Muscarinic Receptor Activation Modulates Ca\(^{2+}\) Channels in Rat Intracardiac Neurons via a PTX- and Voltage-Sensitive Pathway

SEONG-WOO JEONG\(^1\) AND ROBERT D. WURSTER\(^2\)

\(^1\)Department of Physiology and \(^2\)Departments of Physiology and Neurological Surgery, Loyola Stritch School of Medicine, Maywood, Illinois 60153

**Muscarinic Receptor Activation Modulates Ca\(^{2+}\) Channels in Rat Intracardiac Neurons via a PTX- and Voltage-Sensitive Pathway.**

Jeong, Seong-Woo and Robert D. Wurster. Muscarinic receptor activation modulates Ca\(^{2+}\) channels in rat intracardiac neurons via a PTX- and voltage-sensitive pathway. J. Neurophysiol. 78: 1476–1490, 1997. With use of the whole cell patch-clamp technique, effects of the potent muscarinic agonist oxotremorine methiodide (oxo-M) on voltage-activated Ca\(^{2+}\) channel currents were investigated in acutely dissociated adult rat intracardiac neurons. In all tested neurons oxo-M reversibly inhibited the peak Ba\(^{2+}\) current. Inhibition of the peak Ba\(^{2+}\) current by oxo-M was associated with slowing of activation kinetics and was concentration dependent. The concentration of oxo-M necessary to produce a half-maximal inhibition of current and the maximal inhibition were 40.8 nM and 75.9%, respectively. Inhibitory effect of oxo-M was completely abolished by atropine. Among different muscarinic receptor antagonists, methoctramine (100 and 300 nM) significantly antagonized the current inhibition by oxo-M, with a negative logarithm of dissociation constant of 8.3 in adult rat intracardiac neurons. Internal dialysis of neurons with guanosine 5'-O-(thio)triphosphate (GTP\(\gamma\)S, 0.5 mM) could mimic the muscarinic inhibition of the peak Ba\(^{2+}\) current and significantly occlude inhibitory effects of oxo-M. In addition, the internal dialysis of guanosine 5'-O-(2-thiodiphosphate) (GDP\(\beta\)S, 2 mM) also significantly reduced the muscarinic inhibition of the peak Ba\(^{2+}\) current by oxo-M. Inhibitory effects of oxo-M were significantly abolished by pertussis toxin (PTX, 200 and 400 ng/ml) but not by cholera toxin (400 ng/ml). Furthermore, the bath application of N-ethylmaleimide (50 \(\mu\)M) significantly reduced the inhibition of the peak Ba\(^{2+}\) current by oxo-M. The oxo-M shifted the activation curve derived from measurements of tail currents toward more positive potentials. A strong conditioning prepulse to +100 mV significantly relieved the muscarinic inhibition of peak Ba\(^{2+}\) currents by oxo-M and the GTP\(\gamma\)S-induced current inhibition. In a series of experiments, changes in intracellular concentration of bis-(o-aminophenoxyl)-N,N,N',N'-tetraacetic acid and protein kinase activities failed to mimic or occlude the current inhibition by oxo-M. The dihydropyridine antagonist nifedipine (10 \(\mu\)M) was not able to occlude any of the inhibitory effects of oxo-M, and oxo-M (3 \(\mu\)M) failed to reduce the slow tail currents induced by the L-type agonist methyl 2,5-dimethyl-4-[2-(phenylmethoxy)benzoyl]-1H-pyrrrole-3-carboxylate (FPL 64176; 2 \(\mu\)M). However, \(\omega\)-conotoxin (\(\omega\)-CgTX) GVIA (1 \(\mu\)M) significantly occluded the muscarinic inhibition of the Ba\(^{2+}\) currents. In the presence of \(\omega\)-CgTX GVIA (1 \(\mu\)M) and nifedipine (10 \(\mu\)M), oxo-M could further inhibit ~20% of the total Ba\(^{2+}\) current. After complete removal of N-, Q-, and L-type currents with use of \(\omega\)-CgTX GVIA, \(\omega\)-agatoxin IVA, and nifedipine, 70% of the R-type current (~6–7% of the total current) was inhibited by oxo-M (3 \(\mu\)M). In conclusion, the \(\omega\)-muscarinic receptor activation selectively inhibits N-, Q-, and R-type Ca\(^{2+}\) channel currents, sparing L-type Ca\(^{2+}\) channel currents mainly via a PTX- and voltage-sensitive pathway in adult rat intracardiac neurons.

**INTRODUCTION**

Acetylcholine (ACh) is the principal transmitter at both preganglionic and postganglionic synapses in parasympathetic intracardiac ganglia. Activation of the postjunctional \(M_2\) muscarinic receptor in the myocardium exerts negative chronotropic, dromotropic, and inotropic responses (for review see Löffelholz and Pappano 1985). Postsynaptic muscarinic receptors also have important roles in the modulation of ganglionic neurotransmission in mudpuppy, guinea pig, rat, and dog (Allen and Burnstock 1990; Hartzell et al. 1977; Selyanko and Skok 1992; Xi-Moy et al. 1993). With the use of autoradiography techniques, muscarinic receptors have been localized in intracardiac neurons and cardiac myocytes of rat and guinea pig (Hancock et al. 1987; Hassall et al. 1987). However, these radioligand labeling studies did not distinguish specific receptor subtypes present on intracardiac neurons. More recently, the presence of multiple muscarinic receptor subtypes (\(m_1\), \(m_2\), \(m_3\), and \(m_4\)) has been identified with the use of in situ hybridization histochemistry in both rat and neonatal guinea pig (Hassall et al. 1993). Furthermore, Hoover et al. (1994) have found that adult rat intracardiac neurons predominantly express \(m_2\) mRNAs (>4 times as many as are found in atrial myocytes).

Muscarinic receptor activation has been shown to inhibit voltage-activated Ca\(^{2+}\) currents in many types of neurons from both the central and peripheral nervous systems (Allen and Brown 1993; Beech et al. 1991, 1992; Cuevas and Adams 1995; Gähwiler and Brown 1987; Howe and Surmeier 1995; Mochida and Kobayashi 1988; Plummer et al. 1991; Song et al. 1991; Toselli and Lux 1989; Tse et al. 1990; Wanke et al. 1987, 1994; Yan and Surmeier 1996). In general, \(M_2/M_4\) muscarinic receptors were identified as responsible for the inhibition of Ca\(^{2+}\) currents. For example, in neuroblastoma-glioma hybrid (NG108–15) cells transfected with muscarinic receptors, both \(m_2\) and \(m_4\) subtypes are capable of inhibiting Ca\(^{2+}\) currents, but neither \(m_1\) nor \(m_3\) is coupled to Ca\(^{2+}\) channels (Higashida et al. 1990).

Recently Ca\(^{2+}\) channels have been pharmacologically dissected into four different subtypes, i.e., N, Q, L, and R, in adult rat intracardiac neurons (Jeong and Wurster 1997). However, little information is available on the regulation of multiple Ca\(^{2+}\) channels by transmitters in adult rat intracar-
and R-type Ca\textsuperscript{2+} channels were modulated by the M1 muscarinic receptor, which is much less abundant than the M2 in adult rat intracardiac neurons. In addition, the muscarinic agonist inhibited all high-voltage-activated Ca\textsuperscript{2+} channels: L, N, and non-L, and non-N types in neonatal intracardiac neurons.

In the present studies the muscarinic modulation of Ca\textsuperscript{2+} channel currents was investigated in detail in acutely dissociated adult rat intracardiac neurons. Our results suggest that M1 muscarinic receptor activation selectively inhibits N-, Q-, and R-type Ca\textsuperscript{2+} channel currents, sparing L-type Ca\textsuperscript{2+} channel currents mainly via a pertussis toxin (PTX)- and voltage-sensitive pathway in adult rat intracardiac neurons. Some preliminary data have been reported in abstract form (Wurster and Jeong 1996).

METHODS

Dissociation of intracardiac neurons

Intracardiac neurons were enzymatically dissociated as described previously (Jeong and Wurster 1997). Briefly, adult male Sprague-Dawley rats (250–300 g) were anesthetized with pentobarbital sodium (40 mg/kg IP). Both atria were rapidly removed and placed in cold Ca\textsuperscript{2+}- and Mg\textsuperscript{2+}-free Hanks’ balanced salt solution (pH 7.3) supplemented with 2.6 g/l glucose and 10 mM N,2-hydroxyethylpiperazine-N’,N’-2-ethanesulfonic acid (HEPES). After dissection, intracardiac ganglia were cut into small pieces and then incubated in 5 ml oxygenated Hanks’ balanced salt solution containing 1.2 mg/ml collagenase type A, 0.4 mg/ml trypsin, and 1 mg/ml DNAase type I at 35°C for 50 min in a shaking water bath. After incubation, these ganglia were gently triturated with a fire-polished Pasteur pipette. The resulting dissociated neurons were then washed two times and resuspended in minimum essential medium supplemented with 2.6 g/l glucose, 0.11 g/l pyruvic acid, 10% fetal bovine serum, 50 IU/ml penicillin, and 50 μg/ml streptomycin. The cells were plated on poly-l-lysine-covered coverslips (9 × 22 mm) and stored at 37°C in a humidified atmosphere of 95% air-5% CO\textsubscript{2}. All cells were used within 3–8 h after plating, except in experiments in which cells were treated with 200–400 ng/ml PTX or cholera toxin (CTX) for 1 day.

Electrophysiology

A piece of coverslip containing dissociated intracardiac neurons was placed in a recording chamber (300 μl in volume) on an inverted phase-contrast microscope (Nikon, Tokyo, Japan) and superfused at a flow rate of 2 ml/min with the external solution described below. The Ca\textsuperscript{2+} channel currents were recorded in the whole cell configuration of the conventional patch-clamp technique (Hamill et al. 1981) with the use of an Axopatch 1D patch-clamp amplifier (Axon Instruments, Foster City, CA). Patch electrodes were fabricated from thick-walled borosilicate glass capillary (1.5 mm OD, Sutter Instruments, Novato, CA) and fire-polished to final resistances of 1.5–2 MΩ when filled with the internal solution as described below. Series resistance (80–90%) was normally compensated electronically with use of a circuit for serial resistance compensation in the amplifier because of the relatively large capacitance and total current amplitude. The bath was ground with an Ag/AgCl pellet connected via a 3 M KCl/agar bridge. All experiments were performed at room temperature (22–23°C). Voltage protocol generation and data acquisition were performed with the use of pClamp software (version 6.0.2, Axon Instruments, Foster City, CA) and a data acquisition system (Digidata 486DX, PC) for later analysis. For tail current measurement, currents were filtered at 10 kHz and digitized at 10 μs per point. Leakage and capacitive currents were removed by subtracting CdI\textsubscript{2+} (0.1 mM)-insensitive currents from control currents.

Data analysis

The method of Arunlakshane and Schild (1959) was used to determine the affinity of the M1 muscarinic receptor antagonist methoctramine. Noncumulative concentration and response relationships for the inhibition of peak Ba\textsuperscript{2+} currents by oxotremorine methiodide (oxo-M, 3–30 μM) were obtained in the absence or presence of various concentrations of methoctramine (30, 100, and 300 nM). All data points were normalized to the maximal inhibition of current by oxo-M for each concentration of methoctramine and then were fitted with the Hill equation with the use of GraphPad Prism (GraphPad Software, San Diego, CA). With the use of the concentration of oxo-M required to produce a half-maximal inhibition of current (IC\textsubscript{50}) of each curve, the ratio of the IC\textsubscript{50} in the absence or presence of the antagonist was calculated as a concentration ratio. Concentration ratios were plotted on a Scid plot and were fitted with an unconstrained linear regression line. Intercept of the line estimated a value for the negative logarithm of the dissociation constant (pK\textsubscript{d}).

Percent inhibition of currents was quantified with the use of the equation 100 × (1 − I\textsubscript{test}/I\textsubscript{control}). Concentration-response and tail activation data points were well described by least-squares nonlinear regression assuming sigmoidal concentration-response and two-component Boltzmann distribution, respectively, with the use of GraphPad Prism. Averaged data were presented as means ± SE. Statistical significance was determined by an unpaired Student’s t-test. P < 0.05 was considered significant.

Solutions and drugs

The bath solution contained (in mM) 140 NaCl, 5 BaCl\textsubscript{2}, 10 HEPES, 1 MgCl\textsubscript{2}, 10 glucose, and 0.001–0.003 tetrodotoxin, pH adjusted to 7.4 with NaOH, osmolarity 310 mosmol/kg H\textsubscript{2}O. Recording pipettes contained (in mM) 120 CsCl, 40 HEPES, 11 ethylene glycol-bis-(β-aminoethyl) ether)-N,N,N’,N’-tetraacetic acid (EGTA), 1 CaCl\textsubscript{2}, 4 Mg-ATP, 0.3 tris(hydroxymethyl)aminomethane–guanosine 5’-triphosphate (GTP), 14 creatine phosphate, and 0.1 leupeptin, pH adjusted to 7.3 with CsOH, osmolarity 305 mosM/kg H\textsubscript{2}O. In experiments designed to examine the involvement of intracellular Ca\textsuperscript{2+} in the muscarinic modulation, Ba\textsuperscript{2+} and EGTA were replaced with Ca\textsuperscript{2+} and bis-(o-aminophenoxy)-N,N,N’,N’-tetraacetic acid (BAPTA, 0.1 or 20 mM),
respectively. In experiments designed to examine the involvement of G proteins, 0.3 mM GTP was replaced with 0.5 mM guanosine 5'-O-(thio)triphosphate (GTPγS) or 2 mM guanosine-5'-O-(2-thiodiphosphate) (GDPβS).

Drugs were applied to bath via gravity-fed polyethylene tubes connected to a multiway valve (Hamilton, Reno, NA). Drugs used in experiments were obtained as follows: BAPTA from Molecular Probes (Eugene, OR); oxo-M, pirenzepine, methoctramine, hexahydro-sila-dienedifendil hydrochloride, p-fluoro analog (p-FHHSiD), PTX, CTX, methyl 2,5-dimethyl-4-[2-phenylmethyl]-benzoyl]-1H-pyrole-3-carboxylate (FPL 64176), 8-bromo-adenosine 3',5'-cyclic monophosphate (cAMP), and calphostin C from Research Biochemicals International (Natick, MA); N-ethylmaleimide (NEC), trypsin, and DNAse from Sigma (St Louis, MO); collagenase from Boehringer Mannheim (Indianapolis, IN); forskolin, GTPγS, and GDPβS from Calbiochem (La Jolla, CA); nifedipine from ICN Biochemicals (Cleveland, OH); ω-conotoxin (ω-CgTX) GVIA from American Peptide Company (Sunnyvale, CA); and ω-agatoxin (ω-Aga) IVA from Dr. Nicholas A. Sacco-mano at Pfizer (Groton, CT). These drugs were dissolved in distilled water (except for p-FHHSiD, nifedipine, and FPL 64176, which were dissolved in 95% ethanol, and forskolin, which was dissolved in dimethyl sulfoxide). Final concentrations of ethanol and dimethyl sulfoxide were below 0.05 and 0.01%, respectively; these concentrations did not affect Ba2+ currents (n = 5, respectively). For experiments in which ω-Aga IVA was used, cytochrome C (1 mg/ml) was added to the external solution to block nonspecific peptide binding sites (Mintz et al. 1992). Cytochrome C itself also had no significant effect on Ba2+ currents (n = 5).

RESULTS

Effect of oxotremorine on the peak Ba2+ current

As reported previously (Jeong and Wurster 1997), adult rat intracardiac neurons express only high-voltage-activated Ca2+ channels. With the use of 5 mM Ba2+ as a charge carrier, inward Ca2+ channel currents first activated near −30 mV and reached a peak at ~0 mV. Thus, in the present study the effect of a potent muscarinic agonist, oxo-M, was tested on the peak Ba2+ current that was evoked every 10 s by a test pulse to 0 mV from a holding potential of −80 mV. In all tested neurons (n = 285), bath application of oxo-M resulted in a rapid and fully reversible inhibition of Ba2+ currents (Fig. 1A). Inhibition of Ba2+ currents was associated with a significant slowing of the activation kinetics (Fig. 1B, see also Fig. 4A). Thus amplitudes of the peak Ba2+ currents in the presence of oxo-M were measured isochronically with reference to the peak of the control current (Fig. 1B). In addition, the inhibition of the peak Ba2+ current was not attenuated when intracardiac neurons were repeatedly exposed to oxo-M (data not shown).

Concentration-dependent inhibition of Ba2+ currents by oxotremorine

Figure 1C illustrates a noncumulative concentration-response relationship for inhibition of Ba2+ currents by oxo-M ranging from 3 nM to 10 μM. Threshold for the effect of oxo-M occurred at ~3 nM. Maximal inhibition and IC50 for averaged data (3 ≤ n ≤ 7) were 75.9% and 40.8 nM, respectively. These data were well fitted by an equation where \( \text{inhibition of } I_{Na} = 75.9(1 + IC_{50}/[\text{oxo-M}])^{-1} \)

where \([\text{oxo-M}]\) is the concentration of oxo-M and IC50 was 40.8 nM. In all subsequent experiments oxo-M was used at a maximal concentration of 3 μM.

Identification of muscarinic receptor subtype involved in the inhibition of Ba2+ currents by oxotremorine

Atropine (50 μM), a nonselective muscarinic antagonist, completely abolished the inhibitory effect of 3 μM oxo-M on the peak Ba2+ currents (n = 6, data not shown). These data suggest that oxo-M inhibits Ba2+ currents via the muscarinic receptor activation in intracardiac neurons. A recent study on muscarinic receptor activation mediated the modulation of Ca2+ currents in cultured intracardiac neurons from neonatal rats. Thus, in the present study, we characterized the pharmacological subtypes of muscarinic receptors underlying the inhibition of Ba2+ currents by oxo-M with the use of three selective antagonists: pirenzepine, methoctramine, and p-FHHSiD. The effects of these antagonists are summarized in Fig. 2A. Pirenzepine (100 and 300 nM), which can distinguish M1/M3 from M2 and M3 receptors (Bernheim et al. 1992; Caufield and Brown 1991), exerted no significant effects on the inhibition of the peak Ba2+ current by a submaximal concentration (1 μM) of oxo-M (n = 7 for each concentration, P > 0.05). The M1-selective antagonist p-FHHSiD also failed to antagonize the inhibitory effect of oxo-M on the peak Ba2+ current (n = 5 for each concentration, P > 0.05). However, methoctramine, which shows a high selectivity for M2 receptors (Lazareno and Roberts 1989), significantly antagonized the ability of oxo-M to inhibit Ba2+ currents (n = 6 for each concentration, P < 0.05). For example, 3 μM oxo-M only inhibited 27.3 ± 2.8% (means ± SE) and 12.2 ± 1.0% of the total Ba2+ currents in the presence of 100 nM and 300 nM methoctramine, respectively, in comparison with the control inhibition of 74.6 ± 0.9%. Affinity of methoctramine to muscarinic receptors was also determined with the use of a Schild plot analysis. For this analysis, mean concentration-response curves were constructed in the absence or presence of different concentrations (30–300 nM) of methoctramine (4 ≤ n ≤ 8, Fig. 2B). A Schild plot of IC50 values for muscarinic inhibition at different concentrations yielded a pKb of 8.3 with a slope of 1.06 (Fig. 2C). The pKb value for methoctramine is consistent with those (7.8–8.4) acquired from ligand binding assays and functional studies for M2 receptors (Dörje et al. 1991; Hulme et al. 1990; Lazareno and Roberts 1989). Taken together, these data suggest that M2 receptors are responsible for the inhibition of Ba2+ currents by oxo-M in adult rat intracardiac neurons.

Voltage dependence of muscarinic inhibition of Ba2+ currents

To examine the voltage dependence of the effects of oxo-M on Ba2+ currents, cells were held at −80 mV and 50-ms test pulses were delivered every 2 s over the range of −40 to +60 mV. Figure 3A illustrates superimposed Ba2+ current
traces evoked by different test pulses in the absence or presence of oxo-M. Ba\(^{2+}\) current amplitudes measured at the peak of the control currents were plotted as a function of the test potential (Fig. 3B). Both control and modulated currents activated and reversed at around -30 and +60 mV, respectively. Muscarinic inhibition resulted in a shift in the voltage dependence of the Ba\(^{2+}\) currents to more positive potentials (Fig. 3B, n = 6), which is consistent with recent observations in other neuronal cell types such as rat embryonic hippocampal neurons (Toselli and Taglietti 1995).

Maximal inhibition (73%) of Ba\(^{2+}\) currents occurred at 0 mV. Slowing in activation kinetics is also most apparent at 0 mV (Fig. 3A). Muscarinic inhibition was decreased as the test potential became more negative or positive than 0 mV. For example, Ba\(^{2+}\) currents evoked with test pulses to -30 and +40 mV were inhibited in response to oxo-M by 10.2 ± 4.2% and 16.7 ± 5%, respectively. This “bell-shaped” relationship between mean magnitudes of inhibition and different test potentials is summarized in Fig. 3C. Taken together, these results suggest a voltage-dependent inhibition of Ba\(^{2+}\) currents in adult rat intracardiac neurons.

Tail currents and activation curves in the absence or presence of oxotremorine

To investigate further voltage-dependent effects of oxo-M on Ca\(^{2+}\) channel currents, Ba\(^{2+}\) tail currents were recorded during repolarization to -40 mV following 10-ms test pulses to various potentials (between -60 and +120 mV, see Fig. 4A, inset). Tail current amplitudes were measured as the average of the current for 200 μs, beginning 400 μs after the termination of the test pulse (see Bean 1989; Ikeda 1992). Figure 4A illustrates an example of superimposed tail currents in the absence (left) or presence (right) of 3 μM oxo-M. In this example, oxo-M decreased 78.8% of the tail current that followed a test pulse to 0 mV, whereas it decreased only 25.9% of this current after a test pulse to +120 mV. Normalized amplitudes of tail currents to the control tail current amplitude after a test pulse to +120 mV are plotted as a function of test potentials (Fig. 4B). Figure 4C summarizes the oxo-M-induced changes in the amplitudes of tail currents at different test potentials. On average oxo-M inhibited 76.1 ± 4.6% and 17.1 ± 3.6% of Ba\(^{2+}\) tail currents at 0 and +120 mV, respectively (Fig. 4C, n = 4).

In the present study, analysis of tail currents in the absence or presence of oxo-M showed biphasic activation curves in the voltage ranges. In accordance to “willing-reluctant” channel model proposed by Bean (1989), these activation curves are best described by a two-component Boltzmann equation (Fig. 5B) (see also Ikeda 1992)

\[
I(V) = I_w \left[ 1 + \exp \left( \frac{V_{bw} - V}{k_w} \right) \right]^{-1} + I_r \left[ 1 + \exp \left( \frac{V_{br} - V}{k_r} \right) \right]^{-1}
\]

where \(I(V)\) is the tail current amplitude at the test potential \(V\) and \(I_w\), \(V_{bw}\), and \(k_w\) are fractional current amplitude, the half-activation voltage, and slope factors, respectively, for willing (w) and reluctant (r) components. Under control conditions, mean values for \(V_{bw}\), \(V_{br}\), \(k_w\), and \(k_r\) were -6.5 ± 1.8 mV, 57.0 ± 4.6 mV, 5.1 ± 0.5 mV, and 23.6 ± 1.9 mV, respectively (\(n = 4\)). In the presence of 3 μM oxo-M, mean values for \(V_{bw}\), \(V_{br}\), \(k_w\), and \(k_r\) were 2.6 ± 3.6 mV, 52.3 ± 2.7 mV, 4.7 ± 1.8 mV, and 23.6 ± 1.6 mV, respectively (\(n = 4\)). All these parameters were not significantly altered in the presence of the agonist (\(P > 0.05\)). However, oxo-M significantly reduced the mean fraction in the willing mode (\(I_w\)) from 62 ± 4% to 21 ± 3%, while significantly increasing that in the reluctant mode (\(I_r\)) from 38 ± 4% to 62 ± 5% (\(n = 4\), \(P < 0.05\)). These results suggest that oxo-M shifts the tail current activation curve toward more depolarized potentials by transiently converting willing channels to reluctant channels requiring stronger depolarization to activate.

Relief of muscarinic inhibition by strong conditioning prepulses

Voltage dependence of muscarinic inhibition was also demonstrated by employing a double-pulse protocol with
a strong depolarizing conditioning prepulse. This protocol consisted of a 20-ms first test pulse (TP₁) to 0 mV, a 100-ms conditioning prepulse to +100 mV followed by a 5-ms interpulse at −80 mV, and a 20-ms second test pulse (TP₂) to 0 mV (Fig. 5A). In all tested neurons (n = 8), the conditioning prepulse significantly attenuated the muscarinic inhibition of the peak Ba²⁺ currents (P < 0.001). Before a prepulse oxo-M inhibited 72.6 ± 1.6% of peak Ba²⁺ currents, whereas after the conditioning prepulse the inhibition was only 19.1 ± 2.4% (Fig. 5B). In addition to a relief from inhibition, the prepulse restored the fast current activation. The facilitation of current was also evaluated by calculating the ratio of TP₂ to TP₁ Ba²⁺ current amplitudes (called “facilitation ratio”) in the absence or presence of oxo-M (Fig. 5C). In the absence of an agonist, Ba²⁺ currents showed little facilitation by the conditioning prepulse. However, during application of oxo-M, the facilitation ratio significantly increased to 3.19 ± 0.23 from 1.05 ± 0.03 (n = 8, P < 0.001). Because of rapid reinhibition of Ca²⁺ channel currents, this facilitation was significantly reduced (P < 0.05) when the interval between the conditioning pulse and postpulse was increased (Ikeda 1991). For example, facilitation ratios were reduced to 1.99 ± 0.33 (n = 3) and 1.61 ± 0.31 (n = 4) with the use of 10- and 20-ms intervals before the second test pulse, respectively (n = 3).

**G proteins and muscarinic inhibition**

Muscarinic receptors belong to a subfamily of seven transmembrane-spanning proteins that couple to G proteins (Allen and Brown 1993; Caufield and Brown 1991; Wanke et al. 1994). Furthermore, the prepulse relief of inhibition or prepulse facilitation is a hallmark of G-protein-mediated inhibition in neurons (Bean 1989; Elmslie et al. 1990; Grassi and Lux 1989). To investigate the involvement of G proteins in muscarinic inhibition of Ba²⁺ currents in adult rat intracardiac neurons, a group of neurons was alternatively dialyzed with either

---

**FIG. 2.** Effects of different muscarinic antagonists on the inhibition of Ca²⁺ channel currents by oxotremorine. A: effects of the specific muscarinic antagonists (100 and 300 nM) pirenzepine (PIRZ), methoctramine (MTT), and p-FHHSiD on the inhibition of the peak Ba²⁺ current by oxo-M (1 μM). Data represent means ± SE. Number of cells tested is indicated in parentheses. B: rightward shifts of the oxo-M concentration-response curve in the presence of 30, 100, or 300 nM methoctramine, an M₂ muscarinic antagonist. Mean % inhibition at different concentrations of oxo-M was normalized to the maximal inhibition in the absence or presence of antagonist. In each case the curves were drawn according to the Hill equation. C: Schild plot for methoctramine antagonism. Concentration ratios (r) were calculated for 50% inhibition (IC₅₀) of the peak Ba²⁺ current. Concentration ratios for the muscarinic inhibition of peak Ba²⁺ currents were fitted with an unconstrained line to give an estimated value of negative logarithm of the dissociation constant (pKᵦ).
A nonhydrolyzable GDP-analogue, GDPβS, has been known to abolish the G-protein-mediated effects of transmitters by acting as a competitive inhibitor of GTP binding to the alpha subunits of G proteins (Holz et al. 1986; Ikeda 1991; Wanke et al. 1987). Figure 7A illustrates effects of internal application of GDPβS (2 mM) on muscarinic inhibition of peak Ba2+ currents. When GDPβS was dialyzed for 10 min, oxo-M-induced inhibition of the peak Ba2+ currents was significantly attenuated to 16.9 ± 4% compared with a control value of 75.1 ± 1.8% (n = 7, P < 0.001, Fig. 7B) measured in the same group of neurons dialyzed with 0.3 mM GTP. Furthermore, GDPβS removed the slowing of the current activation induced by oxo-M. Dialysis of GDPβS itself had no significant effect on amplitudes of peak Ba2+ currents in the absence of the agonist (n = 7; 3.9 ± 0.5 nA for control neurons vs. 4.2 ± 0.7 nA for GDPβS-dialyzed neurons, P > 0.05).

Involvement of PTX-sensitive G proteins in muscarinic inhibition

In many central and peripheral neurons expressing M1/M4 receptors, it has been reported that muscarinic modulation of Ca2+ currents was mediated by PTX-sensitive Gαi/o-class protein (Allen and Brown 1993; Hille 1994; Howe and Surmeier 1995; Wanke et al. 1987; Yan and Surmeier 1996). To identify the nature of the G protein coupling muscarinic receptors to Ca2+ channels, intracardiac neurons were incubated for 1 day in a medium containing 200–400 ng/ml of
ADP ribosylation of G\textsubscript{i/o} proteins. However, in contrast to that by PTX, the inhibition of peak Ba\textsuperscript{2+} currents from 73.1 ± 2.3% to 15.4 ± 1.9% (Fig. 8D, n = 9, P < 0.001). NEM itself had no significant effects on peak Ba\textsuperscript{2+} currents (see Fig. 8B, n = 9).

**Effect of a Ca\textsuperscript{2+} chelator, BAPTA, on the muscarinic inhibition of Ba\textsuperscript{2+} currents**

Recent studies have shown that the muscarinic inhibition of Ba\textsuperscript{2+} channel currents is partly sensitive to intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) in rat sympathetic superior cervical neurons (Beech et al. 1991; Mathie et al. 1992) and rat striatal neurons (Howe and Surmeier 1995). Thus we tested the effect of [Ca\textsuperscript{2+}], on the muscarinic inhibition of Ba\textsuperscript{2+} channel currents in adult rat intracardiac neurons. Under an experimental condition favoring high [Ca\textsuperscript{2+}], i.e., Ba\textsuperscript{2+} as a charge carrier and 0.1 mM BAPTA, oxo-M inhibited 74.2 ± 2.3% (n = 5) of currents (data not shown). Similarly, oxo-M was able to inhibit 75.1 ± 2.5% (n = 6) of currents under an experimental condition favoring a low [Ca\textsuperscript{2+}], i.e., Ba\textsuperscript{2+} as a charge carrier and 20 mM BAPTA). Taken together, these data strongly suggest that the Ca\textsuperscript{2+}-sensitive pathway is not involved in the muscarinic inhibition of Ba\textsuperscript{2+} channel currents in adult rat intracardiac neurons.

**Effect of cAMP, forskolin, and PKI (5–24) on the muscarinic inhibition of Ba\textsuperscript{2+} currents**

M1/M4 muscarinic receptors have been known to be coupled to the inhibition of adenylyl cyclase (Peralta et al.

---

**FIG. 6.** Effect of guanosine 5'-(thio)triphosphate (GTP\textsubscript{S}) on muscarinic inhibition of Ca\textsuperscript{2+} channel currents. A: superimposed peak Ba\textsuperscript{2+} current traces at the beginning (0 min) and after 10 min of internal dialysis with GTP\textsubscript{S} (0.5 mM). Oxo-M (3 \( \mu \)M) was applied after a steady-state inhibition of the peak Ba\textsuperscript{2+} current by GTP\textsubscript{S} was attained. B: comparison of % inhibition of peak Ba\textsuperscript{2+} currents in neurons dialyzed with guanosine 5'-(thio)triphosphate (GTP, 0.3 mM) and GTP\textsubscript{S} (0.5 mM). C: peak Ba\textsuperscript{2+} currents evoked 10 min after the dialysis of GTP\textsubscript{S} by the double-pulse protocol used in Fig. 6. D: summary of facilitation ratios acquired with the use of a double-pulse protocol. In both B and D, data represent means ± SE. Number of cells tested is indicated in parentheses.

PTX or 400 ng/ml of CTX. PTX has been known to uncouple G\textsubscript{16} proteins from receptors by ADP ribosylation (West et al. 1985). CTX also has been known to ADP ribosylate G\textsubscript{a}\textsubscript{i/o} class proteins. However, in contrast to that by PTX, the ADP ribosylation of G\textsubscript{a}\textsubscript{i/o}-class proteins by CTX inhibits the intrinsic GTPase activity of G\textsubscript{i} (Cassel and Selinger 1977). As summarized in Fig. 8A, PTX treatment significantly (P < 0.001) attenuated the inhibition of peak Ba\textsuperscript{2+} currents by oxo-M. In control neurons incubated in PTX-free medium for 1 day, the mean inhibition of peak Ba\textsuperscript{2+} currents by oxo-M was 76.8 ± 1.7% (n = 13). In neurons treated with 200 or 400 ng/ml PTX for 1 day, the mean inhibition of peak Ba\textsuperscript{2+} currents was significantly attenuated to 15.9 ± 3.3% (n = 7) and 12.1 ± 1.5% (n = 9), respectively. No significant difference in the magnitudes of inhibition was found between two different concentrations of PTX (P > 0.05).

In CTX-treated neurons, however, oxo-M was still able to inhibit the peak Ba\textsuperscript{2+} currents by 75.9 ± 0.8%, which is not different from the value obtained under control conditions (n = 8, P > 0.05). These results strongly suggest that the muscarinic inhibition in adult rat intracardiac neurons is not mediated by CTX-sensitive G\textsubscript{a} but by PTX-sensitive G\textsubscript{16} proteins.

Another test for the involvement of the PTX-sensitive G proteins employs NEM, a sulfhydryl alkylating agent. NEM has been shown to uncouple PTX-sensitive G proteins from receptors by alkylating alpha subunits (Shapiro et al. 1994). After bath application of NEM (50 \( \mu \)M) for 3 min, the inhibition of peak Ba\textsuperscript{2+} current by oxo-M was greatly reduced to 11.6% from 76% in control (Fig. 8B). In the presence of NEM, the peak Ba\textsuperscript{2+} current did not display slowing of the current activation by oxo-M (Fig. 8C). On average, NEM significantly reduced the inhibition of the peak Ba\textsuperscript{2+} currents from 73.1 ± 2.3% to 15.4 ± 1.9% (Fig. 8D, n = 9, P < 0.001). NEM itself had no significant effects on peak Ba\textsuperscript{2+} currents (see Fig. 8B, n = 9).

**FIG. 7.** Effect of guanosine-5'-O-(2-thiodiphosphate) (GDP\textsubscript{S}) on muscarinic inhibition of Ca\textsuperscript{2+} channel currents. A: superimposed peak Ba\textsuperscript{2+} current traces evoked in the absence and presence of oxo-M after 10 min of dialysis with GDP\textsubscript{S} (2 mM). B: summary of % inhibition of peak Ba\textsuperscript{2+} currents by oxo-M in neurons dialyzed with GTP (0.3 mM) and GDP\textsubscript{S} (2 mM). Data represent means ± SE. Number of cells tested is indicated in parentheses.
1988). To test whether muscarinic inhibition of peak \( \text{Ba}^{2+} \) currents was mediated by a decrease in cAMP level or cAMP-dependent protein kinase A (PKA) activity, 1 mM 8-bromo-cAMP, a membrane-permeable cAMP analogue, was applied to adult rat intracardiac neurons. Bath application of 8-bromo-cAMP did not elicit a significant effect either on basal \( \text{Ba}^{2+} \) currents or on the inhibition of currents induced by oxo-M (data not shown). On average, the oxo-M-induced inhibition of peak \( \text{Ba}^{2+} \) currents was 76.2 ± 2.1% and 74.4 ± 4.11% (n = 5) in the absence or presence of 1 mM 8-bromo-cAMP, respectively. Similarly, forskolin (10 \( \mu \text{M} \), n = 4), an adenylyl cyclase activator, also had no significant effect on the oxo-M-induced inhibition of peak \( \text{Ba}^{2+} \) currents. In addition, the internal dialysis of 0.2 mM cAMP-dependent protein kinase inhibitor (PKI) (5–24), a cAMP-dependent PKA inhibitor, had no effect on the oxo-M-induced inhibition of peak \( \text{Ba}^{2+} \) currents (n = 4). Taken together, these data argue against possible roles of cAMP and PKA activation in the muscarinic inhibition of \( \text{Ca}^{2+} \) channel currents in intracardiac neurons. Recently, activation of protein kinase C (PKC) has been shown to be involved in neurotransmitter-induced inhibition of \( \text{Ca}^{2+} \) channel currents in chick embryonic sensory neurons (Diversé-Pierluissi et al. 1995). However, the internal perfusion of a specific PKC inhibitor, calphostin C, did not attenuate the inhibition of peak \( \text{Ba}^{2+} \) currents by oxo-M in adult rat intracardiac neurons (76.3 ± 1.3% for control neurons vs. 77.6 ± 2.5% for calphostin-C-dialyzed neurons, n = 6).

**Modulation of \( \omega\)-CgTx GVIA-sensitive N-type currents by oxotremorine**

As established before (Jeong and Wurster 1997), adult rat intracardiac neurons express only high-voltage-activated \( \text{Ca}^{2+} \) channel currents that comprise at least four different subtypes, the L, N, Q, and R types. To investigate which subtype of \( \text{Ca}^{2+} \) channel currents is modulated by oxo-M, the abilities of specific channel blockers were tested to see whether they occluded the muscarinic inhibition of peak \( \text{Ba}^{2+} \) currents. Figure 9A illustrates effects of the dihydropyridine (DHP) antagonist nifedipine and \( \omega\)-CgTX GVIA on muscarinic modulation of peak \( \text{Ba}^{2+} \) currents. In the presence of 10 \( \mu \text{M} \) nifedipine, the inhibition of peak \( \text{Ba}^{2+} \) currents by oxo-M was still robust, suggesting that L-type \( \text{Ca}^{2+} \) channels might not be modulated by oxo-M. On average, oxo-M inhibited 79.4 ± 1.4% and 77.3 ± 0.8% of the total calcium current, respectively (n = 7, P > 0.05), in the absence or presence of nifedipine (Fig. 9B). Nifedipine itself inhibited the peak \( \text{Ba}^{2+} \) currents by 8.8 ± 0.6% (Fig. 9C). However, the bath application of 1 \( \mu \text{M} \) \( \omega\)-CgTX GVIA significantly blocked the muscarinic inhibition by oxo-M (Fig. 9A, n = 7, P < 0.01). On average, \( \omega\)-CgTX GVIA reduced the inhibition of peak \( \text{Ba}^{2+} \) currents by oxo-M to 18.8 ± 1.7%. These results suggest that N-type \( \text{Ca}^{2+} \) channels are dominant targets for muscarinic modulation in adult rat intracardiac neurons as in other types of neurons (For review see Hille 1994). It is noteworthy that the modulation of peak \( \text{Ba}^{2+} \) currents by oxo-M was not completely eliminated after block of L- and N-type \( \text{Ca}^{2+} \) channels (see Fig. 9B), suggesting that the Q- and R-type channels can be also inhibited by muscarinic receptor activation in intracardiac neurons. In seven cells, combined application of nifedipine (10 \( \mu \text{M} \)) and \( \omega\)-CgTX GVIA (1 \( \mu \text{M} \)) inhibited 76.0 ± 1.9% of peak \( \text{Ba}^{2+} \) currents, leaving a large component (~24%) of the current (Q and R type, Fig. 9C). In the previous findings (Jeong and Wurster 1997), the majority of nifedipine and \( \omega\)-CgTX GVIA-insensitive \( \text{Ca}^{2+} \) channels were of the Q type (19% of the total current) compared with the R type (~7.5% of the total current). Considering that 17.1 ± 1.2% (n = 5) of the total \( \text{Ba}^{2+} \) current was further inhibited...
from a holding potential of −80 mV and amplitudes of slow tail currents were isochronically measured 4 ms after the end of the test pulse (Fig. 10B). In this example oxo-M significantly reduced the peak Ba\(^{2+}\) current and fast tail current in the presence of FPL 64176 (Fig. 10, A and B). However, the amplitude of the slow tail current was not reduced, but rather slightly increased. On average, oxo-M inhibited 39.4 ± 4.6% and 0.6 ± 4.1% of fast and slow tail currents, respectively (Fig. 10C, n = 10). These results strongly suggest that the L-type channel is not modulated by oxo-M in adult rat intracardiac neurons.

Effect of oxotremorine on FPL 64176-sensitive Ba\(^{2+}\) tail currents

Recently, Yassin et al. (1996) showed that in GH\(_3\) cells the muscarinic agonist carbachol modulates neuronal class E Ca\(^{2+}\) channels (\(\alpha_{1E}\)), which have biophysical and pharmacological properties similar to those of R-type Ca\(^{2+}\) channels in cerebellar granule neurons (Zhang et al. 1993). To investigate whether oxo-M also modulates R-type Ca\(^{2+}\) channels in adult rat intracardiac neurons, N-, L-, and Q-type currents were first eliminated by the combined bath application of \(v\)-CgTX GVIA (1 μM), nifedipine (10 μM), and \(v\)-Aga IV A (1 μM). In five cells, nifedipine- and toxin-sensitive (N, L, and Q) and -insensitive (R) currents were 90.4 ± 0.6% and 9.7 ± 0.6% of total current, respectively (Fig. 9A).

Effect of oxotremorine on slow L-type tail currents induced by FPL 64176

As described above, the L-type channel blocker nifedipine had little effect on the muscarinic modulation. To confirm this finding, FPL 64176, an effective L-type channel activator (McKechnie et al. 1989), was utilized. In adult rat intracardiac neurons, FPL 64176 selectively enhanced currents through L-type Ca\(^{2+}\) channels, slowing the deactivation of tail currents and shifting the current-voltage curve to more negative potentials (Jeong and Wurster 1997). The slow component of the tail currents in the presence of FPL 64176 exclusively represents a pure L-type current (McKechnie et al. 1989). Thus the measurement of the slow tail current induced by FPL 64176 provides a useful tool to assess the muscarinic modulation of L-type channels. Figure 10A illustrates the representative effect of 3 μM oxo-M on amplitudes of both fast and slow Ba\(^{2+}\) tail currents in the presence of FPL 64176. The Ba\(^{2+}\) tail currents were obtained during repolarization to −50 mV after a 20-ms test pulse to 0 mV
M2 muscarinic receptors are involved in the inhibition of Ca2+ channel currents

In adult rat intracardiac neurons, the activation of muscarinic receptors inhibited voltage-activated Ca2+ channel currents in a rapid, reversible, and concentration-dependent manner, which is consistent with previous findings in many types of neurons from central and peripheral nervous systems and cell lines (Allen and Brown 1993; Bernheim et al. 1992; Caufield and Brown 1991; Cuevas and Adams 1995; Gäb-wiler and Brown 1987; Howe and Surmeier 1995; Mochida and Kobayashi 1988; Song et al. 1991; Toselli and Taglieti 1995; Tse et al. 1990; Wanke et al. 1994).

Several lines of evidence suggest that M2/M4 muscarinic receptors are responsible for the inhibition of Ca2+ channels. For example, in NG108-15 cells transfected with muscarinic receptors, both m2 and m4 subtypes are capable of inhibiting Ca2+ channel currents but neither m1 or m3 is coupled to Ca2+ channels (Higashida et al. 1990). In situ hybridization histochemistry has shown that adult rat intracardiac neurons express multiple muscarinic receptor subtypes (m1, m2, m3, and m4) (Hassall et al. 1993; Hoover et al. 1994). The present pharmacological studies suggest that the M2 muscarinic receptor underlies the inhibition of Ca2+ channel currents in adult rat intracardiac neurons, in agreement with the findings in rat basal forebrain neurons (Allen and Brown 1993) and rat neostriatal cholinergic interneurons (Yan and Surmeier 1996). In neonatal intracardiac neurons, however, the activation of the M2 muscarinic receptor mediates fast, PTX-sensitive inhibition of Ca2+ channel currents (Cuevas and Adams 1995). Previously we suggested that the expression of voltage-activated Ca2+ channel subtypes might possibly be age-related in rat intracardiac neurons (Jeong and Wurster 1997). Similarly, this age-dependent discrepancy in muscarinic receptor subtypes involved in the inhibition of Ca2+ channel currents in intracardiac neurons may reflect developmental changes in the expression of muscarinic receptor subtypes.

In rat sympathetic neurons and striatal neurons, the M2 muscarinic receptor has been also shown to play a role in the slow, PTX-insensitive and BAPTA-sensitive inhibition of L- and N-type Ca2+ channel currents (Bernheim et al. 1992; Howe and Surmeier 1995). In contrast, the M2 muscarinic receptor in adult rat intracardiac neurons apparently not involved in the inhibition of Ca2+ channel currents, because the potent M1 antagonist pirenzepine failed to antagonize the oxo-M-induced inhibition of Ca2+ channel currents. Instead, the M2 muscarinic receptor seems to be associated with K+ channels such as M currents in intracardiac neurons (Allen and Burnstock 1990; Xi-Moy et al. 1993), in agreement with studies on rat sympathetic neurons and NG108-15 cells (Bernheim et al. 1992; Fukuda et al. 1988).

Muscarinic inhibition of Ca2+ channel currents is predominantly mediated by PTX-sensitive G proteins via a voltage-dependent pathway

In most instances neurotransmitter receptors, including muscarinic receptors, are known to be coupled to voltage-activated Ca2+ channels via PTX-sensitive G proteins (for reviews see Dolphin 1995; Hille 1994). The PTX-sensitive G protein is known to inhibit Ca2+ channels via a membrane-delimited pathway (Hille 1994). In adult rat intracardiac neurons, likewise, G proteins (PTX sensitive) are involved in the muscarinic inhibition of Ca2+ channel currents on the basis of observations in several experiments. First, the internal dialysis of the nonhydrolyzable GTP analogue GTPyS could mimic two features of the muscarinic response: the slowing of activation kinetics and the inhibition of the Ca2+ channel current. Furthermore, the inhibitory action of oxo-M was significantly occluded by the action of GTPyS. Second, the internal dialysis of the nonhydrolyzable GDP analogue GDPβS significantly attenuated the muscarinic response. Third, 1-day incubation of neurons in a PTX-containing medium significantly attenuated the muscarinic response. Fourth, the application of NEM, an uncoupling agent of PTX-sensitive G proteins, significantly eliminated the muscarinic response, as occurred in rat sympathetic neurons and neostriatal cholinergic interneurons (Shapiro et al. 1994; Yan and Surmeier 1996). Some evidence has suggested that the coupling of transmitter receptors to Ca2+ channels is mediated by Goαs, the most abundant G protein in neurons.
In adult rat intracardiac neurons, however, the subunit of PTX-sensitive heterotrimeric G proteins with Ca\(^{2+}\) channels (for reviews see Hille 1994; Wickman and Clapham 1995). First, both oxo-M and GTP\(\gamma\)S induced a significant slowing of the activation kinetics. This slowed activation is caused by the time-dependent relief of inhibition during depolarizations that is more significant with the prolonged test pulse (Ikeda 1991; Kasai 1991). In addition, the slowing in the current activation was most apparent between 0 and +10 mV. Second, the muscarinic inhibition of Ca\(^{2+}\) channel currents occurred most effectively at less depolarized potentials (i.e., 0 mV). For example, oxo-M inhibited 76 and 17% of the Ba\(^{2+}\) tail currents at 0 and +120 mV, respectively. Third, as shown in other studies (Elmslie et al. 1990; Grassi and Lux 1989; Ikeda 1991; Zhu and Ikeda 1993), the strong depolarizing prepulse (e.g., +100 mV) not only restored the fast activation but also relieved the voltage-dependent inhibition of Ca\(^{2+}\) channel currents by oxo-M and GTP\(\gamma\)S. Unlike in dorsal root ganglion neurons (Dolphin and Scott 1987) and superior cervical ganglion neurons (Ikeda 1991), the prepulse relief or facilitation in the present experiments was only observed in the presence of agonist, suggesting little or no tonic inhibition by G proteins under control conditions in adult rat intracardiac neurons. This is supported by the finding that GDP\(\beta\)S alone did not enhance the amplitude of Ca\(^{2+}\) channel currents in adult rat intracardiac neurons. Fourth, the muscarinic receptor activation shifted the tail activation curve toward more depolarized potentials.

In many studies the willing-reluctant model proposed by Bean (1989) has been widely accepted to explain the slowed activation kinetics and the voltage-dependent inhibition of Ca\(^{2+}\) channel currents by many neuromodulators such as adenosine, leuteinizing hormone releasing hormone, muscarine, norepinephrine, prostaglandin, and vasoactive intestinal peptide (Beech et al. 1992; Elmslie et al. 1990; Ikeda 1992; Toselli and Taglietti 1995; Zhu and Ikeda 1993, 1994). According to this model, Ca\(^{2+}\) channels can exist in two interconverting conformational modes, i.e., willing and reluctant modes that are in equilibrium with each other. In the absence of agonists, the Ca\(^{2+}\) channels are dominantly in the willing mode, which allows channels to be easily opened at low membrane potentials. Activation of G proteins by agonists may convert a large fraction of these willing channels to reluctant channels that are slower and less likely to open. On the other hand, a strong depolarizing prepulse may quickly and temporarily promote shifting the reluctant channels to willing channels (Elmslie et al. 1990; Kasai 1992). In adult rat intracardiac neurons, the population of Ca\(^{2+}\) channels in the willing or reluctant mode could be predicted from biphasic tail activation curves. The present study shows that oxo-M decreased the fraction of the willing component from 62 to 21% and increased the reluctant fraction from 38 to 62%, with little effect on the \(V_c\) and \(k\) of either the willing or the reluctant component. In adult rat intracardiac neurons, thus, the voltage dependence of muscarinic inhibition seems to be conceptually well described by the willing-reluctant channel model.

### Muscarinic inhibition of Ca\(^{2+}\) channels is unlikely to be dependent on diffusible second messengers

In many instances a significant fraction of the transmitter-induced inhibition of Ca\(^{2+}\) channel currents has been found to be resistant to relief by strong conditioning prepulses, which implicates the presence of a voltage-independent pathway (Beech et al. 1992; Boland and Bean 1993; Elmslie et al. 1990; Grassi and Lux 1989; Luebke and Dunlap 1994; Zhu and Ikeda 1993). In sympathetic neurons the muscarinic inhibition is known to be mediated by two parallel pathways: 1) the fast, PTX-sensitive, BAPTA-insensitive and voltage-dependent pathway and 2) the slow, PTX-insensitive, BAPTA-sensitive and voltage-independent pathway (Beech et al. 1992). In the present study ~17–19% of the oxo-M-induced inhibition of Ca\(^{2+}\) channel currents remains, even during strong depolarizations (i.e., +120 mV) or after strong conditioning prepulses (i.e., +100 mV). Furthermore, oxo-M could still reduce a fraction (~12%) of total Ca\(^{2+}\) currents in PTX- or NEM-treated neurons. Similar to the situation in rat sympathetic neurons, these observations raise the possibility that the muscarinic inhibition of Ca\(^{2+}\) channel currents might be in part mediated via the slow, PTX-insensitive, BAPTA-sensitive and voltage-independent pathway in adult rat intracardiac neurons. Recently the voltage-independent modulation of Ca\(^{2+}\) channel currents has been also suggested in neonatal intracardiac neurons (Cuevas and Adams 1995). Thus we investigated whether the muscarinic response in adult rat intracardiac neurons is sensitive to intracellular BAPTA and to diffusible second messengers in cascades that are downstream to the muscarinic receptor–G protein coupling. Interestingly, the magnitude of the muscarinic inhibition of Ca\(^{2+}\) channel currents was not changed in experiments in which both high (20 mM) and low (0.1 mM) BAPTA was used.

As mentioned above, generally M\(_2\) muscarinic receptors are also known to inhibit adenylyl cyclase and to decrease cAMP production via PTX-sensitive G protein (Peralta et al. 1988). Several lines of evidence have indicated that voltage-activated Ca\(^{2+}\) channels can be regulated by cAMP-dependent phosphorylation. For example, in cardiac myocytes the L-type Ca\(^{2+}\) current is known to be modulated by the activation of \(\alpha\)-adrenergic or muscarinic receptors via mechanisms involving cAMP-dependent PKA (Fischmeister and Hatzell 1986). Furthermore, cAMP is capable of regulating P-type Ca\(^{2+}\) channels expressed in oocytes following the injection of cerebellar mRNA (Fournier et al. 1993). In contrast, the present study shows that the membrane-permeable cAMP analogue 8-bromo cAMP, forskolin, and the cAMP-dependent PKA inhibitor PKI (5–24) failed to alter basal Ca\(^{2+}\) channel currents or the muscarinic inhibition of Ca\(^{2+}\) channel currents. These results suggest that the M\(_2\) muscarinic-receptor-mediated inhibition of Ca\(^{2+}\) channel currents is independent of the cAMP-dependent pathway in adult rat intracardiac neurons, as occurs in many other neurons (Allen and Brown 1993; Mochida and Kobayashi 1988; Song et al.
The α-adrenergic and M2 muscarinic activation have been known to inhibit Ca2+ channel currents through a PKC-dependent pathway in chick dorsal root ganglion neurons and rabbit superior cervical ganglion neurons, respectively (Diversé-Pierluissi and Dunlap 1993; Mochida and Kobayashi 1988). However, in many cases the muscarinic inhibition does not require PKC activation (Bernheim et al. 1992; Toselli and Lux 1989; Tse et al. 1990; Wanke et al. 1987). Similarly, in the present study PKC inhibition by calphostin C failed to attenuate the muscarinic response. Furthermore, PKC activation by phorbol 12-myristate 13-acetate (PMA) was not able to mimic inhibitory effects of oxo-M on Ca2+ channels (unpublished observation). These results suggest that PKC activation is not necessary for the muscarinic inhibition of the Ca2+ channel currents in adult rat intracardiac neurons. Taken together, the present experimental data strongly suggest that the muscarinic inhibition of Ca2+ channel currents is not mediated by the slow, BAPTA- and diffusible-second-messenger-sensitive pathway. Instead, the present results are likely in agreement with findings in rat sympathetic neurons in which pancreatic polypeptide and substance P inhibit, in part, Ca2+ channel currents via a pathway that is insensitive to PTX, NEM, voltage, BAPTA, and second messengers (Shapiro et al. 1994; Wollmuth et al. 1995). However, the exact mechanism underlying this signaling pathway is still unclear.

N-, Q-, and R-type Ca2+ channel currents were inhibited by muscarinic receptor activation sparing L-type currents

According to pharmacological criteria, high-voltage-activated Ca2+ channel currents have been separated into at least four different subtypes [1] the ω-CgTX GVIA-sensitive N type, 2) the ω-CgTX MVIIC-sensitive Q type, 3) the DHP-sensitive L type, and 4) the DHP- and toxin-insensitive R type] in adult rat intracardiac neurons (Jeong and Wurster 1997). Consistent with studies in other autonomic neurons (Beech et al. 1991, 1992; Plummer et al. 1991; Wanke et al. 1987), the N-type Ca2+ channel was primarily affected by the muscarinic receptor activation in adult rat intracardiac neurons. Although the ω-CgTX GVIA-sensitive N type is the most abundant Ca2+ channel, the ω-CgTX GVIA-insensitive components, i.e., Q-, L-, and R-type currents, constitute a large fraction (~36%) of the total Ca2+ channel current in adult rat intracardiac neurons (Jeong and Wurster 1997). In the present study, thus, we also examined which ω-CgTX GVIA-insensitive channel subtypes could be additionally modulated by the muscarinic receptor activation. In adult rat intracardiac neurons, the L-type Ca2+ channel appears not to be a target of the muscarinic modulation because 1) the DHP antagonist nifedipine did not reduce the magnitude of inhibition caused by oxo-M and 2) the muscarinic receptor activation had no significant effects on prolonged slow tail currents induced by FPL 64176. This conclusion is in agreement with those pertaining to magnocellular cholinergic basal forebrain neurons (Allen and Brown 1993), dorsal root ganglion neurons (Wanke et al. 1994), and neostriatal cholinergic interneurons (Yan and Surmeier 1996) and is also in agreement with other studies in which the L-type Ca2+ channel is insensitive to other transmitters (Elmslie et al. 1992; Plummer et al. 1991; Zhu and Ikeda 1993).

Unlike the present study, however, several studies have shown that the muscarinic receptor is coupled to the DHP-sensitive L-type Ca2+ channel in different types of neurons (Beech et al. 1991, 1992; Howe and Surmeier 1995; Mathie et al. 1992; Toselli and Lux 1989; Wanke et al. 1987). For example, Mathie et al. (1992) and Howe and Surmeier (1995) have demonstrated that the L-type Ca2+ channel is modulated by the muscarinic receptor activation via a BAPTA-sensitive pathway in rat sympathetic neurons and striatal neurons, respectively. In these studies high intracellular concentrations of BAPTA (20 mM) render this pathway inactive, resulting in no inhibition of the L-type Ca2+ channel. As discussed above, however, we found no quantitative differences in the effects of oxo-M on Ca2+ channel currents between conditions favoring high [Ca2+]i, (Ca2+ as the charge carrier and 0.1 mM BAPTA) and low [Ca2+]i, (Ba2+ as the charge carrier and 20 mM BAPTA). These results support the conclusion that the L-type Ca2+ channel is not modulated by the muscarinic receptor activation in adult rat intracardiac neurons.

In the present study oxo-M further inhibited nifedipine- and ω-CgTX-insensitive Ca2+ currents. Although one assumes that the R type is completely blocked by oxo-M (but note that 70% of the R-type current, i.e., ~6–7% of the total current in these experiments, was inhibited by oxo-M), ~13% of the current inhibition is most likely due to the inhibition of the Q type. In addition to N- and Q-type Ca2+ channels, the R type was significantly inhibited by the muscarinic receptor activation in adult rat intracardiac neurons. Until now, information on the transmitter-induced inhibition of the R-type Ca2+ channel has been scarce. Recently, initial attempts have been made to understand the regulation of Ca2+ channels in two different cells expressing neuronal class E (α1E) channels whose biophysical and pharmacological characteristics are similar to those of the R-type Ca2+ channel in cerebellar granule neurons (Zhang et al. 1993). However, conclusions from these two studies of the α1E channel are in conflict with each other. For example, in human embryonic kidney cells 293 (HEK293) cells, activation of somatostatin or opioid receptors exerts no inhibitory effects on α1E channel currents (Toth et al. 1996). In contrast, the α1E channel current is modulated by the activation of muscarinic and somatostatin receptors in GH3 cells (Yassin et al. 1996). Thus actions of transmitters on endogenous R-type Ca2+ channels remain to be resolved in other types of neurons.

Functional implications

The existence of multiple subtypes of Ca2+ channels and their selective modulation implicate different physiological functions of distinct Ca2+ channel subtypes in rat intracardiac neurons (for review see McCleskey 1994; Wheeler et al. 1995). The functional diversity of Ca2+ channels has
been also supported by an asymmetric distribution of different subtypes of Ca\(^{2+}\) channels in the somata/dendrites and presynaptic terminals (Lipscombe et al. 1988; Seabrook and Adams 1989).

A great deal of evidence demonstrates that the Ca\(^{2+}\) influx via high-voltage-activated Ca\(^{2+}\) channels contributes to the generation of the afterhyperpolarization by activating Ca\(^{2+}\)-dependent K\(^+\) channels in intracardiac neurons (Allen and Burnstock 1987; Selyanko and Skok 1992; Xi et al. 1994; Xu and Adams 1992). In whole mount preparation of adult rat intracardiac ganglia, oxo-M increased the firing rate of action potentials by depressing the afterhyperpolarization that was also blocked by CdCl\(_2\) (unpublished observations).

Thus the muscarinic inhibition of Ca\(^{2+}\) channel currents likely influences neuronal excitability postsynaptically by attenuating the afterhyperpolarization (Allen and Burnstock 1987).

Abundant evidence supports the notion that the N- and/or P/Q-type Ca\(^{2+}\) channels generally play a significant role in transmitter release at many synapses (Dunlap et al. 1995; Hille 1994; Wheeler et al. 1995). However, the present studies on the muscarinic modulation of Ca\(^{2+}\) channels were conducted on the cell body. Thus we do not know whether or not the muscarinic inhibition of Ca\(^{2+}\) channels is relevant to the negative feedback regulation of ACh release from preganglionic nerve terminals in adult rat intracardiac ganglia. In neuromuscular synapse of atria, ACh release has been found to be regulated by the endogenous transmitter acting on presynaptic muscarinic receptors (Wetzel and Brown 1985). Recently the inhibition of Ca\(^{2+}\) channels has been shown to be involved in the autoregulation of ACh release by muscarinic receptor activation at the neuromuscular junction in guinea pig atria (Hong and Chang 1995). Thus subtypes of Ca\(^{2+}\) channels and their functional roles in the regulation of ACh release from preganglionic nerve terminals should be elucidated in adult rat intracardiac ganglia by testing sensitivities of synaptic transmission or ACh release to specific Ca\(^{2+}\) channel blockers.

In conclusion, M\(_2\) muscarinic receptor activation mediates the inhibition of high-voltage-activated Ca\(^{2+}\) channels by oxo-M in adult rat intracardiac neurons. The M\(_2\) muscarinic receptors are selectively coupled to N-, Q-, and R-type Ca\(^{2+}\) channels mainly via a PTX- and voltage-sensitive pathway.

This research was supported by National Heart, Lung, and Blood Institute grant HL-27595 and the DePauw Heart Research Fund. This research was supported by National Heart, Lung, and Blood Institute grant HL-27595 and the DePauw Heart Research Fund.

**REFERENCES**


Hancock, J. C., Hoover, D. B., and Hougland, M. D. Distribution of...
MUSCARINIC MODULATION OF CA\(^{2+}\) CHANNELS IN INTRACARDIAC NEURONS


Yan, Z. AND Surmeier, D. J. Muscarinic (m2/m4) receptors reduce N- and P-type Ca\(^{2+}\) currents in rat neonatal cholinergic interneurons through a...


