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M_1 AND M_2 MUSCARINIC RECEPTORS MEDIATE EXCITATION AND INHIBITION OF GUINEA-PIG INTRACARDIAC NEURONES IN CULTURE

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SUMMARY

1. The effects of muscarine upon intracardiac neurones cultured from ganglia within the atria and interatrial septum of the newborn guinea-pig heart were studied using intracellular recording techniques.

2. Muscarine applied to the neuronal soma typically produced a biphasic change in membrane potential which consisted of a small hyperpolarization followed by a depolarization. In addition, muscarine (0·01–10 μM) inhibited the calcium-dependent, after-hyperpolarization (AHP) and greatly increased the number of action potentials that could be evoked by a given depolarizing current.

3. The hyperpolarization was associated with a decrease in input resistance and it reversed to become a depolarization at a potential of −86·5 mV. This response was antagonized by 4-diphenylacetoxy-*N*-methyl-piperidine (4-DAMP; 100 nM) and AF-DX 116 (500 nM), but was unaffected by pirenzepine (0·1–5 μM).

4. Two types of slow depolarization were observed in the presence of muscarine. The most common was associated with an increase in input resistance in the potential range −70 to −40 mV. Pirenzepine (100 nM) selectively antagonized this response, 4-DAMP (100 nM) similarly antagonized the response, but was non-selective. AF-DX 116 (0·5–5 μM) showed no antagonist effect. The less common depolarization (5% of cells) had a long latency and was associated with a decrease in input resistance.

5. Muscarine reduced the duration of the action potential and inhibited the AHP. Cadmium chloride (100 μM) mimicked these actions of muscarine. Application of muscarine immediately following a train of action potentials did not inhibit the AHP, suggesting that muscarine did not directly inhibit the calcium-activated potassium current ($I_{K(Ca)}$). Muscarine-induced depression of the slow AHP was antagonized by 4-DAMP (100 nM) but was not antagonized by either pirenzepine (0·1–0·5 μM) or AF-DX 116 (0·5–5 μM).

6. It is concluded that the muscarine-induced depolarization of guinea-pig intracardiac neurones results from reduction of a potassium conductance similar to the M-conductance, through activation of M_1 muscarinic receptors. The hyper-

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polarization results from an increase in potassium conductance, through activation of M_2 muscarinic receptors.

INTRODUCTION

Extensive studies of neurones from autonomic ganglia have demonstrated a wide range of action directly attributable to muscarinic receptor activation. Within these ganglia, muscarinic receptors have been shown to be involved in the mediation of slow excitatory postsynaptic potentials (slow EPSPs) (Libet, Chichibu & Tosaka, 1968; Koketsu, 1969; Neild, 1978; North & Tokimasa, 1982), as well as slow inhibitory postsynaptic potentials (slow IPSPs) (Hartzell, Kuffler, Stickgold & Yoshikami, 1977; Horn & Dodd, 1981; Gallagher, Griffith & Shinnick-Gallagher, 1982). In addition to these postsynaptic effects, presynaptic inhibition of acetylcholine release involving muscarinic autoreceptors has also been reported (Koketsu & Yamada, 1982; North & Tokimasa, 1982).

Considerable progress in our understanding of how acetylcholine could produce these multiple actions on a single cell came from the discovery that muscarinic receptors were heterogenous and could be distinguished by the selective antagonist pirenzepine (Hammer, Berrie, Birdsall, Burgen & Hulme, 1980). Receptors exhibiting a high affinity for pirenzepine have been classified as M_1, whilst those with a low affinity are termed M_2. The subsequent development of other selective agonists and antagonists has shown clear subdivisions in the M_2 receptor subtype, with M_2 cardiac muscle receptors being distinct from those on smooth muscle and glandular tissue (for review see Birdsall & Hulme, 1987; Bonner, 1989).

Using autoradiographic techniques, we have shown that in a cell culture preparation of guinea-pig atria, muscarinic receptors are present on all intracardiac neurones and atrial myocytes (Hassall, Buckley & Burnstock, 1987). The receptors present on mammalian cardiac muscle have been shown to be of a predominantly M_2 subtype, characterized as having a low affinity for pirenzepine and a high affinity for the antagonist 11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepine-6-one (AF-DX 116) and himbacine (Anwar-UL, Gilani & Gobbin, 1986; Giraldo, Hammer & Ladinsky, 1986). However, a small percentage of the muscarinic receptors in the heart were found to be of the M_1 subtype, a type commonly found within autonomic ganglia (Watson, Yamamura & Roeske, 1983). It is possible therefore that these M_1 receptors may be associated with the intramural neurones of the heart, rather than the cardiac muscle, and that the neuronal muscarinic receptors may be a heterogenous population consisting of M_1 and M_2 receptor subtypes.

In a previous investigation of the electrophysiological properties of guinea-pig intracardiac neurones in culture, we distinguished three distinct cell types which we termed M, AH_s and AH_m cells respectively (Allen & Burnstock, 1987). The M cells displayed non-accommodating tonic firing characteristics when stimulated by intrasomal current injection. The AH_s and AH_m type intracardiac neurones, which accounted for more than 80% of the cells studied, were highly refractory and

displayed pronounced calcium-dependent spike after-hyperpolarizations. In the present study, we have examined the actions of muscarine and utilized a variety of muscarinic receptor antagonists to determine the receptor subtypes involved in mediating these actions in AH_s and AH_m type guinea-pig intracardiac neurones.

METHODS

Experiments were carried out on intracardiac neurones, dissociated from the atria and interatrial septum of newborn guinea-pigs. The mixed cell cultures containing these neurones were prepared and maintained for 5–14 days using the methods developed by Hassall & Burnstock (1986). Prior to starting experiments, the culture chamber was dismantled and the cover-slip bearing the cultured cells was gently rinsed in oxygenated Krebs solution. The cover-slip was then sealed to the underside of a Perspex recording bath using paraffin wax, so that it formed the base of the chamber. The combination of bath and cover-slip were then clamped to the modified stage of an inverted microscope (Zeiss invertoscope D), equipped with conventional phase-contrast optics.

The preparation was perfused at a rate of 4 ml/min with oxygenated Krebs solution, warmed to 36–37 °C using a remote thermostatically controlled heating coil. The Krebs solution had the following composition (mM): NaCl, 117; KCl, 4·7; $MgCl_2$ 1·2; NaH_2PO_4, 1·2; $CaCl_2$, 2·5; $NaHCO_3$, 25 and glucose, 11; gassed with 95% O_2–5% CO_2.

Impalements were made using electrodes with DC resistances of 90–130 MΩ filled with 2 M-potassium citrate solution (pH 7·3). The electrodes were connected to an amplifier with an active bridge circuit that allowed simultaneous current passing and voltage recording (Dagan model 8700 cell explorer). Prior to impalement, electrode resistance and tip offset potentials were nulled to permit estimations of input resistance and membrane potential to be made during the recording. These were checked at the end of each experiment by withdrawing the electrode and passing currents of similar magnitude to those used during the impalement. Input resistance measurements were made by passing brief 50–100 ms current pulses of known intensity across the cell membrane and measuring the amplitudes of the hyperpolarizations evoked. The fractional increase in conductance was calculated as $(R/R')-1$ where R is the input resistance at resting membrane potential and R' that during the muscarinic responses. Predicted peak inhibitory potential amplitude (v) was calculated using the equation: $v = (1-R'/R)(E_{K,i}-E_m)$ where $E_{K,i}$ is the equilibrium potential for the hyperpolarization and E_m is the mean resting membrane potential (see Allen & Burnstock, 1987).

Drugs were applied either by local pressure ejection or bath perfusion. Pressure application was carried out using blunt microeletrodes (3–10 μm diameter tip) containing drugs diluted in Krebs solution. Electrodes were placed 100–500 μm away from the cell surface and the drugs ejected under pressures of 50–100 kPa.

Data were either stored on tape for future analysis (Racal store 4DS) or displayed using a Tektronix storage oscilloscope (model D13) and a Gould pressure ink recorder (model 2200S). Numerical data are expressed as mean ± S.E.M.

Drugs. Muscarine chloride, atropine sulphate, 4-aminopyridine, gallamine triethiodide, tetrodotoxin (TTX), cadmium chloride, acetylcholine chloride were from Sigma. 4-Diphenylacetoxy-*N*-methyl-piperidine methiodide (4-DAMP, was a gift from Dr R. B. Barlow), 11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepine-6-one (AF-DX 116, was a gift from Karl Thomae GmbH), and pirenzepine dihydrochloride monohydrate was from Boots.

RESULTS

The actions of muscarinic agonists and antagonists were studied on more than 200 AH_s and AH_m intracardiac neurones (Allen & Burnstock, 1987). Cells were considered to be suitable for study if, after an initial settling period, they had a stable

membrane potential of at least −45 mv and could produce an action potential with a minimum amplitude of 70 mV. Typically, spike amplitudes were between 85 and 100 mV. Every cell tested ($n = 207$) responded to muscarine.

Application of muscarine (10–50 μM) through a blunt micropipette placed close to

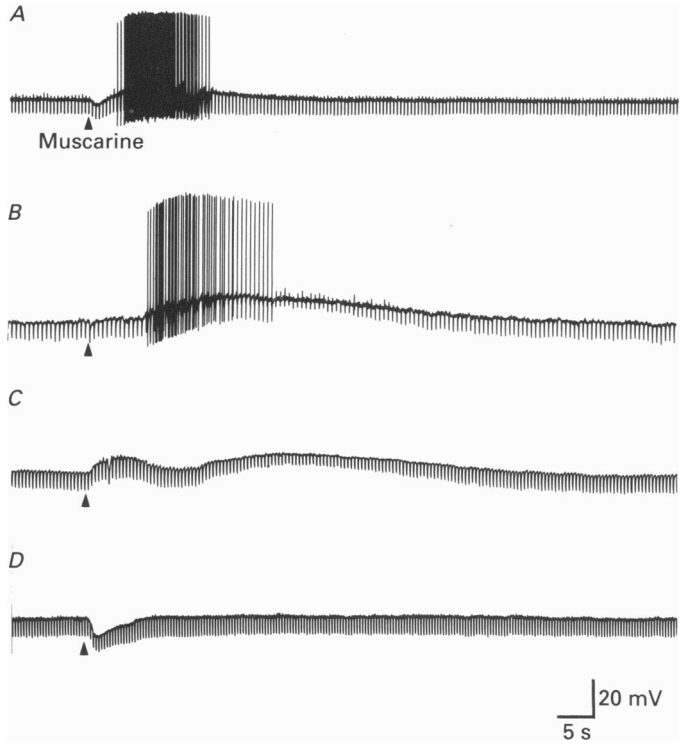

Fig. 1. Examples of the different actions of muscarine upon resting membrane potential and input resistance. Muscarine was applied as indicated by the arrows, using a brief pressure pulse (100–500 ms, 50–100 kPa) applied to a micropipette containing muscarine (10 μM) made up in Krebs solution, positioned 10–20 μm from the soma of the impaled cell. *A*, muscarine elicited a small hyperpolarization associated with a decrease in input resistance that was followed by a slow depolarizaton and an increase in input resistance (resting potential −49 mV). *B*, muscarine evoked, after a longer latency, a second slower type of depolarization associated with a decrease in input resistance (resting potential −58 mV). *C*, muscarine elicited both types of slow depolarization. The different time courses of the two conductance changes can be seen clearly (resting potential −55 mV). *D*, by first hyperpolarizing a cell with DC current injection to a membrane potential of −67 mV, a large pure hyperpolarization to muscarine could be elicited. At the resting membrane potential of −53 mV this cell responded to muscarine in a similar way to the cell shown in *A*.

the neuronal soma evoked a variety of different responses as shown in Fig. 1. All these actions could be abolished by atropine (10 nM) but, with the exception of the evoked firing, were resistant to TTX (330 nM). Different combinations of two of four separate conductance changes were found to underlie the observed actions of muscarine.

Actions of muscarine on resting membrane potential and conductance

The most commonly observed action of muscarine on the resting membrane potential of a cell consisted of a hyperpolarization followed by a more slowly rising, prolonged depolarization (fifty-three out of seventy-four cells; Fig. 1*A*). The

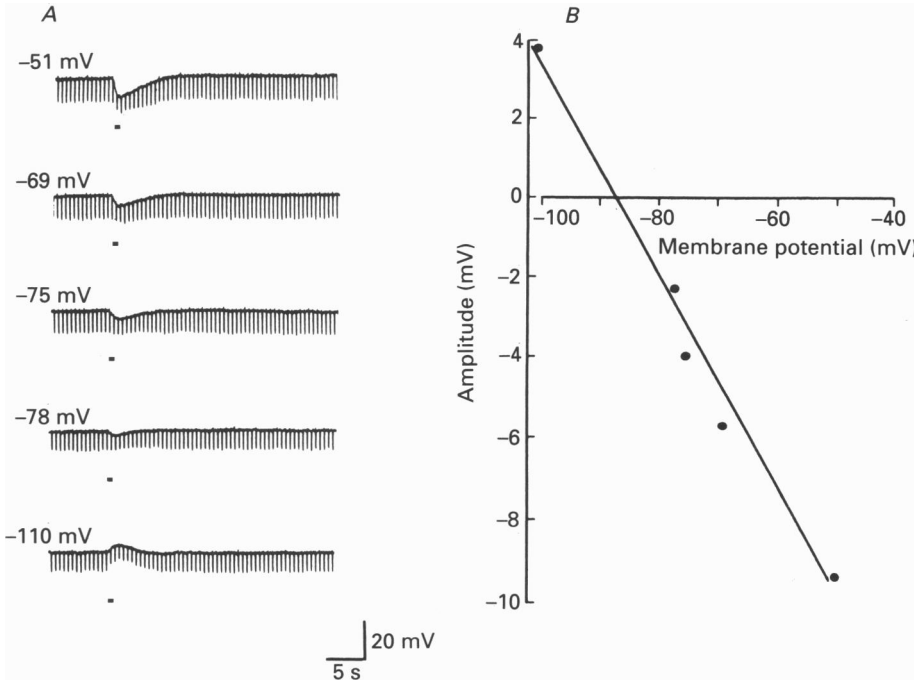

Fig. 2. Voltage dependence of the inhibitory response to muscarine in guinea-pig intracardiac neurones. In all cells tested, the inhibitory response nulled or reversed near the potassium equilibrium potential. *A*, the amplitude of the inhibitory response in an AH_m cell at different membrane potentials. Muscarine (10 μM) was applied by local pressure ejection for the period indicated by the bars. Downward deflections are the result of passing brief 50 ms duration constant hyperpolarizing current pulses (100 pA) in order to monitor changes in membrane input resistance. *B*, a plot of amplitude against membrane potential for the cell shown in *A*. The amplitude was linearly related to membrane potential over the range -51 to -110 mV ($r = 0{\cdot}993$) and reversed at a mebrane potential of $-87{\cdot}4$ mV. Note: in a number of cells which displayed pronounced inward rectification, the amplitude of the inhibitory response was not linearly related to the membrane potential; however, it was always seen to reverse at around -90 mV.

hyperpolarization had a short latency (150–500 ms) and was associated with a decrease in input resistance. The excitatory response was generally associated with an increase in input resistance and reached a peak 5–10 s after the application of muscarine. During this depolarization, cells fired spontaneously or at the termination of hyperpolarizing current pulses (see Fig. 1*A*). A third type of response, a membrane depolarization associated with a fall in input resistance, was observed in less than 5% of the cells (see Fig. 1*B*). In Fig. 1*C*, the different time courses and conductance changes underlying the two muscarine-induced depolarizations can clearly be seen in

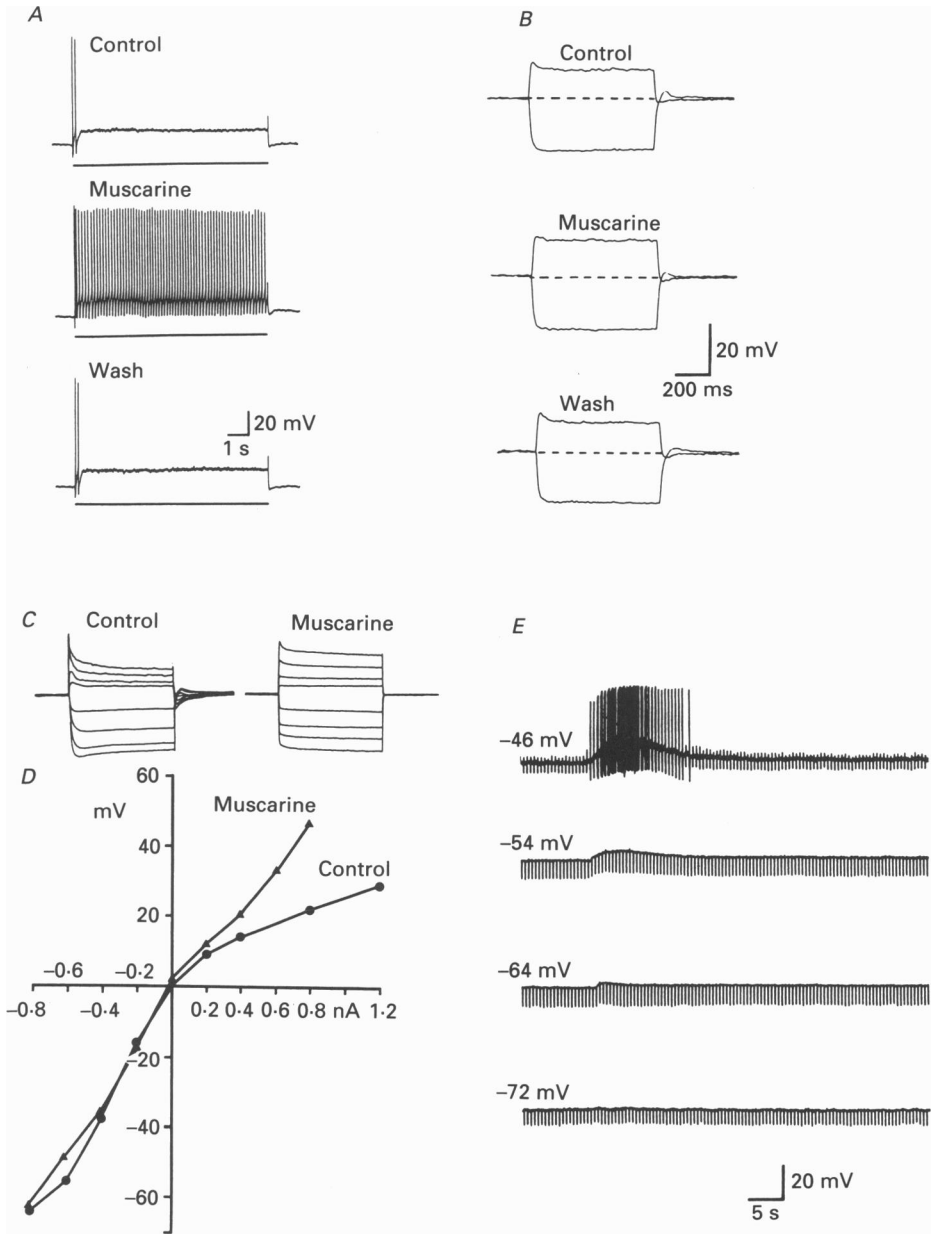

Fig. 3. *A*, the action of muscarine on the firing characteristics of AH type neurones. Prolonged intrasomal current injection (200 pA/10 s) typically elicted firing only at the onset of current injection. Muscarine (10 μM) induced sustained firing during the current stimulus (resting potential −58 mV). *B*, the slow conductance change underlying the refractory behaviour of AH type cells. The cell (membrane potential −58 mV) was stimulated using hyperpolarizing and depolarizing current (±250 pA) in the presence of TTX (330 nM). The cell displayed outward rectification resulting in a sag in the voltage records to depolarizing current. In the presence of muscarine (10 mM) depolarizing offset by passing constant hyperpolarizing current) both the rectification and the voltage sags were reduced. *C*, voltage responses of an AH type neurone to 500 ms current pulses of

a neurone displaying both of these components. Finally, cells that responded to muscarine in the manner shown in Fig. 1*A* frequently displayed a larger initial hyperpolarization if hyperpolarized slightly prior to muscarine application (Fig. 1*D*). The hyperpolarization was resistant to TTX (330 nM), indicating that it probably did not arise from any synaptic interactions. Neither the muscarine-induced membrane hyperpolarizations or depolarizations were inhibited by superfusion with $CdCl_2$ (100 μM; $n = 11$).

Effects of muscarine on current-evoked firing

We have previously described the normal current firing characteristics of AH_s and AH_m neurones when depolarized by current injection (Allen & Burnstock, 1987). Both cell types display pronounced after-hyperpolarizations (AHPs) and this causes them to be strongly refractory. However, in the presence of muscarine (1–10 μM), both of these cell types could fire tonically for prolonged periods (5–30 s) in response to low-stimulus currents (50–200 pA; see Fig. 3*A*). Firing rates ranged from 2 to 12 Hz, although short bursts of firing (up to 250 ms) at 80–90 Hz, could be observed at the onset of depolarizing current injection.

Mechanisms underlying the different responses

Hyperpolarization

In fifty-seven cells with potentials of $-57{\cdot}5 \pm 0{\cdot}13$ mV, the amplitude of the hyperpolarization ranged between -2 and -17 mV (mean $-7{\cdot}3 \pm 0{\cdot}55$ mV, $n = 57$), with a fractional increase in conductance, measured at the peak of the response (see Methods), of $0{\cdot}36 \pm 0{\cdot}04$ ($n = 31$). The hyperpolarization was linearly related to membrane potential and reversed symmetrically near the potassium equilibrium potential. The mean value for the reversal of the hyperpolarization in these cells was $-86{\cdot}55 \pm 1{\cdot}01$ mV ($n = 7$; see Fig. 2) which is very close to E_K (see Allen & Burnstock, 1987). In many cells, hyperpolarization itself resulted in a fall in input resistance; in such cases, the amplitude of the hyperpolarization caused by muscarine was not linearly related to the membrane potential at which it was elicited. It is worth nothing that the incidence of inward rectification in the voltage–current relationship in AH_s and AH_m cells was much higher than previously observed in

varying intensity evoked in the presence of TTX (330 nM). Under control conditions the voltage responses to depolarizing current displayed pronounced sags, whilst in the presence of muscarine this was largely abolished. (Note: in the cell shown muscarine also reduced the fast calcium-dependent voltage transient at the onset of depolarizing current injection and promoted inward rectification at strongly hyperpolarized potentials.) *D*, the steady-state voltage–current relationship for the cell shown in *C*. Under control conditions, marked outward rectification was observed at membrane potentials of less than -65 mV (resting potential -59 mV). Muscarine (10 μM) reduced this rectification and depolarized the cell by approximately 2 mV. *E*, the effect of varying membrane potential on the slow depolarization associated with a fall in membrane conductance. At membrane potentials greater than -70 mV no depolarizations or conductance change could be elicited. Membrane depolarization prior to application of muscarine increased the amplitude of the evoked depolarization. At sufficiently depolarized levels the muscarine-induced depolarization lead to spontaneous spike discharge and a greater tendency for anodal-break current firing.

these cells (Allen & Burnstock, 1987). At present we have no explanation for this difference.

Further evidence that the hyperpolarization resulted from an increase in potassium conductance was derived by comparing the observed amplitude of the response with the value calculated from the observed increase in conductance (see Methods). In thirty-one neurones the predicted peak amplitude of $-6{\cdot}9 \pm 0{\cdot}72$ mV was not significantly different from the observed value of $-7{\cdot}1 \pm 0{\cdot}69$ mV ($P > 0{\cdot}01$).

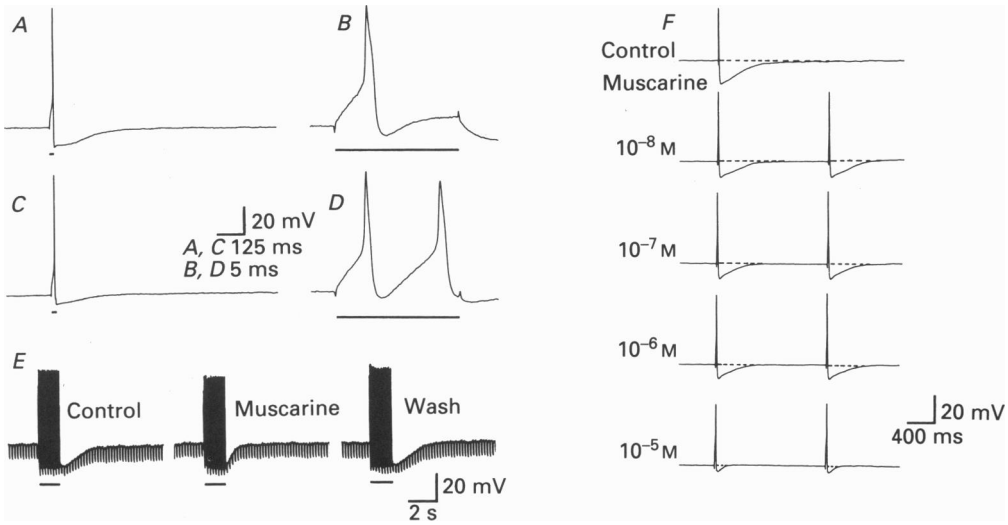

Fig. 4. Muscarine-induced inhibition of the spike AHP and action potential duration in a guinea-pig intracardiac neurone. *A*, control AHP following a single spike and *C*, in the presence of muscarine (1 μM). *B* and *D*, fast records from the same cell (25 ms current stimulation) before and during the application of muscarine (1 μM) respectively. Muscarine reduced the characteristic hump on the falling phase of the action potential, which is associated with calcium entry (see text), but had little effect upon spike amplitude (resting membrane potential -57 mV). *E*, muscarine (10 μM) reversibly reduced the slow AHP and associated increase in membrane conductance following a train of 30 action potentials. Action potentials were evoked by a train of intrasomal current pulses delivered at 20 Hz for 1·5 s (resting membrane potential -56 mV). Note: in *E*, action potential amplitudes were attenuated due to the limited frequency response of the pen recorder. *F*, the amplitude and duration of the slow AHP following a single action potential in the presence of increasing concentrations of muscarine. Top panel, control AHP. Subsequent panels show the effect of superfusion with 0·01–10 μM muscarine respectively. Note: in each case any depolarization produced by muscarine was offset by passing hyperpolarizing DC current. Resting membrane potential -58 mV.

Depolarization associated with a fall in membrane conductance

At the normal resting membrane potential for intracardiac neurones (-55 to -65 mV) the amplitude of the muscarine-induced depolarization rarely exceeded 4–6 mV. The size of the depolarization and the associated increase in input resistance were voltage dependent. At membrane potentials more negative than -60 to -65 mV, the response was almost totally abolished, whilst membrane depolarization

increased its amplitude and frequently led to spontaneous and anodal-break action potential discharge during the action of muscarine (see Fig. 3*E*).

The voltage–current relationship of all AH_s and some AH_m type neurones displayed strong outward rectification which first became evident at about −70 to

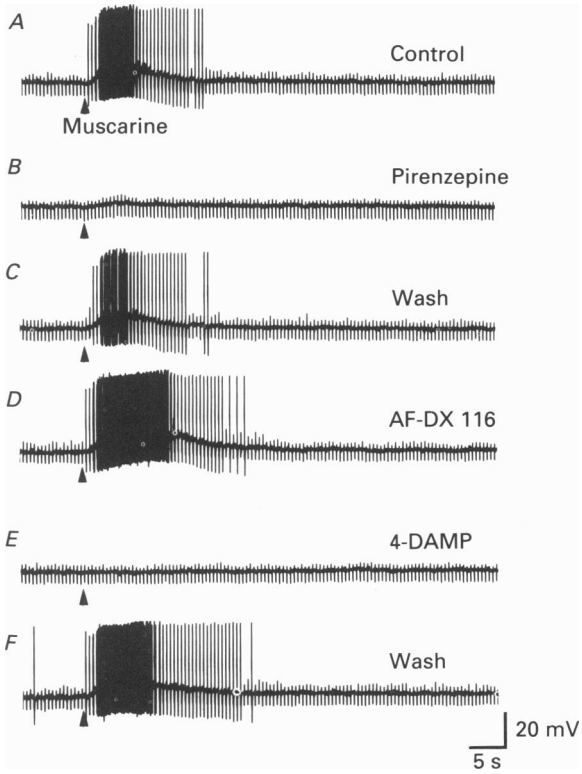

Fig. 5. Antagonism of the muscarine-induced slow depolarization with associated fall in membrane conductance in guinea-pig intracardiac neurones. In order to raise the level of activation of this current, the cell was depolarized slightly using intrasomal current injection. Muscarine (10 μM) was applied by pressure ejection (250 ms pulses) from a blunt micropipette placed close to the neuronal soma. This produced a slow depolarization, a fall in resting membrane conductance and induced spontaneous action potential discharge (see *A*). *B*, pirenzepine (100 nM) reversibly antagonized the depolarization and fall in membrane conductance without inhibiting any of the other muscarinic receptor-mediated effects. *D*, AF-DX 116 at a concentration (500 nM) which antagonized the inhibitory response had no effect upon the depolarization. *E*, 4-DAMP (100 nM) inhibited the depolarization but, unlike pirenzepine, it also antagonized all the other muscarine-induced responses with no selectivity. *C* and *F*, wash-out to control conditions.

−65 mV and which increased with depolarization (see Fig. 3*D*). In addition, during the first 100–150 ms, a time-dependent sag occurred in the voltage response to constant depolarizing current. Likewise, at the offset of the current injection there was a slow relaxation from an initial hyperpolarized potential back to the resting level (see Fig. 3*C*). This behaviour was strongly suggestive of the presence of an outward current in these neurones similar to the M-current (I_m) first described in

bull-frog sympathetic neurones (Brown & Adams, 1980). Application of muscarine (1–10 μM) greatly reduced, although never fully abolished, the slow sag in the voltage records (see Fig. 3*B* and *C*) and also markedly reduced outward rectification in the voltage–current relationship at depolarized potentials (see Fig. 3*D*).

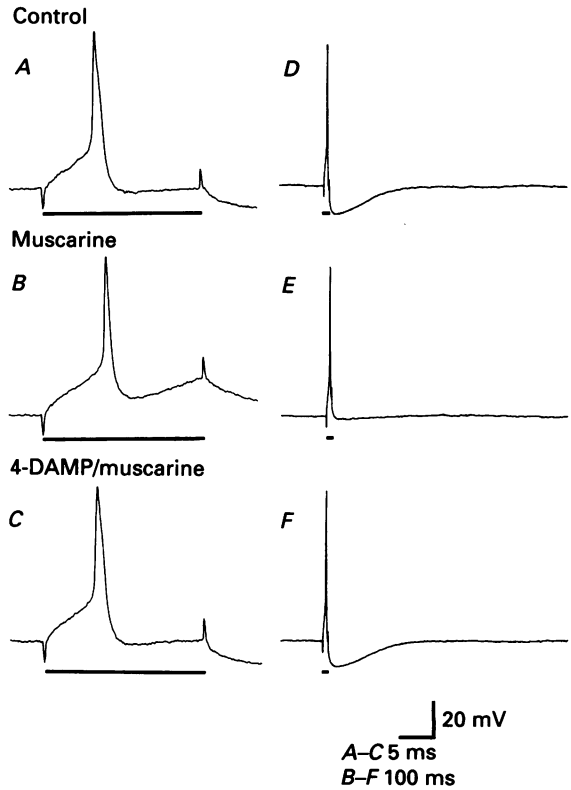

Fig. 6. Antagonism of the muscarine-induced depression of spike width and spike AHP in guinea-pig intracardiac neurones. *A* and *D*, control spike and AHP; *B* and *E*, in the presence of muscarine (10 μM). Note: any depolarization was nulled by passing hyperpolarizing current through the electrode. *C* and *F*, 4-DAMP (100 nM) totally antagonized the effect of muscarine (10 μM) in inhibiting the AHP and in reducing spike width. Bars beneath traces indicate periods of current stimulation. Resting membrane potential −57 mV.

To determine whether the increase in input resistance underlying the slow depolarization arose through inhibition of a tonically operated calcium-activated potassium conductance, we performed a number of experiments in which muscarine was applied in the presence of either low extracellular calcium or 100 μM-$CdCl_2$. In none of the cells tested ($n = 7$) were such procedures observed to inhibit the muscarine-induced increase in input resistance.

Depolarization associated with a rise in membrane conductance

Only seven out of a total of 207 neurones shows this response. This response had a long latency (3–7 s), was unaffected by TTX ($n = 2$), and its amplitude increased

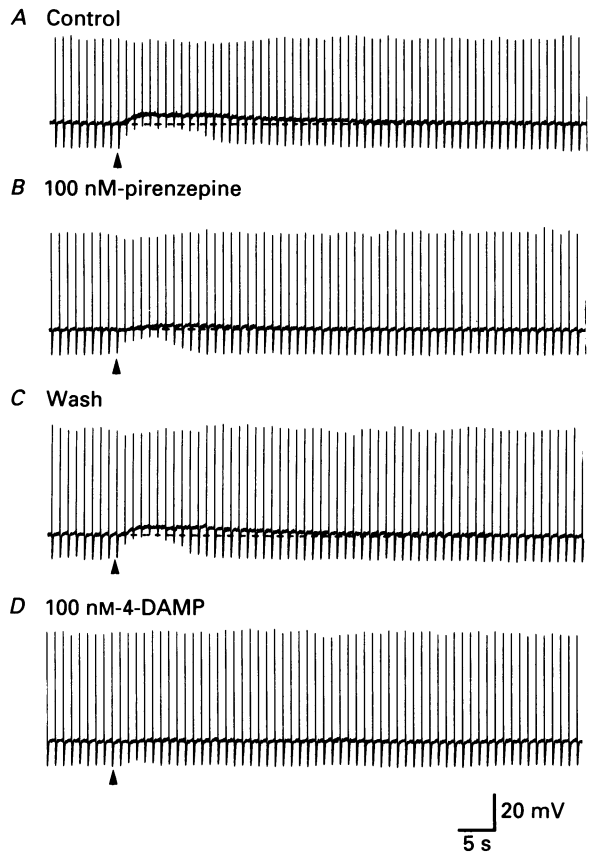

Fig. 7. Antagonism by pirenzepine and 4-DAMP of the muscarine-induced depolarization and inhibition of the spike AHP in guinea-pig intracardiac neurones. Muscarine (10 μM) was applied by local pressure ejection for 150 ms at the points indicated by the arrows. *A*, control; the cell depolarized and there was a concomitant decrease in the amplitude and duration of the AHP. *B*, pirenzepine (100 nM) antagonized the depolarization induced by muscarine, but had no effect on the muscarine-induced decrease in the AHP. *C*, wash-out. *D*, 4-DAMP (100 nM) antagonized both the actions of muscarine but displayed no notable selectivity for any one component. Resting membrane potential −50 mV. Note: spikes were attenuated in the pen recording.

with membrane hyperpolarization. In all cases the response was associated with a large decrease in input resistance.

Effects on the after-hyperpolarization.

An AHP lasting up to 3 s following a single action potential in AH_s and AH_m intracardiac neurones. Both the amplitude and the duration of this response were reduced by muscarine (see Figs 4 and 6). The observed reduction in AHP amplitude in 0·01, 0·1, 1 and 10 μM-muscarine were $23 \pm 12{\cdot}2\%$, $47 \pm 10\%$, $59 \pm 9{\cdot}2\%$ and $73 \pm 11{\cdot}8\%$ ($n = 6$) respectively. The AHP duration (measured as time to half-peak amplitude) was reduced by $7{\cdot}1 \pm 4{\cdot}2\%$, $18 \pm 19{\cdot}7\%$, $26 \pm 21{\cdot}9\%$ and $46 \pm 18{\cdot}2\%$

($n = 6$) respectively. Concomitant with the decrease in AHP amplitude and duration was a reduction in the width of the preceding action potential, an effect which became marked at higher concentrations (1–10 μM (see Figs 4 and 6).

The AHP in intracardiac neurones results from a calcium-activated potassium

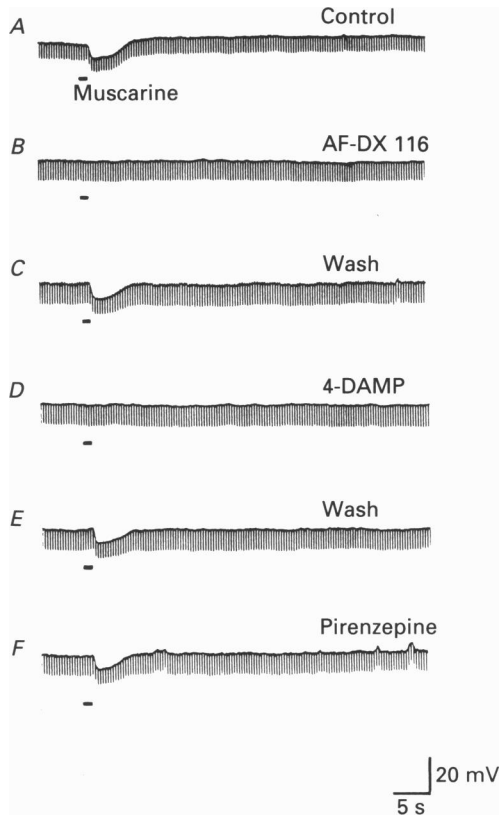

Fig. 8. Antagonism of the muscarine-induced inhibitory potential in guinea-pig intracardiac neurones. *A*, control response to muscarine. *B*, the inhibitory response was completely antagonized by AF-DX 116 (500 nM). At this concentration AF-DX 116 showed a limited degree of selectivity for the inhibitory response and generally required a 2- to 5-fold increase in concentration before it antagonized any of the other muscarinic responses. *C*, wash-out to control conditions. *D*, as with all the other muscarinic response, 4-DAMP (100 nM) potently, but non-selectively, antagonized the inhibitory responses. *E*, wash-out to control conditions. *F*, pirenzepine (100 nM): a concentration that inhibited the slow depolarization) had no effect upon the inhibitory potential. Resting membrane potential −64 mV.

current (Allen & Burnstock, 1987). Possible mechanisms for the inhibition of this current are that muscarine acts to reduce the ability of intracellular calcium to activate the potassium conductance or that muscarine acts to reduce the amount of calcium entering during the action potential. To test the first of these possibilities a brief pulse (100–500 ms) of muscarine was applied at different times before, during and after a train of action potentials evoked by current injection (20 Hz/1·5 s). Muscarine was most effective when applied immediately prior to the train of action potentials. If applied during the train its effect decreased, becoming less effective the

later the delay in its application. When applied immediately following the train of action potentials, it became totally ineffective in inhibiting the AHP. This decrease in the efficacy of muscarine was not due to the latency of its action, as a decrease in the AHP following a single spike could be observed within 50–100 ms of the start of the application of muscarine.

At concentrations of muscarine equal to or greater than 0·1 μM, the observed decrease in the AHP was always associated with a concomitant decrease in the duration of the action potential preceding it. Under control conditions the mean spike duration (measured at half-peak amplitude) was $1{\cdot}44 \pm 0{\cdot}9$ ms ($n = 11$); in the presence of 0·1, 1 and 10 μM-muscarine the durations were $1{\cdot}35 \pm 0{\cdot}1$ ms, $1{\cdot}21 \pm 0{\cdot}09$ ms and $1{\cdot}09 \pm 0{\cdot}08$ ms ($n = 6$) respectively. This suggests that muscarine may reduce the AHP by decreasing the level of calcium entry into the cell during the action potential.

In the presence of TTX (330 nM), muscarine abolished the calcium-dependent action potential in these cells. Cadmium (100 μM) which potently blocks calcium entry during the action potential in intracardiac neurones, mimicked the action of muscarine in reducing spike width as well as AHP amplitude and duration. In the presence of cadmium, muscarine was found to produce no further decrease in either the spike width or the duration of the AH. In intracardiac neurones, superfusion with 4-aminopyridine (1 mM) delays the repolarization phase of the action potential causing an increase in calcium entry which leads to an increase in the duration of the AHP (see Allen & Burnstock, 1987). In the presence of 4-aminopyridine, muscarine (10 μM) abolished the calcium-dependent hump in the repolarization phase of the spike but did not reduce overall spike width to control values. However, the amplitude and duration of the AHP were greatly reduced even though spike duration was 2–3 times longer than under control conditions; this suggested that muscarine was acting to directly inhibit calcium entry rather than indirectly by reducing the period of calcium entry.

Antagonists studies

Pirenzepine (M_1 receptor antagonist)

The muscarine-induced depolarization and increase in the input resistance were antagonized by pirenzepine at concentrations between 50 and 150 nM ($n = 37$; agonist concentration 0·5–10 μM) (see Figs 5 and 7). At these concentrations, pirenzepine did not antagonize any of the other responses to muscarine, indicating that the I_M-like current inhibition was mediated through M_1 receptors. Reduction of the hyperpolarization and the inhibition of the AHP induced by muscarine did not occur until pirenzepine concentrations reached 5–10 μM. This suggests that these responses result from the activation of M_2 muscarinic receptors. The rather high concentrations of pirenzepine result from the necessity to use pipettes containing high concentrations of muscarine (up to 50 μM) to obtain reproducible hyperpolarizations. The muscarinic inhibition of the AHP could be observed by superfusing muscarine (0·1–1 μM); lower concentrations of pirenzepine (0·5–1 μM) were required to antagonize muscarinic inhibition of the AHP.

Gallamine, 4-DAMP and AF-DX 116 (M_2 receptor antagonists)

Gallamine (10 μM) antagonized the hyperpolarization ($n = 9$). At 20 or 30 μM it also blocked the depolarization caused by muscarine. However, gallamine could not be used as a muscarinic antagonist with respect to inhibition of the AHP because it depressed the AHP even in the absence of muscarine.

It was found that 4-DAMP (100 nM) antagonized all the different muscarine-induced conductance changes seen in these neurones ($n = 43$; see Figs 5, 6, 7 and 8).

The compound AF-DX 116, a selective M_2 cardiac receptor antagonist, antagonized the hyperpolarization caused by muscarine at 0·5–1 μM ($n = 10$; see Fig. 8). Antagonism of the other muscarinic responses occurred at slightly higher concentrations in the range 2–5 μM.

DISCUSSION

The muscarinic receptor-mediated responses of intracardiac neurones reported in the present study have a number of similarities to those previously described in other peripheral ganglia and some central neurones. The hyperpolarization resulting from an increase in potassium conductance was similar to the cholinergic membrane hyperpolarizations and slow IPSPs described in mudpuppy cardiac ganglion cells (Hartzell *et al.* 1977), bull-frog C cells (Dodd & Horn, 1983), rat superior cervical neurones (Brown, Fatherazi, Garthwaite & White, 1980), nucleus parabrachialis neurones (Egan & North, 1986) and rat thalamic reticular neurones (McCormick & Prince, 1986)

The slow depolarization and increase in membrane resistance which frequently followed the hyperpolarization seen in both AHP types of intracardiac neurone and the voltage dependence of this response indicated that it resulted from inhibition of resting potassium conductance. Cholinergic slow EPSPs and muscarine-induced depolarizations that result from a decrease in membrane potassium conductance have been described in a number of other neurones (Weight & Votava, 1970; Krnjević, Pumain & Renaud, 1971; Halliwell & Adams, 1982; North & Tokimasa, 1983). Examination of the ionic mechanisms underlying these responses have shown that they result from the inhibition of up to three different types of potassium currents, I_M, $I_{K(Ca)}$ and a background/leak current (Dodd, Dingledine & Kelly, 1981; North & Tokimasa, 1983; Tokimasa, 1984, 1985; Brown, 1988). In the present study we observed muscarinic inhibition of all three conductances. Inhibition of the I_M-like conductance was largely responsible for the generation of the slow depolarization in intracardiac neurones. Inhibition of this conductance during the muscarine-induced depolarization could also be detected as a reduction in the level of outward rectification in the voltage–current relationship at membrane potentials less than −60 mV. During the slow depolarization, these neurones also showed a greater tendency to fire tonically. In intracardiac neurones, as in other cells, part of the action of muscarine in promoting multiple firing can be attributed to the inhibition of the AHP. Inhibition of $I_{K(Ca)}$ appeared to be the predominant factor in determining the degree of tonic firing behaviour in intracardiac neurones. Two pieces

of evidence support this suggestion: firstly, significant inhibition of $I_{K(Ca)}$ took place at muscarine concentrations as low as 50 nM, whilst inhibition of the M-like current (measured as a muscarine-induced depolarization) did not occur until considerably higher concentrations were used (greater than 1 μM); secondly, prevention of calcium entry, and therefore reduction of the level of activation of $I_{K(Ca)}$ using cadmium ions mimicked to a large extent the action that muscarine had upon multiple firing. However, subsequent addition of muscarine in the presence of cadmium further facilitated tonic firing in most cells. Therefore, it would appear that these two currents act together to produce the normal refractory firing characteristics of AHP type intracardiac neurones and are both modulated by muscarinic agonists. The apparently dominant role played by $I_{K(Ca)}$ over I_M in facilitating sustained multiple spike discharge was similar to that seen in hippocampal neurones (Madison & Nicoll, 1984), where cadmium ions also mimicked muscarinic agonists in their ability to promote multiple firing. Interestingly, this differs from what has been found in some other peripheral ganglia, where inhibition of I_M was found to exert a more predominant role in the regulation of tonic firing behaviour (see review by Brown, 1988).

Muscarinic inhibition of the AHP in intracardiac neurones appeared to result from decreased calcium entry during the action potential and not from direct inhibition of $I_{K(Ca)}$. The main evidence for this was the observation that no reduction in $I_{K(Ca)}$ activation occurred in $I_{K(Ca)}$ activation occurred if muscarine was applied immediately after a train of action potentials, whereas reduction in the AHP was observed if muscarine was applied at any point during the spike train: the earlier its application the greater the inhibition. This is in contrast to AH/type 2 enteric neurones, where inhibition of $I_{K(Ca)}$ by muscarinic agonists was not associated with a reduction in spike width (North & Tokimasa, 1983). In intracardiac neurones, inhibition of calcium entry by cadmium and not accelerated spike repolarization resulting from increased activation of the delayed rectifier current $I_{K(V)}$ appeared to be responsible for the fall in calcium entry. Similar inhibition of calcium currents by muscarinic agonists has been suggested to underlie the inhibition of the AHP observed in a number of other neurones (Belluzi, Sacchi & Wanke, 1985; Mochida & Kobayashi, 1986*b*, *c*). In each case a reduction in the calcium-dependent action potential was also seen (see also Wanke, Ferroni, Margaroli, Ambrosini, Pozzan & Meldolesi, 1987).

A slow depolarization with a long latency resulting from an increase in membrane conductance was evoked from a small number of intracardiac neurones. In the present report, no detailed analysis of this phenomenon was possible, but slow depolarizations associated with an increase in conductance have been described in a number of other ganglia and have been suggested to arise as the result of an increase in either a mixed cation conductance (Kuba & Koketsu, 1976; Akasu, Gallagher, Koketsu & Shinnick-Gallagher, 1984) or from an increase in chloride conductance (Mochida & Kobayashi, 1986*a*). Whether the response described in the present study was generated by a similar mechanism will require further studies.

The antagonist experiments performed here indicate that there were both M_1 and M_2 muscarinic receptor subtypes present upon guinea-pig intracardiac neurones. M_1

receptors with a high affinity for pirenzepine were responsible for mediating inhibition of the I_M-like time- and voltage-dependent conductance. Muscarinic inhibition M-currents in other neurones is also predominantly through the mediation of M_1 receptors (Brown, Forward & Marsh, 1980; Brown & Selyanko, 1985; Brown, Gähwiler, Marsh & Selyanko, 1986), however, this is not exclusively the case as M_2 receptor-mediated inhibition of the M-current has recently been described in guinea-pig olfactory neurones (Constanti & Sim, 1987) and rat hippocampal neurones (Dutar & Nicoll, 1988).

Both the slow inhibitory potential and the inhibition of the AHP showed a low affinity for pirenzepine, indicating that these responses were mediated through M_2 muscarinic receptors. M_2 receptors appear to be heterogeneous and two antagonists frequently used to discriminate between these subtypes are AF-DX 116 and 4-DAMP. The antagonist AF-DX 116 shows a high affinity for cardiac muscarinic receptors and a low affinity for those on smooth muscle/gland cells (Giraldo *et al.* 1986; Hammer, Giraldo, Schiavi, Monferini & Ladinsky, 1986), whilst 4-DAMP shows the reverse selectivity (Barlow, Berry, Glenton, Nikolaou & Soh, 1976). On intracardiac neurones, 4-DAMP was found to show almost no selectivity, inhibiting all M_1 and M_2 receptor-mediated actions at concentrations around 100 nM. It was found that AF-DX 116 had a much lower affinity than 4-DAMP, but did show a small degree of selectivity (2- to 5-fold) in antagonizing the hyperpolarization. This suggests that the M_2 receptor subtype responsible for the hyperpolarization may have been of cardiac M_2 type, whilst those responsible for inhibiting the AHP may have been of a smooth muscle/glandular type. However, the very low selectivity showed by these compounds could also indicate that these receptors may belong to yet another subclass of muscarinic receptor for which selective antagonists have yet to be developed.

In conclusion, the wide variety of muscarinic responses displayed by guinea-pig intracardiac neurones suggests that they may play an important role in integrating and/or modulating the action of extrinsic nerves upon the heart. Furthermore, the intracardiac ganglia are well placed to perform this function, as many of these ganglia are concentrated in the nodal regions and at the point of insertion of the great veins (King & Coakley, 1958).

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