Adams *et al.* 1982*b*).

In contrast, the conductance measurements in Fig. 4 suggest that the effect of muscarine on rat ganglion cells is *not* restricted to G_M but that it also depresses that component of conductance previously designated as the leak conductance G_L. This is indicated by the fall in the voltage-insensitive components of G_{in} measured at negative potentials. Depression of G_M would cause an inward current at potentials where I_M dominated the resting membrane current whereas depression of G_L might induce an outward current at more negative potentials. The curves in Fig. 2 are drawn according to this scheme. We have assumed that G_M follows the kinetics previously deduced in rat ganglion cells (Constanti & Brown, 1981), with $\bar{G}_M = 18$ nS in the absence of muscarine, $V_0 = -45$ mV and $A = 8{\cdot}7$ mV. This yields a value of about 9 nS at the holding potential of -46 mV, in agreement with the measured voltage-dependent component of chord conductance. The reversal potential for I_M was -89 mV. G_L is assumed to be a linear conductance and is set at 11·2 nS, from the conductance measurements at hyperpolarized potentials (which also corresponds to the slope of the linear component of the current–voltage curve). V_L was estimated to be about -15 mV as the point of intersection of extrapolations of the linear components of the current–voltage curves recorded in the absence and presence of muscarine. The shift of the current–voltage curves on adding muscarine can be accommodated fairly well by the observed changes in instantaneous input conductance, namely a 55% reduction in $\bar{G}_M$ to 8 nS and a 34% reduction in G_L, to 7·4 nS.

Isolating the outward component of muscarine-induced membrane current (I_{mus})

As indicated above, the inward component of I_{mus} might reasonably be attributed to M-current inhibition. The M-current can be inhibited in other ways: for example, by external Ba^{2+} or by internal Cs^+. If the above interpretation of two components

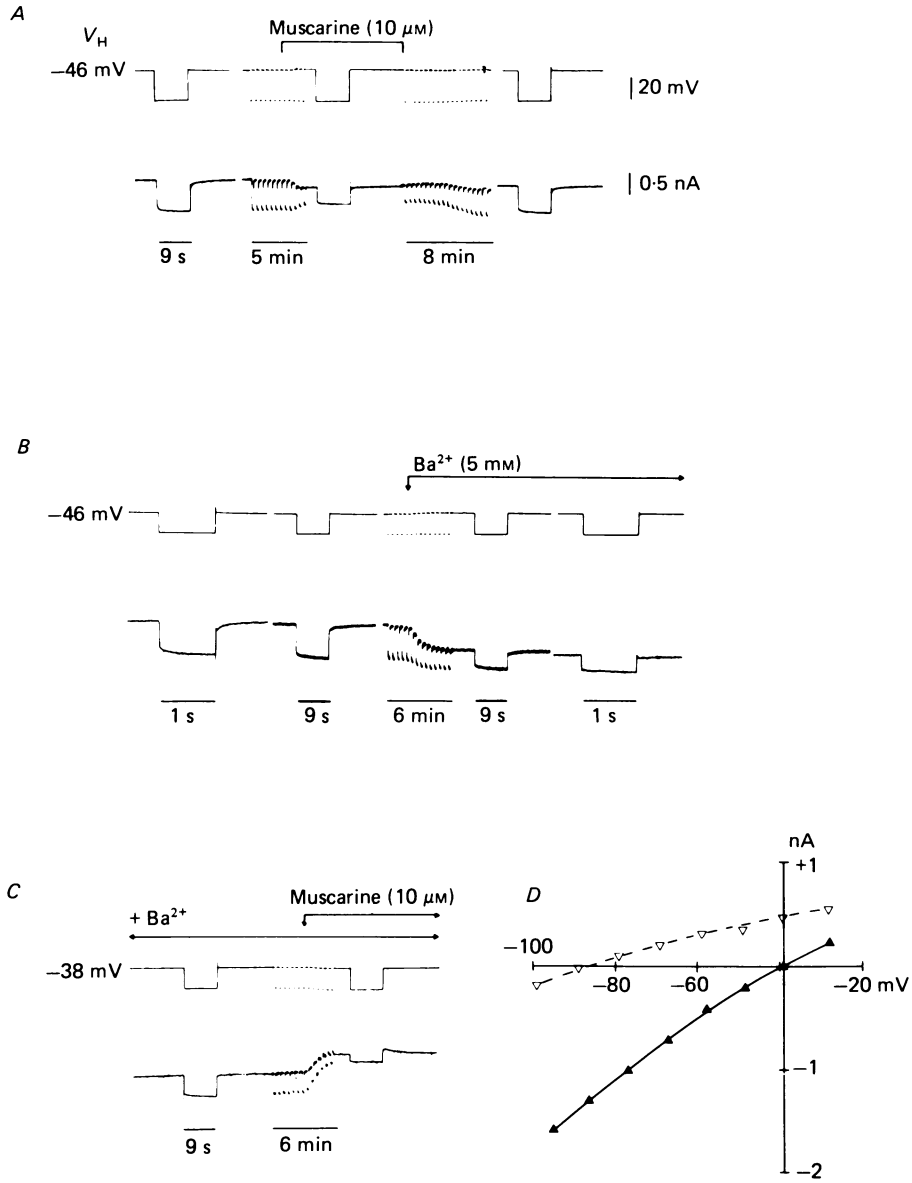

Fig. 5. Effect of muscarine on a rat ganglion cell before and after suppression of I_M by Ba^{2+}. Experimental protocol as in Fig. 2 except that hyperpolarizing voltage commands were 9 s long instead of 1 s. (Shorter, 1 s, commands were also applied in record *B* and recorded at a faster speed.). $CdCl_2$ (0·2 mM) present in the Krebs solution throughout. *A*, muscarine (10 μM) added in normal Krebs solution. *B*, $BaCl_2$ (5 mM) added to the Krebs solution. *C*, muscarine reapplied in the presence of Ba^{2+}. (The holding potential was adjusted to reduce the amount of holding current necessary to clamp the Ba^{2+}-depolarized neurone.) *D*, current–voltage curves before (▲) and after (▽) adding muscarine in Ba^{2+} solution. Currents were measured at the end of the 9 s commands with respect to the holding current at −38 mV before adding muscarine.

of I_{mus} is correct, these procedures should eliminate the inward component arising from I_M inhibition, and muscarine should then induce a simple outward current.

Effect of Ba^{2+}. Fig. 5 shows an experiment to test the effect of Ba^{2+}. The cell was again impaled with a KCl-filled micro-electrode and pre-treated with Cd^{2+}, primarily to preclude entry of Ba^{2+} through Ca^{2+} channels. I_{mus} again comprised two components in Fig. 5*A*: an inward current at the holding potential of −46 mV, and an outward current at the command potential of −73 mV. In contrast, Ba^{2+} (Fig. 5*B*) produced an inward current at both holding and command potentials. This is accompanied by a suppression of the M-current relaxations, as shown by the faster recordings in Fig. 5*B*. Ba^{2+} blocks I_M directly, without involving muscarine receptors. Its effectiveness in Cd^{2+} solution accords with previous observations in frog neurones (Constanti, Adams & Brown, 1981), and suggests that it blocks the M-channels from the outside. The subsequent addition of muscarine, with Ba^{2+} still present and I_M still suppressed (Fig. 5*C*), now evoked a strong *outward* current at both holding and command potentials, even though the holding potential was set at a more depolarized level (to improve clamp stability in the face of the strong inward current in Ba^{2+} solution). This outward current increased in amplitude with increasing membrane hyperpolarization between −30 and −100 mV (Fig. 5*D*) and extrapolated to zero at about 0 mV. As indicated by the declining amplitude of the current excursions induced by voltage commands (Fig. 5*C*), it was accompanied by a large fall in input conductance, from 24 to 8 nS at −38 mV. The generation of a 'pure' outward current by muscarine at holding potentials between −31 and −62 mV, was confirmed in each of eight other cells treated with Ba^{2+}.

Cs^+-filled cells. An alternative method of pre-inhibiting I_M is to inject Cs^+ into the neurone, by using a Cs^+-filled micro-electrode (Adams *et al.* 1982*a*). Fig. 6 illustrates the effect of muscarine on a neurone impaled with a CsCl-filled electrode. No I_M relaxations are evident during voltage jumps, only the slow relaxations referred to above (see Selyanko, 1984) and previously suppressed by adding Cd^{2+}. (No Cd^{2+} was added in Fig. 6.) Although the holding potential was very positive (−19 mV) muscarine produced an outward current, accompanied by a 50% fall in input conductance. As seen from the current responses to depolarizing voltage commands, I_{mus} reversed as the membrane was depolarized, the reversal potential (V_{rev}) being +2 mV. Outward muscarine currents were recorded in all cells impaled with CsCl electrodes when held between −6 and −45 mV (Fig. 4*C*). Reversal potentials measured from the intersection of current–voltage curves ranged from −8 to +17 mV (mean +2·3 mV, Table 2; cf. Fig. 10 below).

Ionic basis of outward I_{mus}

The above experiments using Ba^{2+} or CsCl-filled electrodes suggest that the outward current previously observed at hyperpolarized potentials in cells impaled with KCl electrodes is due to the inhibition of a component of resting membrane current which is quite separate from the M-current and which has a reversal potential near to 0 mV. What ionic species carries this current?

Three groups of observations suggest that there may be a substantial involvement of *Cl^- current*. These observations concern (1) effects of muscarine recorded using micro-electrodes filled with *acetate* salts of K^+ and Cs^+; (2) comparisons of the reversal

potential for the muscarine-induced current with that for the (presumed) Cl^- current induced by γ-aminobutyric acid (GABA); and (3) effects of reducing external $[Cl^-]$.

K acetate electrodes. Fig. 7 illustrates the response to muscarine of a cell impaled with a K-acetate-filled micro-electrode. The response is qualitatively similar to that observed using KCl electrodes in two respects. *First*, a net inward current is evoked at a depolarized holding potential (Fig. 7*A a* and *c*), associated with a reduction of

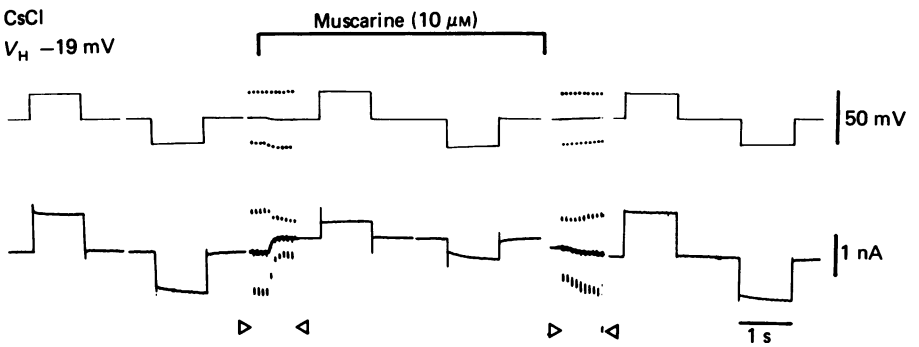

Fig. 6. Effect of muscarine (10 μM) recorded in a neurone impaled with a CsCl-filled micro-electrode. Following impalement Cs^+ was ejected with positive current and inhibition of K^+ currents assessed by progressive spike-broadening. During impalement the cell progressively depolarized to a final steady value of −8 mV and was clamped at −19 mV. The record shows the effect of muscarine (10 μM) applied at $V_H = -19$ mV, with superimposed 1 s voltage jumps of −30 mV. The recorder speed was slowed sixty times between ▷ and ◁.

the M-current relaxations. (This cell was not pre-treated with Cd^{2+}, so the I_M relaxations appear as a fast component preceding the Cd^{2+}-sensitive slow component: cf. Brown & Selyanko, 1983.) *Secondly*, current–voltage curves determined from the current amplitudes attained at the end of short (1 s) hyperpolarizing commands (Fig. 7*B a* and *c*) showed a clear cross-over, at between −55 and −60 mV in this cell, implying reversal of I_{mus} from inward to outward as the membrane is hyperpolarized, just as with KCl electrodes (cf. Fig. 2). However, a striking *difference* from KCl-impaled cells was noted when the membrane potential was shifted to hyperpolarized levels for prolonged periods by adjusting the clamp holding potential: even when set to −80 mV, well beyond the cross-over potential of the previous current–voltage curve, addition of muscarine did *not* produce the expected outward current (Fig. 7*A b*). This is very different from the results obtained with KCl-filled micro-electrodes, where the outward current predicted from the current–voltage curve was directly confirmed by changing the holding potential (see Fig. 1 in Brown & Selyanko, 1983 for an illustration of this).

The results shown in Fig. 7 were confirmed in all cells impaled with K acetate electrodes. Current–voltage curves obtained in ten cells using short commands from depolarized holding potentials where I_{mus} was inward crossed at potentials between −53 and −85 mV (mean ± S.E. of mean, −63 ± 4·3 mV; Table 1); yet, in five of these, no clear outward current was detected on shifting the holding potential beyond the cross-over potential, to between −76 and −90 mV (Fig. 3*B*).

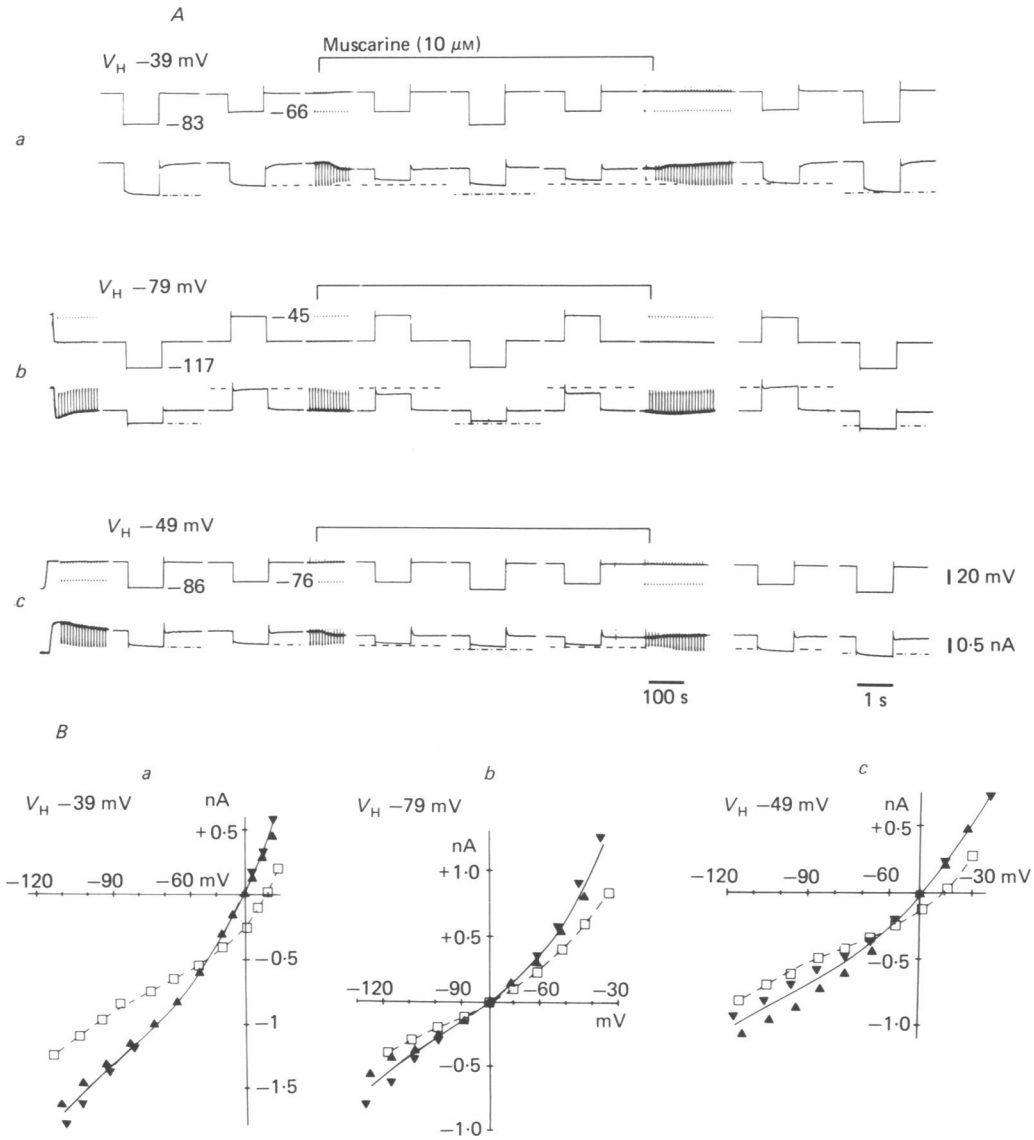

Fig. 7. Effect of muscarine on a cell impaled with a K-acetate-filled micro-electrode (no Cd^{2+}). *A* shows sample records, *B* current–voltage curves. The holding potential (V_H) was initially set at −39 mV (*a*), then adjusted to −79 mV (*b*) and finally reset to −49 mV (*c*). The change in steady current associated with the change in clamp potential is shown at the start of records *b* and *c* in *A*. Current–voltage curves were obtained before (▲), during (□) and after (▼) addition of 10 μM-muscarine at the three holding potentials; currents are plotted with respect to the steady holding current at each holding potential.

The *reason* for this behaviour can be seen from the current–voltage curves in Fig. 7*B*: on hyperpolarizing the cell the cross-over potential itself shifts, to become equal to the new holding potential (Fig. 7*B b*). Hence, even though muscarine is still effective in reducing the conductance of the cell, as judged from the decreased current amplitude produced by voltage commands either side of the holding potential (shown

in record *b* of Fig. 7*A*), no net change in holding currents occurs. This suggests that the change in holding potential induces a shift in the transmembrane gradient of the ionic species mediating the outward component of I_{mus}. Since the appearance of this effect is associated with the use of acetate instead of Cl^- in the electrode, a plausible corollary is that the outward current induced by muscarine is due to the inhibition of an outward Cl^- current.

TABLE 1. Effects of muscarine (10 μM) on rat superior cervical ganglion cells impaled with micro-electrodes filled with 3 M-KCl or 4 M-K acetate. Numbers are means ± S.E. of mean (number of cells in parentheses)

	KCl	K acetate
*I_{mus} (−40 mV) (nA)	−0·24 ± 0·06 (13)	−0·45 ± 0·17 (10)
*I_{mus} (−80 mV) (nA)	+0·58 ± 0·11 (14)	+0·16 ± 0·05 (10)
†V_M (mV)	−84·5 ± 2·1 (8)	−89·7 ± 3·0 (10)
‡V_{mus} (mV)	−50·4 ± 2·7 (14)	−63·0 ± 4·3 (10)

* Muscarine-induced change in net membrane current, measured from current–voltage curves, at −40 and −80 mV command potential.

† Reversal potential for the M-current relaxations.

‡ Inversion potential for I_{mus}, measured from the cross-over of the current–voltage curves constructed from hyperpolarizing voltage commands delivered in the absence and presence of muscarine.

We reason that passage of sustained negative current through acetate-filled electrodes leads to a loss of intracellular Cl^- through a patent muscarine-sensitive Cl^- conductance and that this effect is less pronounced with KCl-filled electrodes because it is partly compensated by the ejection of Cl^- from the electrode. Thus, 1 nA of current for 1 min (about that required for a 40 mV hyperpolarization in Fig. 7), if carried half by Cl^-, would correspond to the injection of about 80 mM-Cl^- into a ganglion cell of equivalent radius (12 μm), so rapid changes in intracellular [Cl^-] are very plausible. Some indication of ionic shifts within this time scale may be gleaned from the holding current drifts during the first 1–1·5 min after changing the holding potential in records *a* and *c* of Fig. 7*A*. Thus the slow outward creep in record *b* after changing the holding potential from −39 to −79 mV we interpret to reflect the declining outward Cl^- current consequent upon a loss of intracellular Cl^-; the slow inward creep in record *c* might then reflect an increasing outward Cl^- current as Cl^- is reaccumulated on depolarizing to −49 mV.

It should be emphasized that shifts in internal [Cl^-] would be negligible ($\leqslant$ 2 mM) during *short* (1 s) potential changes, and hence would not affect the cross-over potential measured using such pulses. The data in Table 1 suggest that this cross-over potential may be some 13 mV more negative using K acetate electrodes than with KCl electrodes. This is *not* the Cl^- equilibrium potential (E_{Cl}) but a compound equilibrium potential for the depression of two ionic conductances, and hence will show a smaller shift than that for E_{Cl} alone.

Cs acetate electrodes. As pointed out above the current–voltage cross-over potential in cells impaled with K^+-filled electrodes is a complex function of two muscarine-induced currents. The effect of the intracellular anion on the muscarine-induced outward current can best be recorded in isolation by suppressing the muscarine-sensitive K^+ current (I_M) with Cs^+. Fig. 8 illustrates the response of a cell impaled with

a Cs acetate electrode to muscarine. As with CsCl electrodes (Fig. 6), I_{mus} is outward at the holding potential of −24 mV and is accompanied by a reduced conductance. The outward current was increased by hyperpolarization but reversed to an inward current on depolarizing the cell beyond −10 mV.

In six cells impaled with Cs acetate electrodes (four with external Cd^{2+}, two without) and held at membrane potentials between −27 and −15 mV, the reversal potential for I_{mus} (V_{mus}) was −20±2·6 mV (mean±S.E. of mean; Table 2). This is about 22 mV negative to that (+3±2 mV) recorded with CsCl electrodes; the difference was significant ($P < 0·02$).

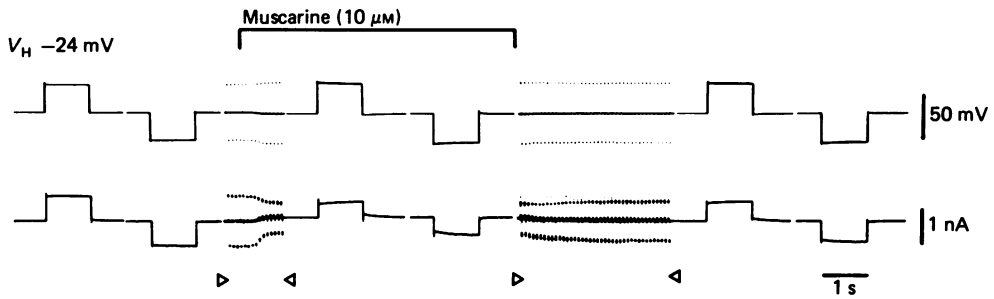

Fig. 8. Action of muscarine (10 μM) on a cell impaled with a Cs-acetate-filled micro-electrode. Holding potential −24 mV; commands −32 mV.

The data in Table 2 also indicate some other differences between cells impaled with Cs acetate and CsCl electrodes. First, the resting input conductance (as measured by the slope of the current–voltage curves over the range −40 to 0 mV, where they were approximately linear) was about 3 times greater in CsCl-impaled cells. Notwithstanding, the proportionate reduction in input conductance produced by muscarine was not significantly different; hence muscarine produced a larger absolute fall in input conductance in CsCl-impaled cells than in Cs-acetate-impaled cells. Thus, substitution of acetate for chloride in the impaling micro-electrode both shifts the reversal potential for the muscarine-induced current to a more negative value and reduces the conductance change. As a result the *total* outward current produced by muscarine in Cs-acetate-impaled neurones is very much smaller than that in CsCl-impaled cells (see also Fig. 3).

Reversal potential for γ-aminobutyric acid (GABA). GABA increases the Cl^- conductance of rat sympathetic neurones (Adams & Brown, 1975). The reversal potential for the GABA-induced membrane current therefore forms a convenient measure of the Cl^- equilibrium potential, applicable under recording conditions comparable to those used for testing the action of muscarine. GABA was applied by rapid bath perfusion at different holding potentials and the peak amplitude of the GABA-induced current recorded, in the manner illustrated in Fig. 9. Constancy of the GABA current was ensured by superimposing short voltage commands to estimate the conductance change. The GABA current varied approximately linearly with holding potential within ±20 mV of its reversal potential, and showed a smooth inversion from outward to inward on hyperpolarizing the cell. The mean reversal

potential (V_{GABA}) estimated by interpolation was $+4{\cdot}3 \pm 1{\cdot}47$ mV (mean ± S.E. of mean, $n = 7$) in neurones impaled with CsCl-filled electrodes, and $-15{\cdot}6 \pm 2{\cdot}61$ mV ($n = 5$) in cells impaled with Cs-acetate-filled electrodes (Table 2). The difference (19·9 mV) was highly significant ($P < 0{\cdot}001$). Values for V_{GABA} were closely similar to those for the reversal potential for the muscarine-induced outward current recorded with the corresponding Cs electrodes. V_{GABA} could not be measured directly using K^+ electrodes because the large outward K^+ currents compromised the clamp at positive potentials. Previous experiments under current clamp (Adams & Brown, 1975) suggested a more negative value (−42 mV) using K acetate electrodes, but these were obtained at much lower ambient temperatures.

TABLE 2. Effects of muscarine (10 μM) on neurones impaled with micro-electrodes filled with CsCl or Cs acetate. Numbers give means ± S.E. of mean (number of cells in parentheses)

	CsCl	Cs acetate
*I_{mus} (−40 mV) (nA)	+1·22 ± 0·20 (12)	+0·18 ± 0·03 (4)
†G_i (−40 mV) (nS)	52·8 ± 8·0 (12)	17·7 ± 2·8 (4)
‡ΔG_{mus} (−40 mV) (nS)	−26·7 ± 4·2 (12)	−6·3 ± 1·6 (4)
§V_{mus} (mV)	+2·3 ± 2·0 (12)	−20·0 ± 2·6 (6)
‖V_{GABA} (mV)	+4·3 ± 1·5 (7)	−15·6 ± 2·6 (5)

* Muscarine-induced current at −40 mV, measured from current–voltage curves.

† Slope of the control current–voltage curves between −40 and 0 mV.

‡ Change in slope conductance at −40 mV produced by muscarine.

§ Reversal potential for I_{mus}, measured from the intersection of control and test current–voltage curves.

‖ Reversal potential for the GABA-induced current (measured in a separate sample of cells).

External Cl^- concentration. To further assess the role of Cl^- movements in generating outward I_{mus}, external NaCl was replaced with Na acetate, so reducing external Cl^- concentration ($[Cl^-]_o$) from 154·6 to 21·6 mM. Fig. 10 illustrates the effect of such a procedure on the current–voltage curves obtained in a cell impaled with a CsCl-filled micro-electrode. In normal (154·6 mM-Cl^-) solution its resting potential was −2 mV; under voltage clamp at −10 mV, muscarine produced an outward current of about 0·5 nA and reduced the input conductance at 0 mV from 52 to 16 nS (−69 %). The reversal potential for I_{mus} was −1 mV; i.e. very near rest potential. On replacing NaCl with Na acetate, the neurone depolarized to +17 mV (corrected for −10 mV junction potential shift at the reference electrode) and the reversal potential for I_{mus} shifted 25 mV, to +24 mV. Both the resting input conductance (36 nS) and the conductance drop produced by muscarine (−40 %) were less than in normal Krebs solution. On replacing external Cl^-, the cell hyperpolarized to −6 mV and the reversal potential for I_{mus} shifted back to near the resting potential. The input conductance was now very much larger (142 nS) and was reduced 64 % by muscarine.

In one other cell in which the appropriate sequences of perfusion-fluid changes could be completed, acetate replacement produced a smaller depolarization of +2 mV and shifted the reversal potential for I_{mus} +14·5 mV, from −3 to +11·5 mV. In five cells (including the above two), cell input conductance was reduced by 34 ± 7·7 % (S.E. of mean) on replacing NaCl with Na acetate; changes in resting membrane potential were variable.

External Na^+. An alternative explanation for the muscarine-induced outward current is that muscarine inhibits an inward cation current. A reversal potential near zero implies a substantial contribution of Na^+ to any such cation current. To test this, effects of muscarine were tested in solutions containing 118 and 11·8 mM-Na^+,

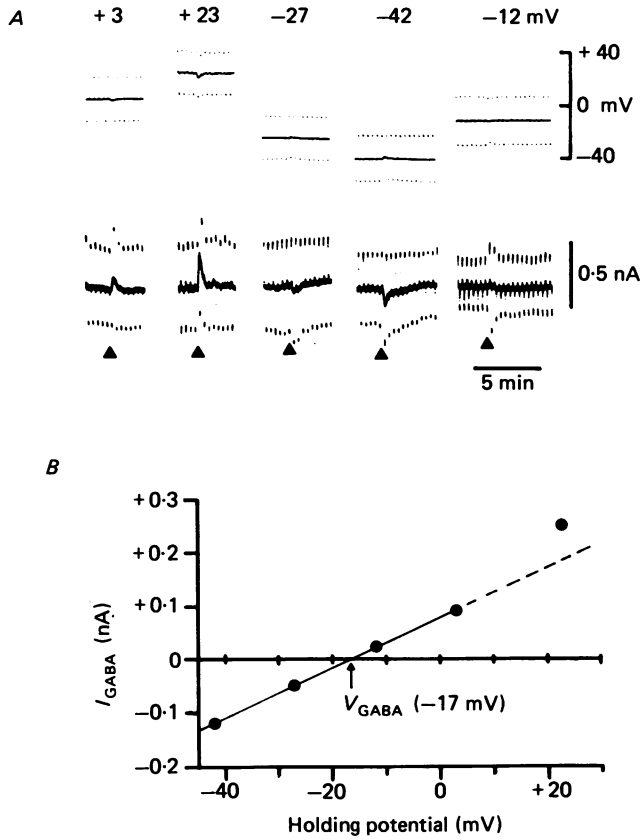

Fig. 9. Reversal potential for the current induced by γ-aminobutyric acid (GABA) in a neurone impaled with a Cs-acetate-filled electrode. Records in *A* show membrane currents (lower trace) induced by 20 s bath application of 1 mM-GABA, recorded at different holding potentials in the sequence indicated. Upward and downward reflexions from steady current levels are current deflexions produced by −18 mV voltage commands from the holding potential: note that these increase in magnitude during GABA ionophoresis, indicating an increased input conductance. Peak GABA-induced currents (I_{GABA}) are plotted against holding potential in *B*. Currents inverted from inward (−) to outward (+) at −17 mV (V_{GABA}).

by replacement with glucosamine (see Methods). We were unable to maintain cell impalements during the transition from Na^+–Krebs to glucosamine–Krebs solution, and hence compared responses of separate neurones in the two solutions. This limited our ability to detect small changes in reversal potential or current amplitude. Notwithstanding, it was clear that the action of muscarine in glucosamine–Krebs solution was essentially similar to that in Na^+–Krebs solution. Thus, Fig. 11 shows

representative responses in two cells in glucosamine–Krebs solution, one impaled with a KCl electrode and the other with a CsCl electrode. I_M relaxations were preserved in KCl-impaled neurones in glucosamine solution, and were inhibited by muscarine. Nevertheless, very little inward current is seen at the holding potential (−38 mV)

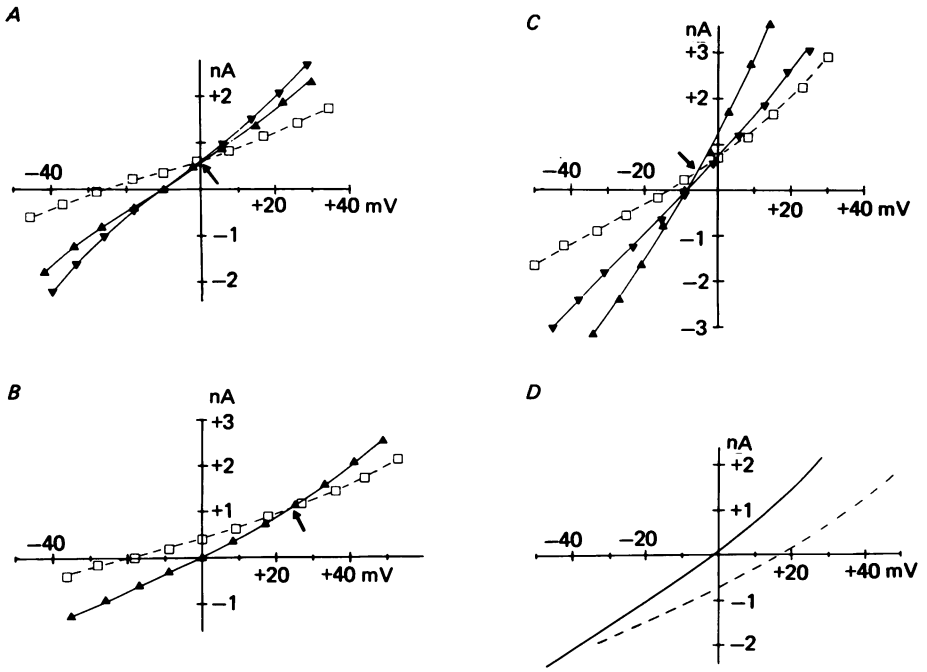

Fig. 10. Effect of reducing external [Cl^-] (by replacing NaCl with Na acetate) on the action of muscarine (10 μM) on a neurone impaled with a CsCl-filled micro-electrode. Plots show current–voltage curves constructed before (▲), during (□) and after (▼) perfusion with muscarine solution, *A* in normal Krebs solution (154·6 mM-Cl^-), *B* in acetate–Krebs solution (21·6 mM-Cl^-), and *C* on returning to normal Krebs solution. Plots were constructed from current deflexions produced by 1 s voltage jumps, as in Fig. 3. Holding potentials were: *A*, −10 mV; *B*, 0 mV; *C*, −9 mV; zero current potentials (E_{rest}) were: *A*, −3 mV; *B*, +17 mV; *C*, −6 mV. Arrows show reversal potentials for the muscarine-induced currents. All potentials were corrected for changes in junction potential at the reference electrode. The plot in *D* shows the resting instantaneous current–voltage curves in 154·6 mM-Cl^- (continuous line) and 21·6 mM-Cl^- (dashed line) determined from the amplitudes of the initial current deflexions, with currents plotted as deviations from zero current. The curves are approximately linear and cross the zero-current line near to, but not exactly at, the reversal potential for I_{mus}. (The difference in zero-current potentials and reversal potentials may reflect the influence of small residual time-dependent components of current on the steady-state current–voltage curves.)

and a strong outward current is apparent at more negative potentials. The CsCl-impaled neurone shows a clear outward current response to muscarine at the holding potential (−16 mV), reversing near 0 mV. Table 3 summarizes the results obtained in this series of tests. Neither the conductance changes produced by muscarine nor the reversal or cross-over potentials for I_{mus} were significantly different in Na^+–Krebs solution and glucosamine–Krebs solution.

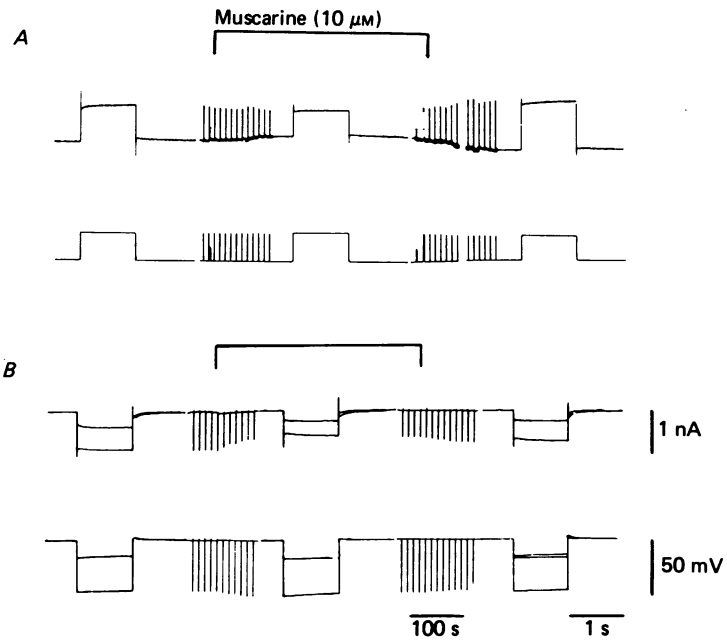

Fig. 11. Effects of muscarine (10 μM) on two neurones impaled with CsCl- (*A*) and KCl-filled (*B*) micro-electrodes and bathed in Krebs solution containing 11·8 mM-Na^+ and 131 mM-Cl^-. Holding potentials: *A*, −16 mV; *B*, −38 mV. Expanded sweep records in *B* are superimposed pen tracings of currents evoked by two hyperpolarizing commands. Note that muscarine produced an outward current at the holding potential in *A* and at the command potentials in *B*. Reversal potentials from current–voltage curve intersections were: *A*, 0 mV; *B*, −35 mV.

TABLE 3. Effects of muscarine (10 μM) recorded in 118 mM and 11·8 mM-Na^+ solution. Numbers are means ± S.E. of mean (number of cells in parentheses)

Electrode		118 mM-Na^+	11·8 mM-Na^+
KCl	*G/G (%)	−47 ± 4·4 (5)	−42 ± 3·1 (3)
	†V_{mus} (mV)	−45 ± 2·4 (5)	−40 ± 3·3 (3)
CsCl	*G/G (%)	−52 ± 5·0 (3)	−35 ± 5·7 (3)
	†V_{mus} (mV)	−9 ± 2·0 (3)	−9 ± 6·0 (3)

* Muscarine-induced change in input slope conductance as a fraction of resting input conductance measured near −40 mV (KCl electrodes) and −20 mV (CsCl electrode).

† Cross-over/reversal potentials for I_{mus} determined from the intersection of current–voltage curves.

Making the simplest possible assumptions that G_L is a linear mixed cation ($Na^+ + K^+$) conductance, with K^+ equilibrium potential (E_K) about −90 mV and Na^+ equilibrium potential (E_{Na}) about +60 mV (Brown & Scholfield, 1974; Ballanyi, Grafe & ten Bruggencate, 1983), then a tenfold reduction in external Na^+ concentration ($[Na^+]_o$) without compensatory changes in internal Na^+ concentration ($[Na^+]_i$) might be expected to produce a hyperpolarizing shift in V_{mus} of about 36 mV as recorded

with a CsCl electrode. Even with the limited number of tests made, such a shift should have been very obvious but was not. Because of the continued presence of I_M, the cross-over potential in KCl-impaled neurones is not a true reversal potential for either I_M or I_L. Assuming G_L to be about half $\bar{G}_M$ and muscarine to reduce each by about

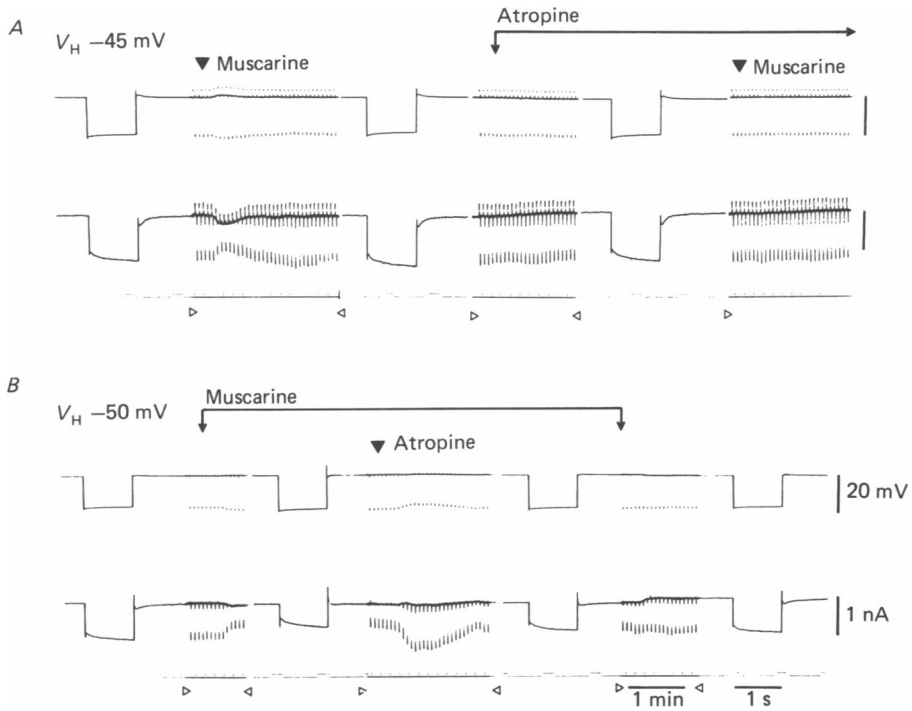

Fig. 12. Effect of atropine (1 μM) on muscarine-induced membrane current. (Two cells from separate ganglia, both impaled with a KCl-filled micro-electrode.) In *A* 10 μM-muscarine was applied for two periods of 30 s (▼) before and after adding atropine to the perfusing solution; in *B* atropine was applied for 30 s during continuous perfusion with 10 μM-muscarine. Note that atropine inhibited both the inward current produced by muscarine at the holding potential and the outward current produced by muscarine at the command potential.

half, a −36 mV shift in V_L would translate to a −9 mV shift in cross-over potential. This is just within the limits of variation in the control measurements; notwithstanding there was no indication for such a shift in glucosamine–Krebs solution. These tests cannot exclude a lesser contribution of Na^+ to a mixed ($Na^+ + Cl^-$) current.

Pharmacology of I_{mus}

Both inward and outward components of I_{mus} were inhibited by atropine (Fig. 12). This indicates that both components could be attributed to activation of 'muscarinic' receptors, and not to some previously unidentified action of muscarine on 'non-muscarinic' receptors.

It may be noted in Fig. 12*B* that, on adding atropine in the presence of muscarine, a transient increase in the amplitude of the current excursions produced by

hyperpolarizing voltage command occurred, *beyond* that seen before adding muscarine. A similar effect was often seen on washing out muscarine (e.g. Fig. 6) and also as a delayed response to repetitive preganglionic nerve stimulation (A. A. Selyanko & D. A. Brown, unpublished observation). This 'wash-out' current appears as a delayed inward current with a relatively positive reversal potential, accompanied by an increase in membrane conductance. If one effect of muscarine is to reduce a steady outward Cl^- current, this could lead to a temporary gain in intracellular Cl^- and consequent secondary increase in outward Cl^- current and Cl^- conductance on washing. Its appearance on adding atropine probably results from the exceptionally fast offset of muscarinic receptor activation; it is unlikely to indicate a 'tonic' level of receptor-activated outward I_{mus} since atropine did not produce this effect if given before muscarine (Fig. 12*A*).

Muscarinic receptors have recently been subclassified into 'M_1' and 'M_2' receptors: both are antagonized by atropine, but the former are about 20 times more sensitive to pirenzepine (Hammer, Berrie, Birdsall, Burgen & Hulme, 1980; Hammer & Giachetti 1982). Previous experiments have indicated that the depolarizing action of muscarine on rat superior cervical ganglia has the pharmacological sensitivity to antagonists (including pirenzepine) appropriate to an M_1 receptor subtype (Brown, Forward & Marsh, 1980). However, ligand-binding studies indicate the presence of both subclasses in ganglion homogenates (Hammer & Giachetti, 1982). Tests on the synaptic equivalent of I_{mus} (Brown & Selyanko, 1984) indicate that both inward and outward components of I_{mus} are antagonized *pari passu* by pirenzepine, implying that both are effected through the same receptor subtype.

We also tested various substances which might block Cl^- conductance, to see whether these replicated or inhibited the outward component of I_{mus} in rat ganglion cells. These included penicillin G (3 mM, two cells), furosemide (100 μM, one cell), the disulphonic stilbene derivative 4,4′-diisothiocyanostilbene-2,2′-disulphonate (DIDS; 1 mM, one cell) and zinc chloride (0·1 and 1 mM, two cells). Penicillin (3 mM) had no clear effect. The others all produced a small outward current but did not clearly affect the action of muscarine.

The outward current produced by muscarine persisted in tetrodotoxin (TTX; 1 μM), in Ca^{2+}-free solutions with 12 mM-Mg^{2+}, or in solutions containing added Cd^{2+} (0·2 mM) or Co^{2+} (4 mM) and hence was probably due to a direct effect on the ganglion cells. It was not replicated by 100 μM-noradrenaline.

Observations on unclamped neurones

The presence of an outward component to I_{mus} implies that muscarine might hyperpolarize rat ganglion cells under certain circumstances. In previous experiments on unclamped rat neurones, muscarine produced a depolarization when applied at the normal resting potential (Brown & Constanti, 1980). As pointed out in the Introduction, a cross-over in the voltage–current curves was observed in these experiments but the corollary to this, that muscarine might hyperpolarize a neurone which had already been hyperpolarized beyond the cross-over potential, was not directly tested.

Fig. 13 shows the results of such a test using a KCl electrode. At the initial resting potential of −40 mV, muscarine produced a depolarization of 6 mV and increased

the apparent input resistance. On increasing the membrane potential to −45 mV, the depolarization produced by muscarine was reduced to 2·5 mV. The voltage deflexions produced by constant-current injections were also increased to such an extent as to produce a clear cross-over in the voltage current relation (cf. Brown &

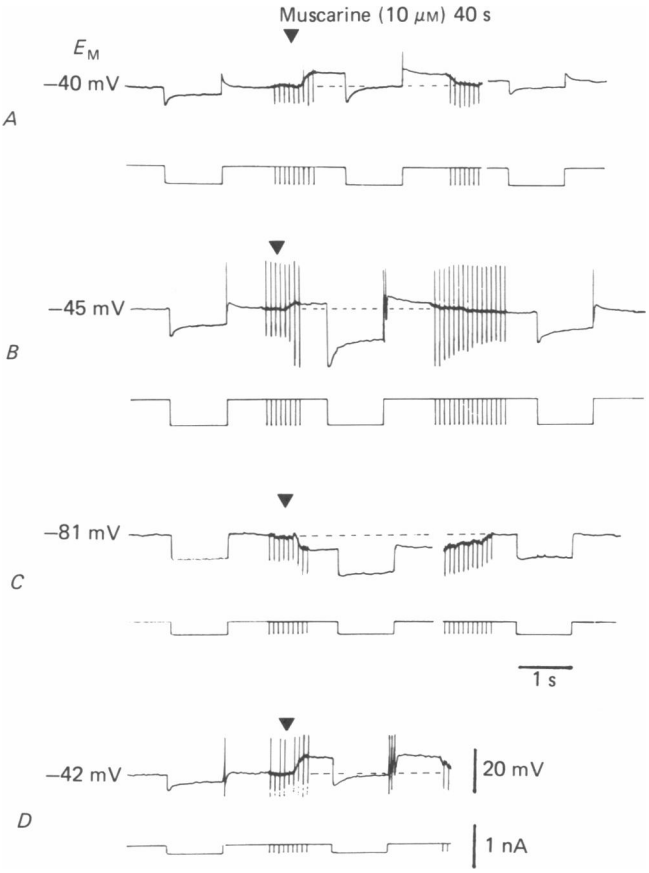

Fig. 13. Potential changes produced by muscarine in an unclamped neurone impaled with a KCl-filled micro-electrode. Muscarine (10 μM) was applied for 40 s periods at ▼ in the sequence *A*–*D*. Records *A* and *D* were obtained at the resting potential (−40 and −42 mV respectively), records *B* and *C* during the passage of steady hyperpolarizing current to gain membrane potentials of −45 and −81 mV respectively. Downward deflexions in the voltage records are responses to hyperpolarizing current pulses, the magnitude of which are shown in the lower records.

Constanti, 1980). When sufficient steady current was injected to bring the membrane potential to −81 mV (record *c*), muscarine now produced a *hyper*polarization. The voltage excursions produced by current injections no longer showed the pronounced 'sag' characteristic of the time-dependent rectification produced by M-channel closure (see Adams *et al.* 1982*b*), showing that the membrane potential was now out of the M-current range. On relieving the steady current the potential reassumed a value of −42 mV (trace *d*) and muscarine again depolarized the cell.

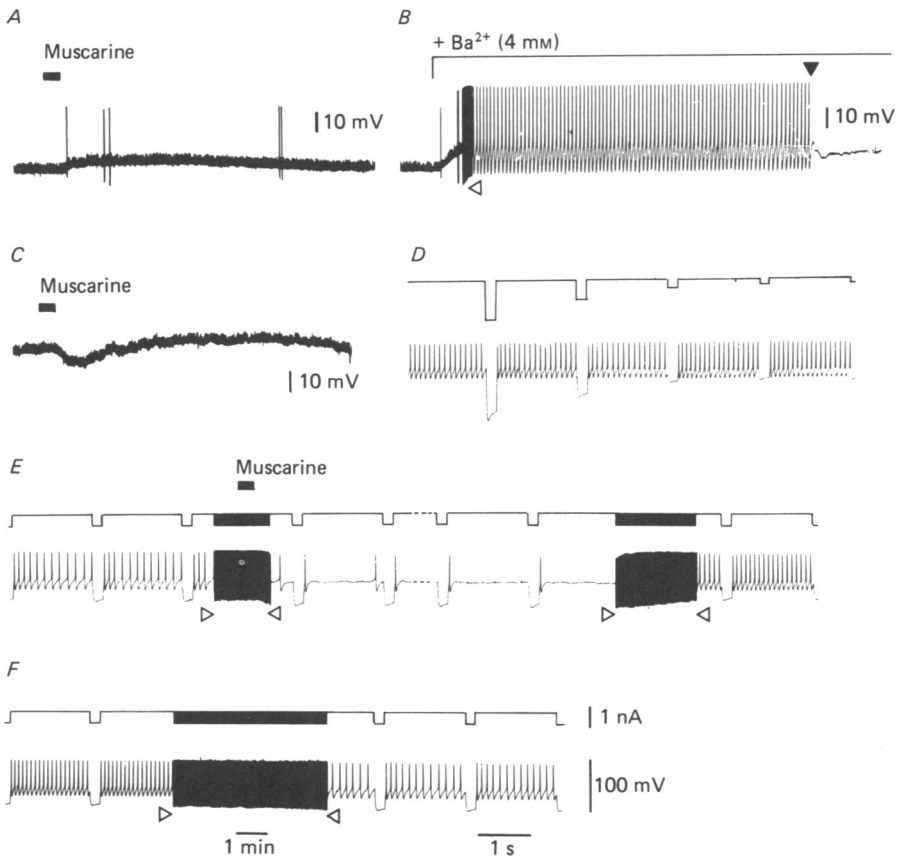

Fig. 14. Effects of muscarine recorded under 'current clamp' before (*A*) and after (*C–F*) adding Ba^{2+} to the bathing medium. The cell was impaled with a KCl-filled micro-electrode and had an initial resting potential of −50 mV. Muscarine (20 μM) was added at the bars for 30 s. *A*, muscarine added in normal Krebs solution at −50 mV. *B*, 4 mM-$BaCl_2$ added to the perfusion fluid and left in throughout the rest of the experiment. Ba^{2+}-induced spiking was arrested by 0·1 nA hyperpolarizing current at ▼. *C*, muscarine reapplied in Ba^{2+} solution after the potential was reset to the original value of −50 mV. *D*, current was relieved until Ba^{2+}-induced spiking recommenced (lower trace), and 'regularized' by intermittent 200 ms hyperpolarizing pulses (upper trace). Note that the steady firing frequency was not changed by varying the amplitude of the intermittent current injections. *E* and *F* are continuous and show the action of a brief (30 s) application of muscarine on Ba^{2+}-induced spiking. Note that the voltage amplification was reduced in *D–F*. The recorder was slowed 100 times between ▷ and ◁.

A second prediction from the present voltage-clamp experiments is that muscarine will also hyperpolarize a ganglion cell in which I_M has been inhibited by means other than voltage. Fig. 14 shows a test of this prediction using Ba^{2+} to inhibit I_M. The cell had an initial resting potential of −50 mV when impaled with a KCl-filled micro-electrode and bathed in normal Krebs solution. Under these conditions 20 μM-muscarine depolarized the cell by 5 mV and initiated a few random spikes (panel *a*); a normal response to I_M inhibition. $BaCl_2$ (4 mM) was then added to the

perfusion fluid for the rest of the experiment (panel *b*). This produced a 10 mV depolarization and induced sustained regular spiking at ~ 14 Hz, which was arrested by passing hyperpolarizing current. On resetting the membrane potential to the original (pre-Ba^{2+}) value of −50 mV, muscarine now produced an initial *hyper*-polarization of about 6 mV (panel *c*); the expected manifestation of the outward component of I_{mus} seen in voltage-clamped neurones in Ba^{2+} solution (cf. Fig. 5). (The hyperpolarization is followed by a delayed, sustained depolarization. This is *not* due to I_M inhibition but results from a secondary increase in membrane conductance following wash-out of muscarine: see below and p. 356.)

Our next question was whether this hyperpolarization had any effect on the *excitability* of the cell. To test this, the injected current was adjusted until the cell resumed its regular Ba^{2+}-induced spiking (panel *d*). Regularity was ensured by interrupting the spiking with intermittent 200 ms hyperpolarizing pulses (otherwise the firing tends to gradually subside, leaving the cell in a depolarized state). Application of muscarine now transiently *inhibited* the spike discharges. During this period of inhibition the voltage deflexions produced by the hyperpolarizing currents were noticeably increased, reflecting the decline in input conductance. (The inhibition of spiking was not due to these larger hyperpolarizations *per se*: panel *d*.) Subsequently spiking accelerated and the voltage deflexions diminished: this is the delayed 'wash-out' inward current noted above. Finally the cell resumed its initial firing rate.

The experimental situation represented in this experiment is highly abnormal, in that these particular ganglion cells do not normally exhibit repetitive spiking *in vitro*. However, they permit a clear demonstration that the outward component of I_{mus}, in isolation, is essentially inhibitory in its effect on cell excitability, notwithstanding the fact that it is accompanied by a *decrease* in membrane conductance. The firing rate of cells treated with Ba^{2+} is exquisitely sensitive to changes in membrane potential, so, even though there is no 'resting' potential in the spontaneously firing state, it seems likely that the inhibition seen in Fig. 14 results primarily from the small membrane hyperpolarization. Even though the outward component of I_{mus} is quite small (about 1 nA at −40 mV in Cl^--loaded cells: Table 2), it is sufficient to account for this inhibition since the input conductance of the cell was low (12 nS) in Ba^{2+} solution; an equivalent inhibition of spike discharges to that produced by muscarine could be obtained by injecting less than 0·1 nA of hyperpolarizing current.

DISCUSSION

The present experiments show that stimulation of muscarinic acetylcholine receptors can inhibit two components of membrane ionic current in rat sympathetic neurones: the voltage-dependent K^+ current, I_M; and a previously undetected component of inward current which (for convenience) we may provisionally term I_X. Inhibition of I_X may be readily observed at potentials where I_M is normally deactivated, or when I_M is already suppressed by external Ba^{2+} or internal Cs^+. Under these conditions, muscarine then produces a net *outward* (hyperpolarizing) current, instead of the inward (depolarizing) current normally associated with I_M inhibition (cf. Constanti & Brown, 1981; Adams *et al.* 1982*b*). This outward current is

accompanied by a fall in membrane conductance, showing that it results from the inhibition of a normal component of ionic conductance rather than activation of an additional conductance.

We suggest that I_X might be predominantly a Cl^- current. Three observations lend themselves to this interpretation. *First*, in Cs^+-filled neurones the reversal potential for the outward component of I_{mus} resulting from I_X inhibition was about 23 mV more negative with acetate as the anion in the intracellular electrode than with Cl^- as the accompanying anion. *Secondly*, the reversal potentials were close to those for the membrane currents induced by GABA in neurones impaled with CsCl and Cs-acetate-filled electrodes: GABA probably activates a Cl^- conductance in these cells (Adams & Brown, 1975) as in other neurones. *Thirdly*, the reversal potential for I_{mus} in CsCl-impaled cells showed a positive shift on reducing external $[Cl^-]$ from 155 to 22 mM with acetate as the replacing anion.

Although these observations suggest a major role for Cl^- as the current carrier for I_K, some difficulties ensue if it were regarded as a pure Cl^- current. First, the intracellular Cl^- concentrations predicted from the reversal potentials of outward I_{mus} (72 and 167 mM at $[Cl^-]_o = 155$ mM in cells impaled with Cs acetate and CsCl electrodes respectively) are appreciably higher than those recently estimated in unimpaled rat ganglion cells (32 mM at $[Cl^-]_o = 128$ mM; Galvan, Dorge, Beck & Rick, 1984) and in K_2SO_4-impaled cells (40 mM at $[Cl^-]_o = 128$ mM; Ballanyi, Grafe, Reddy & ten Bruggencate, 1984). Even if due to Cl^- loading, this would have appreciable osmotic consequences. Secondly, the shift in reversal potential on reducing $[Cl^-]_o$ fell appreciably short of that expected for Nernstian dependence on $[Cl^-]_o$, although this may be compounded by secondary Cl^- shifts, perhaps exacerbated by the slow penetration of the replacing anion acetate as the protonated acid (Sharp & Thomas, 1981). Na-replacement tests did not indicate a *major* contribution of a Na^+ current to I_X but were of insufficient accuracy to exclude a partial contribution by Na^+.

For example, a 10% contribution of a Na^+ current (I_{Na}) to I_X might be anticipated if E_{Cl} with an acetate-filled electrode was in fact the same as that (−27 mV) recorded by Ballanyi *et al.* (1984) with a sulphate-filled electrode, instead of the −20 mV I_X reversal potential recorded with a Cs acetate electrode. Assuming the same partial conductances (G_{Cl}, G_{Na}) in CsCl-impaled cells ($E_{Cl} \approx -2{\cdot}75$ mV ($[Cl^-]_i \approx 140$ mM at $[Cl^-]_o = 155$ mM)) a tenfold reduction of $[Na^+]_o$ would shift I_X by only −4 mV; undetectable by our tests to date. The reversal potential for I_X would then shift +44 mV when $[Cl^-]_o$ was reduced from 155 to 22 mM, instead of the anticipated value of +50 mV. At 25% contribution by G_{Na}, I_X would shift by −9 mV and +29 mV for the same changes in $[Na^+]_o$ and $[Cl^-]_o$ respectively.

The parallel inhibition of I_M and I_X readily explains the apparent reversal of the muscarine-induced current in rat ganglion cells with membrane hyperpolarization, as shown in Fig. 2. In cells impaled with KCl-filled electrodes this cross-over potential was about −50 mV (Table 1). Hence, the normal depolarization resulting from I_M inhibition can be readily inverted to a hyperpolarization by modest membrane hyperpolarization, as shown in Fig. 13. (Indeed, in a few cells, muscarine produced an outward current when the cell was clamped at its inital resting potential.) A comparable inversion with membrane hyperpolarization has been noted in rabbit ganglion cells impaled with KCl-filled micro-electrodes (Kobayashi & Libet, 1970;

Ivanov & Skok, 1981). In cells impaled with acetate-filled electrodes the cross-over potential was about 13 mV more negative. This may be ascribed to two factors: the negative shift in the reversal potential for I_X itself, from +3 to −20 mV; and a threefold reduction in the conductance G_X in acetate-impaled cells (see Table 2). As a result, the outward current in K-acetate-impaled cells is much smaller, and, because of secondary Cl^- shifts, is unlikely to lead to overt hyperpolarization during prolonged membrane hyperpolarization: indeed, no net outward current could be generated on applying muscarine during imposed hyperpolarization of acetate-impaled neurones.

The threefold difference in resting membrane conductance in cells impaled with CsCl and Cs acetate electrodes is too great to explain in terms of Goldman rectification, and recalls the observations of Chesnoy-Marchais (1983) on the dependence of an inward Cl^- current on internal Cl^- concentration in *Aplysia* neurones. In the present treatment we have assumed a linear (voltage independent) conductance G_X since the current–voltage curves in Cs-impaled cells did not show pronounced rectification at positive potentials (e.g. Fig. 10*D*) and the effects of external Cl^- on membrane conductance illustrated in Fig. 10 were not always reproducible. Notwithstanding, the present experiments imply that I_X makes a substantial contribution to the resting membrane currents, and hence to the resting potential, of rat ganglion cells, and that this contribution may vary with the anion in the micro-electrode. Thus, if I_X and I_M were the only currents contributing to the resting potential, we calculate from the data in Fig. 2 and Table 2 that a cell impaled with a K-acetate-filled micro-electrode should have a 12 mV more negative resting potential and a twice greater slope resistance at its resting potential than a cell impaled with a KCl-filled micro-electrode. We have not made systematic measurements in the present experiments, but this would accord with our general impression and with the electrophysiological folklore that acetate electrodes yield 'better cells'.

Frog neurones

Previous experiments on voltage-clamped bull-frog ganglion cells impaled with KCl-filled electrodes yielded little or no evidence for a cross-over in the current–voltage curves during the application of muscarine (Adams *et al.* 1982*b*). This suggests either that the contribution of I_X to the leak current was relatively small in these experiments or that it was relatively insensitive to muscarine. However, others have reported a current–voltage cross-over during the muscarinic slow e.p.s.p. (Kuba & Koketsu, 1976) or reversal of the slow e.p.s.p. on hyperpolarization (Weight & Votava, 1970) in at least a proportion of unclamped bull-frog ganglion cells. This was initially attributed to reversal of the muscarine-sensitive K^+ current, but might equally have resulted from the same cause as that we have demonstrated in rat neurones.

Weight & Padjen (1973) have also described an acetylcholine-induced hyperpolarization of frog ganglion cells accompanied by an increased input conductance. Since this was abolished by replacing NaCl with sucrose, Weight & Padjen (1973) attributed it to a fall in Na^+ conductance. In the present experiments outward I_{mus} persisted in low-Na^+ solution, as pointed out above.

Physiological significance of I_X inhibition

There seems little reason to doubt that the principal effect of muscarine on rat ganglion cells, as on frog cells (cf. Adams *et al.* 1982*b*) is to depolarize the neurones and increase their excitability, as a consequence of inhibiting the outwardly rectifying K^+ current, I_M (see Brown & Constanti, 1980; Constanti & Brown, 1981). On the other hand, it is equally clear from Fig. 14 that, under conditions where I_M is already inhibited, the further inhibition of I_X is potentially capable of inhibiting neuronal activity. It seems possible, therefore, that a parallel inhibition of I_X might serve to limit the degree of excitation resulting from I_M inhibition, so long as the reversal potential for I_X is positive to the mean membrane potential attained during I_M inhibition. This is not implausible since, as pointed out above, E_{Cl} is strongly positive to the resting potential even in unimpaled neurones. The precise roles of I_M and I_X in the response to muscarine would be easier to evaluate if we could find a selective way of inhibiting each component of the response: so far we have not been successful.

Finally, we would point out that the effects of muscarine described in this paper can be fully reproduced when the muscarinic receptors are activated by stimulating the preganglionic nerve, that is, the synaptic slow e.p.s.p. in the rat ganglion is also a composite potential generated by the parallel inhibition of I_M and I_X (Brown & Selyanko, 1984).

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