Calcium Channel Currents in Acutely Dissociated Intracardiac Neurons From Adult Rats

SEONG-WOO JEONG1 AND ROBERT D. WURSTER2
1Department of Physiology and 2Departments of Physiology and Neurological Surgery, Loyola Stritch School of Medicine, Maywood, Illinois 60153

Jeong, Seong-Woo and Robert D. Wurster. Calcium channel currents in acutely dissociated intracardiac neurons from adult rats. J. Neurophysiol. 77: 1769–1778, 1997. With the use of the whole cell patch-clamp technique, multiple subtypes of voltage-activated calcium channels, as indicated by measuring Ba2+ currents, were pharmacologically identified in acutely dissociated intracardiac neurons from adult rats. All tested neurons that were held at ~80 mV displayed only high-voltage-activated (HVA) Ca2+ channel currents that were completely blocked by 100 μM CdCl2. The current density of HVA Ca2+ currents was dependent on the external Ca2+ concentration. The Ba2+ (5 mM) currents were half-activated at ~16.3 mV with a slope of 5.6 mV per e-fold change. The steady-state inactivation was also voltage dependent with half-inactivation at ~33.7 mV and a slope of ~12.1 mV per e-fold change. The most effective L-type channel activator, FPL 64176 (2 μM), enhanced the Ba2+ current in a voltage-dependent manner. When cells were held at ~80 mV, the saturating concentration (10 μM) of nifedipine blocked ~11% of the control Ba2+ current. The major component of the Ca2+ channels was N type (63%), which was blocked by a saturating concentration (1 μM) of ω-conotoxin GVIA. Approximately 19% of the control Ba2+ current was sensitive to ω-conotoxin MVIIC (5 μM) but insensitive to low concentrations (30 and 100 nM) of ω-agatoxin IVA (ω-Aga IVA). In addition, a high concentration (1 μM) of ω-Aga IVA occluded the effect of ω-conotoxin MVIIC. Taken together, these results indicate that the ω-conotoxin MVIIC-sensitive current represents only the Q type of Ca2+ channels. The current that was insensitive to nifedipine and various toxins represents the R-type current (7%), which was sensitive to 100 μM NiCl2. In conclusion, the intracardiac neurons from adult rats express at least four different subtypes (L, N, Q, and R) of HVA Ca2+ channels. This information is essential for understanding the regulation of synaptic transmission and excitability of intracardiac neurons by different neurotransmitters and neural regulation of cardiac functions.

INTRODUCTION

Parasympathetic intracardiac ganglia play an important role in the regulation of various cardiac functions including heart rate, contractile force, and atrioventricular conduction. Numerous clusters of neurons within these ganglia receive synaptic input from preganglionic fibers that originate in the medulla oblongata. Axons of these intracardiac neurons, in turn, terminate among atrial and some ventricular muscle fibers and, in particular, within the sinoatrial and atrioventricular nodal regions. The synaptic transmission between preganglionic fibers and intracardiac neurons is primarily mediated by acetylcholine (Dennis et al. 1971; Lane et al. 1976; Roskoski et al. 1977; Xi-Moy et al. 1993). In addition, increasing evidence suggests that this cholinergic synaptic transmission is under the influence of a variety of putative adrenergic and peptidergic neurotransmitters (Hardwick et al. 1995; Jacobowitz 1967; Konopka et al. 1992; Moravec et al. 1990; Steele et al. 1994). Therefore it has been proposed that intracardiac ganglia can function as a “cardiac brain” through which diverse extrinsic and intrinsic neuronal inputs are integrated before being transmitted to the cardiac tissues, particularly to the sinoatrial node (Parsons et al. 1987; Randall and Wurster 1994). In mammalian parasympathetic ganglia, Ca2+ channels are important modulators of synaptic transmission as well as spike frequency (Allen and Burnstock 1987; Seabrook and Adams 1989). Abundant evidence demonstrates that multiple types of Ca2+ channels contribute to the total Ca2+ current and play various roles in different neuronal tissues (McCleskey 1994; Wheeler et al. 1995). Therefore information about the nature of Ca2+ currents in intracardiac neurons is fundamental and critical for the understanding of the modulation of synaptic transmission and excitability, and, eventually, for the parasympathetic regulation of cardiac functions.

Voltage-activated Ca2+ channels in intracardiac neurons have been primarily studied in adult amphibians, e.g., bullfrog (Clark et al. 1990) and mudpuppy (Merriam and Parsons 1995). Xu and Adams (1992) first described the electrophysiological properties of voltage-activated Ca2+ channels in mammalian intracardiac neurons. In this study intracardiac neurons from neonatal rats have been found to have three different types of channels (L, N, and non-L, non-N types). Both L- and N-type currents displayed considerable overlapping pharmacological profiles, i.e., a maximum concentration of nifedipine blocks >60% of total currents, as much as that by ω-conotoxin GVIA (ω-CgTX GVIA). Until now, no information has been available concerning voltage-activated Ca2+ channels in acutely dissociated intracardiac neurons from mammalian adults, whose electrophysiological properties may be not the same as those of fetal or neonatal cells (Hirst and McLachlan 1986; Scott 1982). In the present study we pharmacologically identified multiple subtypes of Ca2+ currents by the application of different kinds of toxins and drugs in acutely dissociated intracardiac neurons from adult rats.

METHODS

Isolation of intracardiac neurons

Adult male Sprague-Dawley rats (250–300 g) were anesthetized with pentobarbital sodium (40 mg/kg ip). The whole atria were
rapidly removed and placed in cold Ca\(^{2+}\) - and Mg\(^{2+}\) -free Hank’s balanced salt solution (HBSS) (pH 7.3). The HBSS was supplemented with 2.6 g/l glucose and 10 mM N-(2-hydroxyethyl) piperazine-N’-(2-ethanesulfonic acid) (HEPES). The intracardiac ganglia were dissected from fat pads on the dorsal surfaces of atria under a dissecting microscope (×40). The ganglia were cut into small pieces and then incubated in 5 ml oxygenated HBSS containing 1.2 mg/ml collagenase type A (Boehringer Mannheim, Indianapolis, IN), 0.4 mg/ml trypsin, and 1 mg/ml DNASE type I (both from Sigma Chemical, St Louis, MO) at 35°C for 50 min in a shaking water bath. After incubation, the tissues were gently triturated with a fire-polished Pasteur pipette. The dissociated neurones were 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-coated coverslips (9 × 22 mm) and stored at 37°C in a humidified atmosphere of 95% air-5% CO\(_2\). All cells were used within 3–8 h of plating to avoid poor space-clamp conditions, which may be induced due to elongated neurones, and to avoid possible phenotypic changes of Ca\(^{2+}\) currents in culture.

**Electrophysiology**

A piece of coverslip containing the dissociated intracardiac neurones was placed in a recording chamber (volume ~ 300 μl) on an inverted phase-contrast microscope (Nikon, Tokyo) and superfused through a gravity-fed polyethylene tube with a normal physiological solution containing (in mM) 140 NaCl, 3 KCl, 10 HEPES, 2 CaCl\(_2\), 1 MgCl\(_2\), and 10 glucose, pH adjusted to 7.4 with NaOH, at a flow rate of 2 ml/min. The Ca\(^{2+}\) 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 Instrument, Novato, CA) and fire-polished to final resistances of 1.5–2.5 MΩ when filled with the internal solution described below. After access to each cell was gained, the membrane capacitance and series resistance were measured by application of a 10-mV hyperpolarizing step from a holding potential of ~80 mV and calculated according to the following equation (Bénitah et al. 1993): 

\[
C_m = \tau_i / \Delta V_m(1 - I_f/I_h),
\]

where \(C_m\) is the membrane capacitance, \(\tau_i\) is the time constant of membrane capacitance, \(I_h\) is the maximum capacitive current value, \(\Delta V_m\) is the amplitude of voltage steps, and \(I_f\) is the amplitude of steady-state current. Series resistances were calculated as \(R_s = \Delta V_m/\Delta I_f\). Averaged data are presented as means ± SE. Statistical significance was determined by an unpaired Student’s t-test. P < 0.05 was considered significant.

**Results**

Identification of intracardiac neurons from adult rats

The acutely dissociated cells from rat intracardiac ganglia consist of neurones, glia (satellite cells), fibroblasts, and a few endothelial cells. The intracardiac neurones are easily distinguished from other cells by their large size (15–25 μm diam), round or ovoid shape, and Na\(^{+}\) - and Ca\(^{2+}\) -dependent action potentials in the amplifier current-clamp mode (data not shown). The glial cells (~5 μm diam) expressed not voltage-activated Ca\(^{2+}\) currents but delayed rectifier K\(^+\) currents (data not shown).

**General characteristics of** \(\text{Ca}^{2+}\) **channel currents in intracardiac neurones**

Voltage-clamp recordings of inward Ca\(^{2+}\) currents were obtained from acutely dissociated neurones that had no or only a few processes. These neurones exhibited currents that
expressed fast activation and deactivation judged from the time course of the tail currents. After whole cell access, the Ca²⁺ currents initially increased and attained a steady state within 2–3 min. During current measurements for >30 min, no significant rundown in the peak current was detected (data not shown). Therefore no correction for rundown was necessary for drug-induced inhibition and stimulation. The nature of Ca²⁺ currents was verified in three ways: 1) substitution of Ca²⁺ with Ba²⁺ for a charge carrier in the external solution; 2) application of CdCl₂, an inorganic Ca²⁺ channel blocker; and 3) demonstration of Ca²⁺ dependency of the peak current by varying extracellular Ca²⁺ concentration ([Ca²⁺]). Representative current traces were obtained with the use of depolarizing voltage steps between −60 and +60 mV from a holding potential of −80 mV with the use of either 5 mM Ca²⁺ or 5 mM Ba²⁺ as the charge carrier (Fig. 1, A and B, respectively). The inward Ca²⁺ channel currents were first detected at around −20 and −30 mV, and peak currents occurred at +10 and 0 mV for Ca²⁺ and Ba²⁺, respectively (Fig. 1D). Both Ca²⁺ and Ba²⁺ currents displayed very slow inactivation during the 100-ms voltage step with an internal solution containing 11 mM EGTA. With replacement of Ca²⁺ with Ba²⁺, the peak current density increased ~1.4-fold, from 43.6 ± 5.2 pA/pF (n = 20) for Ca²⁺ to 59.5 ± 4.7 pA/pF (n = 14) for Ba²⁺ (see Fig. 1C). In all ranges of applied voltages, both Ca²⁺ and Ba²⁺ currents were completely blocked by 0.1 mM CdCl₂ (n = 7) (Fig. 1B). Increased [Ca²⁺], enhanced the peak current density in a hyperbolic manner (n = 15) (Fig. 1C). The curve was fitted by nonlinear regression in accordance with the equation

\[ I_{c,a,n} = I_{c,a,n} (1 + K_a/[Ca^{2+}])^{-1} \]  

where \( I_{c,a,n} \) was 67.0 pA/pF and the association constant (Kₐ) of the Ca²⁺ binding site was 2.61 mM. This saturation phenomenon of Ca²⁺ currents is thought to be due to the existence of saturable binding sites for Ca²⁺ within the Ca²⁺ channel (Carbone and Lux 1987) and has been also reported in other parasymptomatic neurons (Aibara et al. 1992; Hirst et al. 1985). Figure 1D illustrates a rightward shift in the current-voltage (I-V) relationship when [Ca²⁺]ₐ was increased from 2 to 5 mM (n = 15). This shift is mostly due to the screening effect of surface charges on the membrane. The intracellular Ca²⁺ currents from the adult rats had no low-voltage-activated (LVA, T-type) Ca²⁺ current. This was verified by the lack of a hump or inflection between −60 and −40 mV in the I-V curve (Fig. 1D) and no shift in the voltage dependence of currents after a change in the holding potential to −40 mV (data not shown).

The voltage dependence of the activation and inactivation of currents was assessed in an external solution containing 5 mM Ba²⁺ (Fig. 2). The activation curve was constructed by plotting the normalized chord conductance as a function of the membrane potential. The activation curve was fitted by the Boltzmann equation

\[ g/g_{\text{max}} = \frac{1 + \exp(V - V_h/k)}{1 + \exp(V - V_h/k)}^{-1} \]

where \( V_h \), the half-activation voltage, was −16.3 mV, and \( k \), a slope factor, was 5.6 mV per e-fold change in conductance (n = 9) (Fig. 2B). To evaluate the steady-state inactivation of Ba²⁺ currents, cells were held at different holding potentials between −110 and +30 mV for 15 s, followed by a voltage step to 0 mV for 100 ms. Currents for the voltage steps were normalized to the current elicited from a holding potential of −110 mV. Although the steady-state inactivation curve was fitted by the simple Boltzmann equation

\[ I_{\text{in, max}} = \frac{1 + \exp[(V - V_b/k)]^{-1}}{(1 + \exp[(V - V_b/k)]^{-1})} \]

where \( V_b = -33.7 \) mV and slope (k) = −12.1 mV per e-fold change (n = 11), some data points deviated from the curve (see Randall and Tsien 1995). In addition, there were some nonactivating currents (due to the L type) after holding at high voltages (between 0 and +30 mV) for 15 s (Fig. 2B).

**Pharmacological identification of subtypes of Ca²⁺ channel currents**

The subtypes of high-voltage-activated (HVA) Ca²⁺ channels in intracardiac neurons were pharmacologically identified by the application of different kinds of toxins and drugs (Adams et al. 1993; Randall and Tsien 1995). In all of the following experiments, 5 mM Ba²⁺ was used as the charge carrier for the following reasons: 1) to have large currents for the convenience and accuracy in dissecting each Ca²⁺ channel subtype, 2) to eliminate changes in inactivation kinetics due to Ca²⁺-dependent Ca²⁺ channel inactivation (Randall and Tsien 1995), and 3) to block voltage-dependent K⁺ channels.

**L-type current**

First, FPL 64176 (2 μM), the most effective L-type channel activator developed to date (McKechnie et al. 1989), was tested to see whether it enhanced the Ba²⁺ currents at different test potentials. Similar to descriptions in other tissues (Kunze and Rampe 1992; Randall and Tsien 1995; Zheng et al. 1991), FPL 64176 dramatically increased current amplitudes ~16.5-fold (0.14 ± 0.02 nA to 2.3 ± 0.6 nA), 12.7-fold (0.37 ± 0.1 nA to 4.7 ± 0.6 nA), 3.6-fold (1.7 ± 0.4 nA to 6.1 ± 0.7 nA), and 1.4-fold (4.0 ± 0.4 nA to 5.7 ± 0.6 nA) at test potentials of −30, −20, −10, and 0 mV, respectively (n = 9), resulting in a leftward shift in the voltage dependence of the I-V curve (Fig. 3, A and B). This effect may be explained by the enhanced channel open probability and mean open time, which are more evident at low membrane potentials (Kunze and Rampe 1992; Zheng et al. 1991). This activator also prolonged the tail current decay on deactivation and slowed the activation and inactivation kinetics of the Ba²⁺ current (Figs. 3A and 4B). These data demonstrate the presence of an L-type current in intracardiac neurons from adult rats.

To quantify the relative proportion of L-type current, the dihydropyridine (DHP) antagonist nifedipine was tested. Figure 4A illustrates a concentration-response curve for nifedipine (0.3–30 μM). The curve was best fitted by assuming one binding site with an equation

\[ %B = B_{\text{max}}(1 + I_{c,50}/[\text{nifedipine}])^{-1} \]

where \( B_{\text{max}} \) is a maximal blockade of 14.1% and \( I_{c,50} \) is the concentration of nifedipine for half-maximal blockade, 1.47 μM (8 ≤ n ≤ 28 for each data point). These values are
FIG. 1. General characteristics of Ca\(^{2+}\) channel currents in acutely dissociated intracardiac neurons from adult rats. 

A: Ca\(^{2+}\) currents evoked by depolarizing voltage steps over the range of −60 to +60 mV from a holding potential of −80 mV. External Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{o}\)) = 5 mM. B: Ba\(^{2+}\) currents evoked by the same protocol as in A. [Ba\(^{2+}\)]\(_{o}\) = 5 mM. Note the Ba\(^{2+}\) currents were completely blocked by 100 μM CdCl\(_2\). Data in A and B were obtained in the same cell. In both A and B, the current traces at voltage steps to −60, −50, −40, and +60 mV were not presented. C: dependence of Ca\(^{2+}\) channel current density (pA/pF) on [Ca\(^{2+}\)]\(_{o}\). Each data point represents mean ± SE (n = 14). Note that the averaged density of Ca\(^{2+}\) channel current was increased ~1.4-fold when Ba\(^{2+}\) (●) was a charge carrier at the same concentration. Curve: best fit of data points with the use of a nonlinear regression with Eq. 1 (see text). D: current-voltage (I-V) relationships of Ca\(^{2+}\) channel currents when 2 mM Ca\(^{2+}\) (●), 5 mM Ca\(^{2+}\) (■), and 5 mM Ba\(^{2+}\) (▲) as charge carriers. The currents were evoked by the same protocol as in A. Each data point represents mean ± SE (n = 15).

different from the maximal inhibition of 67% and IC\(_{50}\) of 3.4 μM acquired in rat neonatal intracardiac neurons held at the same holding potential (−80 mV) (Xu and Adams 1992).

Nifedipine is known to antagonize noncompetitively the effects of FPL 64176 (Zheng et al. 1991). Therefore FPL 64176 can be used as a good tool for determining the saturating concentration of nifedipine to elicit a complete blockade of the L-type current (Randall and Tsien 1995). In the presence of nifedipine (10 μM), FPL 64176 (2 μM) could not enhance the peak L-type current that was evoked by a voltage step to 0 mV (n = 4) (Fig. 4B). Therefore in further experiments a saturating concentration (10 μM) of nifedipine was used for the complete blockade of the L-type current. Only a small portion (10.6 ± 0.5%, n = 17) of the control Ba\(^{2+}\) current was blocked by 10 μM nifedipine when intracardiac neurons were held at −80 mV (see Fig. 5B).

**N-type current**

The N-type current was identified with the use of ω-CgTX GVIA isolated from the venom of the fish-hunting snail, *Conus geographus* (Olivera et al. 1984). To find a saturating concentration of ω-CgTX GVIA for the complete blockade of the N-type current, three different concentrations (0.3, 1, and 3 μM) were tested (Fig. 5A). From a series of experi-
FIG. 2. Voltage dependence of activation and inactivation of the Ba\(^{2+}\) current. A: activation curve fitted by Eq. 2 (see text), with half-activation voltage \((V_1) = -16.3\) mV and \(k = 5.6\) mV per e-fold change \((n = 9)\). B: steady-state inactivation curve fitted by Eq. 3 (see text), with \(V_1 = -33.7\) mV and slope of \(-12.1\) mV per e-fold change \((n = 11)\). Insert: representative current records showing the degree of inactivation of Ca\(^{2+}\) channels when a neuron was held at different voltages from \(-110\) to \(+30\) mV for 15 s before stepping to 0 mV for 100 ms. In both A and B, 5 mM Ba\(^{2+}\) was used as a charge carrier. \(G/G_{\text{max}}\), normalized chord conductance; \(I/I_{\text{max}}\), normalized currents.

ments \((n \approx 3)\), 61.1 ± 2.4%, 64.5 ± 1.4%, and 64.4 ± 4% of the control Ba\(^{2+}\) currents were blocked by successive applications of 0.3, 1, and 3 \(\mu\)M of \(\omega\)-CgTX GVIA. The results indicate that 1 \(\mu\)M \(\omega\)-CgTX GVIA was enough to produce a complete blockade of the N-type current. In 23 neurons, 1 \(\mu\)M \(\omega\)-CgTX GVIA irreversibly blocked 63.3 ± 0.6% of the control Ba\(^{2+}\) currents (Figs. 5B and 6). Consequently, the N type is the major voltage-activated

FIG. 3. Activation of L-type Ca\(^{2+}\) channel currents by FPL 64176. The Ba\(^{2+}\) currents were evoked by depolarizing voltage steps over the range of \(-60\) to \(+60\) mV from a holding potential of \(-80\) mV. A: representative current records. B: \(I-V\) relationships in the absence (●) and presence (■) of 2 \(\mu\)M FPL 64176, an L-type channel activator \((n = 9)\).
calcium channel in adult rat intracardiac neurons. Although the blocking effect of ω-CgTX GVIA has been considered to be specific for N-type current, Xu and Adams (1992) have shown that a significant overlap exists in the currents that were blocked by nifedipine and ω-CgTX GVIA in rat neonatal intracardiac neurons (see above). Furthermore, ω-CgTX GVIA at micromolar concentrations has been reported to reduce L-type current in chick sensory neurons (Aosaki and Kasai 1989). Therefore we tested to see whether the order of the application of nifedipine and ω-CgTX GVIA affected the degree of the current blockade. With the application of nifedipine (10 μM) first followed by ω-CgTX GVIA (1 μM), 11.5 ± 0.9% (n = 9) and 62.6 ± 0.9% (n = 9) of the control Ba2+ current, respectively, were blocked. With a reversed order, nifedipine and ω-CgTX GVIA blocked 9.7 ± 0.4% (n = 8) and 64.1 ± 0.8% (n = 8) of the control Ba2+ current, respectively. Although ω-CgTX GVIA tended to block a small portion of L-type current, there was no significant difference (P > 0.05) between the different orders of drug application, indicating little or no overlap in the currents blocked by nifedipine and ω-CgTX GVIA in intracardiac neurons from adult rats. Successive application of both nifedipine and ω-CgTX GVIA left ~26% (n = 17) of the control Ba2+ current unblocked. The size of nifedipine- and ω-CgTX GVIA-resistant currents was larger than 10–15% of the total current in rat sympathetic neurons (Re- gan et al. 1991) and parasympathetic neonatal intracardiac neurons (Xu and Adams 1992). The remaining currents were completely and reversibly blocked by 0.1 mM CdCl2 (n = 9; Fig. 5B).

Nature of non-L- and non-N-type currents

Among the different subtypes of Ca2+ channels that were identified pharmacologically and by molecular biological techniques, the P/Q (α1A) and R (α1H) types might be candidates for nifedipine- and ω-CgTX GVIA-insensitive currents in intracardiac neurons. Therefore we tested first whether there are P/Q-type currents in rat intracardiac neurons with the use of ω-CgTX MVIIC from Conus magus, which has been known to block N-, P-, and Q-type currents (Hillyard et al. 1992; Sather et al. 1993; Zhang et al. 1993). Figure 5C illustrates the effect of ω-CgTX MVIIC applied after the complete blockade of L- and N-type currents by nifedipine and ω-CgTX GVIA. Application of 5 μM ω-CgTX MVIIC blocked 19.1 ± 1.4% (n = 8) of the control Ba2+ current. These results suggest that rat intracardiac neurons have ω-CgTX MVIIC-sensitive P/Q-type currents. The waveform of P/Q-type currents acquired by subtraction showed inactivation during the voltage step (data not shown). The speed of the current blockade was much slower (time constant = ~60 s) than those by nifedipine and ω-CgTX GVIA. The combination of nifedipine, ω-CgTX GVIA, and ω-CgTX MVIIC left a residual current (Fig. 5C) that has been named the R type (Zhang et al. 1993). In rat intracardiac neurons this residual current contributed 7 ± 0.8% (n = 8) of the control Ba2+ current. The R-type current was sensitive to 100 μM NiCl2 (Fig. 5C, n = 4) similar to doe-1 and αH currents expressed in Xenopus oocytes (Soong et al. 1993; Zhang et al. 1993) and the residual current in rat cerebellar granule neurons (Randall and Tsien 1995).

Dissection of ω-Cgtx MVIIC-sensitive P/Q-type currents

Previously, P-type currents have been known to be highly sensitive to ω-Aga IVA, isolated from the venom of the spider, Agelenopsis aperta, with IC50 = ~2 nM in cerebellar Purkinje cells (Mintz et al. 1992). In the following experiments, therefore, we tested to see whether low or high concentrations of ω-Aga IVA blocked a portion of the control Ba2+ current. When 30 nM ω-Aga IVA, a concentration sufficient to eliminate P-type currents, was applied, no current was blocked (n = 4, data not shown). A supramaximal concentration (100 nM) of ω-Aga IVA for P-type currents also failed to block any Ba2+ currents after several minutes of application (Fig. 6, A and B, n = 7). To test the possible...
FIG. 5. Effect of \(\omega\)-conotoxin GVIA (\(\omega\)-CgTX GVIA) and \(\omega\)-conotoxin MVIIIC (\(\omega\)-CgTX MVIIIC) on \(\text{Ca}^{2+}\) channel currents. A: determination of a saturating concentration of \(\omega\)-CgTX GVIA. The saturation of blocking current was assessed by successive application of increasing concentrations of \(\omega\)-CgTX GVIA (0.3, 1, and 3 \(\mu\)M). B: blockade of \(N\)-type currents by a saturating concentration of \(\omega\)-CgTX GVIA. \(\omega\)-CgTX GVIA (1 \(\mu\)M) was applied after nifedipine (10 \(\mu\)M), which blocked a small portion (\(L\) type) of currents. Inset: current traces obtained at different time points (labeled a–d). In both A and B, the peak \(\text{Ba}^{2+}\) current was evoked every 10 s by a depolarizing step to 0 mV for 100 ms. C: blockade of the total \(\text{Ca}^{2+}\) current by \(\omega\)-CgTX MVIIIC (5 \(\mu\)M). Nifedipine (10 \(\mu\)M), \(\omega\)-CgTX GVIA (1 \(\mu\)M), \(\omega\)-CgTX MVIIIC (5 \(\mu\)M), and NiCl\(_2\) (100 \(\mu\)M) were successively applied. The peak \(\text{Ba}^{2+}\) current was evoked every 10 s by a depolarizing step to 0 mV for 100 ms. Inset: current traces obtained at different time points (labeled a–f).
FIG. 6. Nature of \( \omega \)-CgTX MVIIC-sensitive currents. A: effect of a low concentration (0.1 \( \mu \)M) of \( \omega \)-agatoxin IVA (\( \omega \)-Aga IVA) on the total \( \text{Ba}^{2+} \) current. After \( \omega \)-Aga IVA, \( \omega \)-CgTX GVIA (1 \( \mu \)M), nifedipine (10 \( \mu \)M), and \( \omega \)-CgTX MVIIC (5 \( \mu \)M) were successively applied. Inset: current traces obtained at different time points (labeled a–e).

B: effect of a high concentration (1 \( \mu \)M) of \( \omega \)-Aga IVA on the \( \omega \)-CgTX MVIIC-sensitive currents. Time course of inhibition of the total \( \text{Ba}^{2+} \) current was obtained from another cell by successive applications of \( \omega \)-Aga IVA (0.1 and 1 \( \mu \)M), \( \omega \)-CgTX GVIA (1 \( \mu \)M), nifedipine (10 \( \mu \)M), and \( \omega \)-CgTX MVIIC (5 \( \mu \)M). Inset: current traces obtained at different time points (labeled a–f) and the waveform (labeled b–c) of inactivating \( \omega \)-Aga IVA-sensitive current. In both A and B, the peak \( \text{Ba}^{2+} \) current was evoked every 10 s by a depolarizing step to 0 mV for 100 ms.

Reduction of the effective concentration of \( \omega \)-Aga IVA due to nonspecific binding to surfaces of glass or polyethylene tubing, we increased the concentration of cytochrome C in the external solution 10-fold. Cytochrome C (1 mg/ml) itself blocked 6.3 \( \pm \) 2.1% of the control \( \text{Ba}^{2+} \) current (\( n = 6 \)), but did not change the effectiveness of \( \omega \)-Aga IVA (100 nM) (\( n = 3 \)). However, in the presence of \( \omega \)-Aga IVA at 100 nM, 19.1 \( \pm \) 1.4% of the control \( \text{Ba}^{2+} \) current was slowly blocked by the successive addition of 5 \( \mu \)M \( \omega \)-CgTX MVIIC after block of L- and N-type currents (Fig. 6A, \( n = 6 \)). Taken together, these results strongly suggest that rat intracardiac neurons do not have P-type currents but rather have Q-type currents. Randall and Tsien (1995) have reported that high concentrations (>1 \( \mu \)M) of \( \omega \)-Aga IVA are able to block Q-type currents. We also tested the effect of the high concentration of \( \omega \)-Aga IVA on Q-type currents. Application of 1 \( \mu \)M \( \omega \)-Aga IVA occluded the effect of \( \omega \)-CgTX MVIIC (5 \( \mu \)M) by blocking Q-type currents (\( n = 4 \)) (Fig. 6B). The speed of blockade by \( \omega \)-Aga IVA (1 \( \mu \)M) was similar to that by \( \omega \)-CgTX MVIIC (5 \( \mu \)M). The \( \omega \)-Aga IVA-sensitive current showed inactivation during the 100-ms voltage step to 0 mV as described above (Fig. 6B, inset), indicative of non-P-type current (Mintz et al. 1992; Randall and Tsien 1995; Usowicz et al. 1992).

DISCUSSION

In comparison with sympathetic neurons, mammalian parasympathetic neurons have not been very intensively studied with the use of electrophysiological approaches because of their relatively inaccessible locations. Consequently, most voltage-clamp studies on ionic currents of intracardiac neurons have utilized the amphibian preparations (Clark et al. 1990; Merriam and Parsons 1995), which may be easily isolated. A few studies on ionic currents have been made in mammalian intracardiac neurons. However, those studies have been confined to neurons from neonates (Xu and Adams 1992) because of the difficulties in dissociating adult intracardiac neurons. In the present study we successfully dissociated intracardiac neurons from adult rats. Acutely dissociated adult intracardiac neurons were found to be a highly homogeneous population in terms of their...
cholinergic properties (data not shown) as well as their expression of multiple subtypes of voltage-activated Ca\(^{2+}\) currents. In addition, these dissociated intracardiac neurons had suitable morphological characteristics (e.g., lack of numerous, large, long processes) for the voltage-clamp recording of ionic currents. Taken as a whole, the preparation from adult rats is a good model system for studying the physiology and pharmacology of Ca\(^{2+}\) channel currents of parasympathetic intracardiac neurons.

**L- and N-type Ca\(^{2+}\) channel currents**

The present study first demonstrates that intracardiac neurons from adult rats express at least four different subtypes of HVA Ca\(^{2+}\) channels: L-, N-, Q-, and R-types, based on pharmacological criteria as applied to other types of neurons (Adams et al. 1993; Randall and Tsien 1995). In general agreement with studies on most autonomic neurons (see Adams and Harper 1995 for review), intracardiac neurons have no LVA (T-type) Ca\(^{2+}\) currents. Our results show that the predominant component of the Ca\(^{2+}\) channels in intracardiac neurons is the ω-CgTX GVIA-sensitive N type (∼63%), similar to findings in other mammalian autonomic neurons including neonatal intracardiac neurons (Aibara et al. 1992; Carrier and Ikeda 1992; Xu and Adams 1992). Applications of a non-DHP agonist, FPL 64176, and a DHP antagonist, nifedipine, revealed the presence of L-type current in intracardiac neurons. However, the L-type current contributed to only a small portion (11%) of the total current, similar to the situation in adult rat pelvic neurons (Zhu et al. 1995). This contribution was much lower than that in neonatal intracardiac neurons (Xu and Adams 1992), although neurons in both cases were held at same holding potentials (∼80 mV). In addition to this dissimilarity, we found that the Ca\(^{2+}\) channels of adult intracardiac neurons display some other properties of Ca\(^{2+}\) channels that were different from those in neonates, e.g., 1) little or no overlap between selectivities of nifedipine and ω-CgTX GVIA, 2) a higher sensitivity (a lower IC\(_{50}\)) to nifedipine, and 3) relatively greater contributions of non-L and non-N types to total Ca\(^{2+}\) current. In the present experiments on adult intracardiac neurons, different orders of application of nifedipine (10 μM) and ω-CgTX GVIA (1 μM) revealed no significant overlap, indicating selective blockades of L- or N-type Ca\(^{2+}\) channels by each drug, respectively. Although the origins of these differences are unclear, they may be due to age-dependent changes in the density and pharmacology of subtypes of calcium currents (Hamill et al. 1991; Hirst and McLachlan 1986) and/or due to changes related to the more prolonged culture duration used by Xu and Adams (1992).

**Q-type Ca\(^{2+}\) channel currents**

Some studies on parasympathetic neurons have revealed three different types of Ca\(^{2+}\) channels, i.e., DHP-sensitive, ω-CgTX GVIA-sensitive, and both DHP- and ω-CgTX GVIA-resistant Ca\(^{2+}\) channels (Aibara et al. 1992; Xu and Adams 1992). However, the nature of Ca\(^{2+}\) channels resistant to both blockers has remained undissected. After blockade of L- and N-type currents in intracardiac neurons, these resistant currents (26%) were easily identified with the use of two additional toxins: ω-Aga IVA and ω-CgTX MVIIIC. Several studies indicate that P-type currents are highly sensitive to ω-Aga IVA (IC\(_{50}\) = ∼2 nM) in cerebellar Purkinje cells (Mintz et al. 1992), whereas Q-type currents are highly sensitive to ω-CgTX MVIIIC but weakly sensitive to ω-Aga IVA (IC\(_{50}\) = ∼200 nM) (Hillyard et al. 1992; Sather et al. 1993; Zhang et al. 1993). With the use of these criteria, our results suggest that intracardiac neurons express Q-type currents that share similar properties with ω-Aga current expressed in Xenopus oocytes (Sather et al. 1993). It is known that Q-type currents show inactivation, whereas P-type currents do not, during voltage steps (Sather et al. 1993; Usowicz et al. 1992). In our experiment, the finding that the waveform of ω-CgTX MVIIIC-sensitive current showed inactivation during a voltage step supports pharmacological identification of Q-type currents.

**R-type calcium channel currents**

R-type calcium currents contributed only 7% of the total current in adult rat intracardiac neurons. The identity of an R-type current distinct from others is supported by several lines of evidence: 1) the robust current after blockade of L-, N-, and Q-type currents with the use of nifedipine, ω-CgTX GVIA, and ω-CgTX MVIIIC or even with the addition of ω-Aga IVA; 2) the fastest inactivation kinetics among the different types of waveforms (data not shown); and 3) the Ni\(^{2+}\) sensitivity of the current. These characteristics of R-type currents in intracardiac neurons seem to be similar to those of the ω-Aga clone, a mammalian neuronal counterpart to doe-1 in the marine ray (Soong et al. 1993; Zhang et al. 1993). However, the more precise dissection of R-type currents awaits the development of a selective blocker in future.

**Summary**

The parasympathetic intracardiac neurons from adult rats functionally express at least four distinct subtypes of HVA Ca\(^{2+}\) channels: L, N, Q, and R types, which contribute ∼11, 63, 19, and 7% of the whole current, respectively. Recently we have found that the Ca\(^{2+}\) channel currents were modulated by cholinergic, adrenergic, and some peptidergic receptor activation (Wurster and Jeong 1996). In addition, N-, Q-, and R-type currents are found to be selectively modulated by muscarinic activation (unpublished observations). However, the physiological roles of multiple subtypes of Ca\(^{2+}\) channels are yet unclear in parasympathetic intracardiac neurons. Therefore the present study provides the essential basis for further studies of the role of each subtype in the actions of neurotransmitters in synaptic transmission and in the control of excitability of these neurons that regulate cardiac functions.

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Address for reprint requests: R. D. Wurster, Depts. of Physiology and Neurological Surgery, Loyola Stritch School of Medicine, 2160 S. First Ave., Maywood, IL 60153.

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S. W. JEONG AND R. D. WURSTER1778 

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