Muscarinic M₂ Receptors on Cardiac Ganglion Neurons of the Guinea-pig Heart

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Summary: Intracellular recordings were made from neurons of the cardiac ganglion of the guinea-pig heart. Application of muscarinic agonists produced a depolarization in the majority of neurons. The change in membrane conductance underlying the depolarization consisted of an initial low-conductance phase followed by a high-conductance phase. The first component was probably due to suppression of a potassium conductance, and the second was likely generated by activation of a chloride conductance. Both components of the depolarization were blocked by pirenzepine with an antagonist dissociation constants (K_D) of 200-900 nM, suggesting that both are mediated through a muscarinic M₂ receptor.

Key words: cardiac ganglion neurons — muscarinic receptors — potassium conductance — chloride conductance — pirenzepine

Introduction

Acetylcholine mediates two types of responses in sympathetic and parasympathetic neurons. The nicotinic depolarization is characterized by a shorter time course (of tens of milliseconds) and is selectively blocked by curare or other ganglion blocking agents such as hexamethonium. Activation of muscarinic receptors produces either depolarizing or hyperpolarizing responses with duration of several seconds or minutes and is antagonized by atropine (Libet, 1964; Nishi, 1974; Hartzell et al. 1977; Gallagher et al. 1982; Dodd and Horn, 1983).

In cardiac ganglion neurons, nicotinic responses have been well documented in the frog (Dennis et al. 1971) and mudpuppy (Hartzell et al. 1977) heart. Nishi et al. (1978) have shown that cardiac ganglion neurons of the guinea-pig are en-

dowed with two types of nicotinic receptors, one presumably subsynaptic, the other extrasynaptic. On the other hand, the muscarinic action of acetylcholine in these neurons has been less well analysed except for the muscarinic slow inhibitory postsynaptic potential (IPSP) described by Hartzell et al. (1977).

There are now many reports that muscarinic receptors comprise at least two distinct subtypes, the M₁ and M₂ subtype, which are characterized by high and low affinity, respectively, for pirenzepine. Both types have the same affinity for atropine (Birdsall and Hulme, 1985).

The purpose of the present experiments was to describe the action of muscarinic agonists on cardiac ganglion neurons of the guinea-pig heart as well as to determine their ionic mechanism and the muscarinic receptor subtypes which mediate them.

Materials and Methods

Adult male guinea-pigs (200-400g) were lightly anesthetized with ether and stunned. The heart was rapidly removed from the chest and placed in Krebs solution of the following composition (mM): NaCl, 117; KCl, 4.7; CaCl_2 , 2.5; NaH_2PO_4 , 1.2; NaHCO_3 , 25; glucose 11; gassed with 5% CO_2 and 95% O_2 . The intramural ganglia were dissected from the atrium. Single ganglia embedded in the subendocardial connective tissue were pinned in a shallow tissue bath similar to that described by Nishi and North (1973). Neurons in the ganglia were visualized using Nomarski optics (Zeiss) at a total magnification of 500.

Intracellular recordings were made with glass microelectrode filled with 3M potassium chloride or 2M potassium acetate (DC tip resistance 30-60M Ω). Depolarizing and hyperpolarizing currents were injected through the recording electrodes by means of a bridge circuit (WPI, M701).

Different solutions were applied to the preparation by using a three-way tap so that the flow rate (3 ml/min) did not change. Low chloride solutions were made by substituting NaCl with Na-isethionate. Low sodium solutions were made by replacement of NaCl with Tris-(hydroxymethyl) aminomethane-Cl. When switching the perfusing solution, the new solution reached the bath with a delay of 30-50 s, due to the volume of the connecting tubing. Acetylcholine and other muscarinic agonists were applied by either superfusion or pressure ejection. γ -Aminobutyric acid (GABA) was applied by pressure ejection. For pressure ejection, muscarinic agonists and GABA were dissolved in the superfusion solution at concentrations of 100 μM -10mM. Pressure pipettes had tip diameters of 2-5 μM and were positioned within 50 μM of the impaled cell. Pressure pulses of 10-30 psi for 10-100 ms were typically used.

Selected cell bodies were injected ionophoretically with horseradish peroxidase (HRP) by rectangular depolarizing currents of 400 ms duration at 2 Hz for 20-30 min. HRP was dissolved in 0.05M Tris/HCl containing 0.2M KCl (pH=7.2). After standing in Krebs solution for 20 hrs, the preparation was fixed with glutaraldehyde/paraformaldehyde mixtures and then reacted for HRP by the diaminobenzidine (DAB) method (Brown and Fyffe, 1984).

Drugs used were: acetylcholine chloride (ACh), atropine sulphate, barium chloride (Wako), bethanechol chloride (Sigma), cobalt chloride, γ -aminobutyric acid (GABA) (Wako), hexamethonium chloride (Tokyo Kasei), ($\pm$)-muscarine chloride (Sigma), pirenzepine dihydrochloride monohydrate (Boehringer) and tetrodotoxin (TTX) (Sankyo).

Results are expressed as their mean $\pm$ S.E. of the mean for the number of observations stated. Lines were fitted to points by eye.

Results

General observations

Intracellular recordings were made from 58 cardiac ganglion neurons in the present study. Resting membrane potentials ranged from -50 - -70 mV, input resistance ranged from 20-50 M Ω , and the time constants ranged from 2-10 ms. These electrophysiological properties are similar to those described in previous experiments on frog parasympathetic (Dennis et al. 1971) and sympathetic (Nishi and Koketsu, 1960) neurons.

Intracellular injection of horseradish peroxidase (HRP) in six neurons revealed that the cardiac ganglion neurons are oblong and monopolar cells having their major axis ranging from 30 to 50 μM and their minor axis ranging from 20 to 40 μM . Most of the cell bodies gave rise to one axon which often had varicosities.

These observations are quite similar to those made in the frog (McMahan and Kuffler, 1971).

Muscarinic responses

Most of the neurones (44 of 52 neurones, 84.6%) were depolarized by the application of ACh (100 μ M–10 mM), muscarine (100 μ M–10 mM) and bethanechol (1–100 mM). Although the responses produced by the three muscarinic agonists were qualitatively similar, bethanechol was approximately ten times less potent than ACh or muscarine. Superfusion of ACh (3 mM for 30 s) caused an early, faster depolarization followed by a slower long-lasting depolar-

ization (Fig. 1A). The fast depolarization was selectively depressed by hexamethonium (200 μ M) (Fig. 1B), suggesting that this component of the response is due to the activation of nicotinic ACh receptors. The slower component of the ACh-evoked depolarization was associated with an initial increase followed by a decrease in membrane input resistance. This biphasic change in input resistance was clearly observed when the membrane potential was restored to control by passing inward current through the recording electrode (Fig. 1C). Bethanechol (1–100 mM) and muscarine (100 μ M–10 mM) produced depolarizations of slow time course and associated

Fig. 1. Depolarizations produced by superfusion of acetylcholine (ACh) (3 mM; horizontal bar) onto cardiac ganglion neurons. In *B* and *C*, the Krebs solution contained 200 μ M hexamethonium (C₆). Downward deflections are electrotonic potentials caused by current pulses of constant amplitude (0.6 nA in *A*, *B*; 0.35 nA in *C*). The electrotonic potentials shown in this and following figures appear biphasic due to the occurrence of a slow depolarizing response following the turning off of the current. *A*, application of acetylcholine produced an initial fast depolarization followed by a slow depolarization. Both were associated with a decreased membrane input resistance. *B*, in the presence of hexamethonium, the initial fast portion of the depolarization was depressed. The remaining slow depolarization was now associated with an initial increase, followed by a decrease, in membrane input resistance. *C*, the input resistance change was clearly seen when the membrane potential was shifted back to control (indicated by arrows) by passing inward current through the recording electrode.

with a sequence of increase-decrease in neuron input resistance, similar to the slow component of the ACh depolarizing response. This suggests that the latter is a muscarinic response. The amplitude and duration of the muscarinic depolarization, as well as the associated biphasic input resistance change were not altered by TTX ($0.5 \mu\text{M}$) and Co^{2+} (3 mM) containing Krebs solution, although the action potentials were abolished (see Fig. 3). This suggests that the effects of the muscarinic agonists are the result of a direct action of the drugs on the soma membrane from which the recordings were made. On the other hand, the membrane input resistance again increased on the decay phase

of the response in some neurons. This is probably due to the release of some other transmitters from neighboring neurons, since this very late increase in input resistance was abolished by TTX ($0.5 \mu\text{M}$) or Co^{2+} (3 mM) containing solutions (Fig. 3).

A few neurons (5 of 52 neurons, 9.6%) were hyperpolarized by the muscarinic agonists. The hyperpolarization was associated with a decrease in input resistance and was reversibly antagonized by atropine ($0.5\text{--}3 \mu\text{M}$). Because of the paucity of neurons showing this muscarinic hyperpolarization, a further analysis of the ionic mechanism and pharmacology of the response was not performed.

Fig. 2. Muscarinic depolarizations induced by superfusion of acetylcholine in the presence of hexamethonium ($200 \mu\text{M}$). *A*, the components of the slow response associated with the low- and high-conductance were both increased in amplitude when the concentration of acetylcholine was increased. The downward deflections are electrotonic potentials evoked by constant current pulses (0.45 nA). *B*, dose-response curve for muscarinic depolarization in the absence and in the presence of pirenzepine ($3 \mu\text{M}$). The peak amplitude of the low-conductance component (closed circles) and the high-conductance component (open circles) of the depolarization was plotted against the agonist concentration. The dose-response curve was shifted to the right by pirenzepine with an antagonist K_D of 890 nM for the low-conductance component and of 750 nM for the high-conductance component at a response level of 10 mV .

Fig. 3. Effects of changes in the composition of the Krebs solution on the muscarinic depolarization. Acetylcholine (10 mM) was applied by superfusion in the presence of hexamethonium (200 μ M). The downward deflections are electrotonic potentials produced by constant current pulses (0.3 nA). *A*, addition of Ba^{2+} depolarized the membrane and increased the membrane input resistance. The muscarinic depolarization was induced after the membrane potential was shifted back to control by passing constant inward current. The initial low-conductance depolarization was depressed but the late, high-conductance, component of the depolarization was not affected. *B*, when the external Na^+ concentration was reduced to 14 mM, spike initiation was suppressed but the muscarinic depolarization was not significantly changed. *C*, in the presence of Co^{2+} (3 mM) and TTX (0.5 μ M), spikes were suppressed, but the muscarinic depolarization was not altered. Note that the increase in membrane input resistance in the decay phase of the response is abolished by Co^{2+} .

Muscarinic receptor subtypes

The amplitude of the muscarinic depolarization was dependent on the concentration of ACh applied (Fig. 2A). Atropine (1–10 μ M) or pirenzepine (1–3 μ M) reversibly antagonized the muscarinic depolarization. The agonist dose-response curve was shifted to the right by the presence of pirenzepine (3 μ M) (Fig. 2B). The dissociation constant (K_D) of pirenzepine for the muscarinic depolarization was estimated to be 200–900 nM ($n=3$).

Ionic mechanism

When the muscarinic depolarization was induced in Ba^{2+} (2 mM)-containing solution ($n=5$), the initial component was slightly depressed but the slower component of the depolarization was not affected (Fig. 3A).

Addition of Ba^{2+} also depolarized the membrane by about 5–10 mV and increased the input resistance. Lowering Na^+ (14 mM) concentration did not alter the muscarinic depolarization except for preventing action potential initiation ($n=5$) (Fig. 3B). Co^{2+} (3 mM) and TTX (0.5 μ M)-containing solution also did not change the muscarinic depolarization except for the abolition of the action potentials ($n=5$) (Fig. 3C).

These results, taken together with the evidence that the depolarizations evoked by muscarinic agonists were associated with an initial increase, followed by a decrease, in input resistance, suggest that the muscarinic depolarization may be related to changes in potassium and chloride conductance rather than sodium or calcium conductance. We have further in-

Fig. 4. *A*, muscarinic depolarization produced by superfusion of acetylcholine (3 mM) in the presence of hexamethonium (200 μ M) at various levels of membrane potential. At resting membrane potential (–57 mV), the muscarinic response is composed of an initial low-conductance component followed by a high-conductance component. The amplitude of the former was reduced and that of the latter increased when the membrane was hyperpolarized by passing steady inward current through the recording electrode. *B*, effect of membrane potential on responses to GABA. GABA was applied by pressure (20 psi, 150 ms), at the time indicated by closed triangles, from a pipette containing GABA (1 mM). The recordings were made from the same neurone and at the same membrane potential level as in *A* by using KCl-filled electrode. The downward deflections in *A* and *B* are electrotonic potentials evoked by constant current pulses (0.3 nA).

vestigated this possibility in two ways. First, by using KCl filled recording electrodes, we have compared the effect of changes in membrane potential on the two components of the muscarinic depolarization with that on the GABA-induced depolarization which is known to be mediated by an increased chloride conductance in both central (Andersen et al. 1980; Kelly et al. 1969) and peripheral (Cherubini and North, 1984; Gallagher et al. 1978) neurons. Then, the same series of experiments were performed by using K-acetate filled electrodes. Fig. 4 shows the recording by KCl filled electrodes. The amplitude of the low-conductance component of the depolarization was reduced

by membrane hyperpolarization and nullified at about -90 mV, whereas, the high-conductance component of the depolarization was augmented in amplitude by membrane hyperpolarization (Fig. 4A). Pressure application of GABA induced a depolarization accompanied by a decreased membrane input resistance in the same neuron (Fig. 4B). The GABA-induced depolarization was increased in amplitude by membrane hyperpolarization as was the high-conductance component of the muscarinic depolarization. When recorded with K-acetate filled electrodes (Fig. 5), the muscarinic depolarization was associated almost exclusively with an increase in input resistance. The late, low-membrane

Fig. 5. *A*, muscarinic depolarization induced by superfusion of acetylcholine (3 mM) in the presence of hexamethonium (200 μ M) recorded with a K-acetate filled electrode. Hyperpolarization influences the two components of the muscarinic depolarization differently. The early, low-conductance component decreases in amplitude with hyperpolarization and reverses at -95 mV. The late, high-conductance component is hardly detectable at resting potential and increases in amplitude with hyperpolarization. Thus, at -95 mV the muscarinic response has become a hyperpolarization-depolarization sequence. *B*, GABA-induced depolarization. GABA was applied by pressure ejection (1 mM, 20 Psi, 150 ms; indicated by closed triangles). Note that the GABA-depolarization became much smaller than those shown in Fig. 4. The recordings were made from the same neuron and at the same membrane level as in *A*. The downward deflections are electrotonic potentials recorded by passing current pulses of constant amplitude (0.3 nA).

resistance component of the muscarinic depolarization, which was clearly seen when the recordings were made with KCl filled electrodes, became difficult to detect at the resting membrane potential but increased in amplitude with hyperpolarization (Fig. 5A). When the membrane was hyperpolarized, the early, high-resistance component of the depolarization was reduced in amplitude and reversed polarity at about -90 mV. At -95 mV, the muscarinic response was characterized by a hyperpolarization, associated with low-conductance, followed by a depolarization, associated with high-conductance. GABA produced a very small depolarization at resting potential. The depolarization increased in amplitude with membrane hyperpolarization, but at every membrane potential level the GABA response was much smaller than when recorded with KCl-electrodes (Fig. 5B).

Lowered Cl^- solution (12.5 mM) did not influence the high-conductance phase of the muscarinic depolarization ($n=5$). The GABA-induced depolarization was transiently augmented within 1-2 minutes after switching to the low Cl^- solution, but returned to control as superfusion with the low Cl^- solution continued. The long duration of the muscarinic response compared with the GABA response only permitted testing at times when the GABA response was back to control. This lack of effect of low Cl^- solution may be explained by previous observations by Gallagher et al. (1978) that the foreign anions used as Cl^- substitutes may impair the Cl^- channels.

Discussion

Muscarinic responses

The present results indicate that muscarinic agonists produce membrane depolarization in a majority of cardiac ganglion neurons both by reducing membrane potassium conductance and by increasing

membrane chloride conductance. The following evidence supports this conclusion. The muscarinic depolarization had two components, an early component accompanied by an increase in membrane input resistance followed by a later component associated with a decreased input resistance. The first component was reduced in amplitude by hyperpolarization and reversed its polarity at a membrane potential close to the potassium equilibrium potential. This component was partially blocked by Ba^{2+} , but was not affected by low Na^+ solution or by Co^{2+} . The later component increased in amplitude with membrane hyperpolarization and was smaller when recorded with a K-acetate electrode than when recorded with a KCl electrode. It was not affected by Ba^{2+} or Co^{2+} or by low Na^+ solution. These characteristics of the later depolarization agree well with those of the GABA-induced depolarization.

Muscarinic depolarization due to inactivation of a potassium conductance has been reported from many other central (Dodd et al. 1981; Krnjević et al. 1971) or peripheral (Adams et al. 1982; Brown and Selyanko, 1985; Kobayashi and Libet, 1970) neurons. Previous investigations on sympathetic neurons (Adams et al. 1982; Hashiguchi et al. 1982) have shown that the mechanism is inhibition of a voltage-dependent K^+ current termed the M-current. However, the contribution of the M-current inhibition to the muscarinic depolarization seems to be a minor one in cardiac ganglion neurons because reversal of the response was found around the potassium equilibrium potential. The M-channel in sympathetic neurons is already shut at this membrane potential (Adams et al. 1982). In this respect, the muscarinic response in cardiac ganglion neurons is similar to that of myenteric neurons (Morita et al. 1982).

Muscarinic depolarization due to an activation of membrane conductance has been described in frog (Akasu et al. 1984;

Kuba and Koketsu, 1976) and rat (Brown and Selyanko, 1985) sympathetic neurons. An increased conductance to Na⁺ or Na⁺ and Ca²⁺ has been suggested in frog, whereas an increased conductance to Cl⁻ in rat. The fact that the muscarinic depolarization persists in low Na⁺ solution or in Co²⁺-containing solution in the present experiments might deny the former possibility. A Ca²⁺-activated K⁺-conductance underlying a slow post-spike after-hyperpolarization has recently been reported in cultured intracardiac neurons of the guinea-pig (Allen and Burnstock, 1987). Inactivation of this conductance also seems not to be account for the muscarinic depolarization because of the lack of effect of Co²⁺ and Ba²⁺.

Muscarinic receptor subtypes

We have demonstrated that the muscarinic receptors producing the muscarinic depolarization on the soma membrane of the cardiac ganglion neurons are of the M₂ type. This is suggested by the evidence that pirenzepine produced a parallel shift to the right of the dose-response curve for the muscarinic depolarization with K_D of 200-900 nM. It has been previously generalized that M₁ receptors are found in neuronal tissue and M₂ receptors in cardiac or smooth muscle (Bidsall and Hulme, 1985). This generalization can be no longer accepted, although M₁ receptor involvement in mediating muscarinic depolarization has been suggested in hippocampal (Brown et al. 1986) and sympathetic (Ashe and Yarosh, 1984) neurons. Several exception to the generalization have been demonstrated. It has been shown in the guinea-pig enteric nervous system (North et al. 1985), that presynaptic muscarinic receptors, activation of which inhibits the release of transmitters, are of the M₂ type, whereas postsynaptic muscarinic receptors, activation of which induces membrane depolarization, are of the M₁ type. In the central nervous system,

activation of M₂ receptors produces a membrane hyperpolarization both in neurons of nucleus parabrachialis (Egan and North, 1986) and of thalamic nucleus reticularis (McCormick and Prince, 1986). Moreover, M₂ receptor activation has been described to bring about a membrane depolarization in rat locus coeruleus neurons (Egan and North, 1985).

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