Muscarinic and Nicotinic ACh Receptor Activation Differentially Mobilize Ca\(^{2+}\) in Rat Intracardiac Ganglion Neurons

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INTRODUCTION

Intracellular calcium regulates an array of cellular events associated with neuronal function including excitability, exocytosis, synaptic plasticity, gene expression, and cell death (see reviews by Berridge 1998; Meldolesi 2001). The spatial and temporal characteristics of the Ca\(^{2+}\) signal are regulated by distinct signaling pathways linked to activation of cell-surface receptors. However, the precise mechanisms underlying changes in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(]_{i}\)) after cholinergic receptor activation in mammalian autonomic neurons remain unknown.

The neuronal nicotinic acetylcholine receptor (nAChR) is a pentameric ligand-gated cation channel, and numerous nAChR subtypes with different pharmacological and functional properties are expressed in the central and peripheral nervous systems (McGehee 1999). In rat intracardiac neurons, the predominant nAChR subtype contains the α3 subunit in combination with β2 and/or β4 and α5 subunits (Poth et al. 1997). These neurons also express the α7 subunit, which can form a functional homomeric pentamer that may correspond to the α-bungarotoxin-sensitive component of the whole cell ACh-evoked current (Cuevas and Berg 1998). Activation of neuronal nAChRs mediates rapid excitatory synaptic transmission in rat intracardiac neurons (Fieber and Adams 1991; Selyanko and Skok 1992). Although the relative Ca\(^{2+}\) permeability of nAChR channels has been studied in these neurons (Adams and Nutter 1992; Nutter and Adams 1995), the relative contributions of extracellular and intracellular Ca\(^{2+}\) and the involvement of Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) and voltage-gated Ca\(^{2+}\) channels to the elevation of [Ca\(^{2+}\)\(]_{i}\) in these neurons remain to be determined.

Mammalian autonomic neurons also express muscarinic ACh receptors (mAChR) that have been shown to activate intracellular second-messenger pathways and subsequent membrane responses (Simeone et al. 1996; Sorimachi et al. 1995). Agonist binding to M1 and/or M3 mAChR subtypes activates phospholipase C (PLC), which generates the second messenger, inositol 1,4,5-trisphosphate (IP\(_3\)), and subsequent Ca\(^{2+}\) release from intracellular IP\(_3\)-sensitive Ca\(^{2+}\) stores (see Caulfield and Birdsell 1998). Interestingly, in adult rat sympathetic neurons, it has been suggested that increases in [Ca\(^{2+}\)\(]_{i}\) evoked by the nonselective cholinergic agonist carbamylcholine are not dependent on intracellular stores (Foucart et al. 1995). Activation of the M1 receptor has been shown to inhibit muscarinic-sensitive K\(^+\) currents in rat autonomic neurons (Bernheim et al. 1992; Cuevas et al. 1997; Xi-Moy and Dun 1995), whereas, the M3 receptor has been reported to induce a nonselective cation current in rat dorsolateral septal neurons (Hasuo et al. 1996). In contrast, stimulation of M2 and M4 receptors activate G proteins, which in turn produce cAMP stimulating the activation of protein kinase A (PKA) and phosphorylation of PKA-dependent enzymes. Expression of mRNAs encoding for M1-M4 receptor subtypes has been detected in intracardiac neurons in vitro and in situ (Hassall et al. 1993: Hoover et al. 1994). The G-protein-coupled M2 receptor increases the K\(^+\) conductance in mammalian intracardiac neurons (Allen and Burnstock 1990; Xi-Moy et al. 1993), whereas M4 receptor activation inhibits the voltage-dependent N- and L-type Ca\(^{2+}\) channels (Cuevas and Adams 1997). However, the intracellular signaling mechanism(s) by which mAChR
activation changes [Ca\(^{2+}\)], in intracardiac neurons has not been addressed.

In the present study, the mobilization of Ca\(^{2+}\) via n- and mAChR activation and the relative contributions of intra- and extracellular Ca\(^{2+}\) were investigated in isolated rat intracardiac neurons. A preliminary report of some of these results has been published (Beker and Adams 2001).

**METHODS**

**Preparation**

Parasympathetic neurons from neonatal rat intracardiac ganglia were isolated and placed in tissue culture. The procedures for isolation of the intracardiac neurons have been described previously (Xu and Adams 1992) and were in accordance with guidelines of the University of Queensland Animal Experimentation Ethics Committee. Briefly, Wistar rats (3–10 days old) were killed by decapitation, the heart was excised and placed in a saline solution containing (in mM) 140 NaCl, 3 KCl, 2.5 CaCl\(_2\), 0.6 MgCl\(_2\), 7.7 glucose, and 10 histidine (pH to 7.2 with NaOH). Atria were removed and incubated for 1 h at 37°C in saline solution containing collagenase (0.9 mg/ml, Worthington-Biochemical, Freehold, NJ). After enzymatic treatment, clusters of ganglia were dissected, transferred to a sterile culture dish containing high glucose culture media (Dulbecco’s modified Eagle media), 10% (vol/vol) fetal calf serum, 100 units/ml penicillin and 0.1 mg/ml streptomycin, and triturated using a fine-bore Pasteur pipette. The dissociated neurons were plated on laminin-coated 24-mm glass cover slips and incubated at 37°C for 24–48 h under a 95% O\(_2\)-5% CO\(_2\) atmosphere.

**Microfluorimetric measurements**

Measurement of [Ca\(^{2+}\)] in response to application of cholinergic agonists and bath-applied drugs were carried out in rat intracardiac neurons using single-cell photometry. Neurons were loaded for 1 h at room temperature in physiological saline solution containing 5 μM fura-2 acetoxymethylester (1 mM fura-2/AM in DMSO stock solution), 0.5% wt/vol bovine serum albumin, 0.02% pluronic-127. After incubation with fura-2/AM, the cells were washed in PSS and allowed 30 min to recover before the experiments were carried out. The coverslip containing the neurons was fixed between two sealed rings forming the bottom of the recording chamber and the neurons were monitored using a ×40 oil-immersion objective (Nikon, Fluor 40/1.3 n.a.). A 75-W xenon arc lamp (PTI OC-4000 Optical Chopper, Photon Technology International, South Brunswick, NJ) supplied alternating 340- and 380-nm illumination via a fiber-optic cable and a 450-nm dichroic mirror (Nikon DM 400). Emission fluorescence (510-nm band-pass filter) was collected by a Hamamatsu R 928 photomultiplier tube through a variable aperture set around the cell image. The output of the photomultiplier tube was digitized using a PTI interface and sampled at 5 Hz using Felix 1.1 software (PTI) run on a Pentium computer.

The control experiments involving mecamylamine and yarondine were recorded with an Olympus OSP-3 photometry system including a fast filter changing unit with a Xenon UV-light source added to an IMPT-inverted microscope with UV-opts. The fluorescence emission was detected by a photomultiplier via a detection pinhole of variable size and position (Utenweiler et al. 1995).

Changes in intracellular free Ca\(^{2+}\) concentration (Δ[Ca\(^{2+}\)]) were estimated from the ratio of the intensities of the emitted 510-nm fluorescence following the excitation with 340- and 380-nm light, R(340/380). This ratio was converted to approximate Ca\(^{2+}\) concentrations using the equation

\[
[\text{Ca}^{2+}] = K_d \times \frac{R - R_a}{R_a - R} \times \frac{S_a}{S_d}
\]

The ratio of the fluorescence intensities (R = F\(_{340\text{-nM}}\)/F\(_{380\text{-nM}}\)) was determined during experiments, and a calibration procedure was used to determine the constants for the other numerical values in this equation using fura-2 pentapotassium salt and standard Ca\(^{2+}\)-EGTA solutions (Grynkiewicz et al. 1985). Measurements of solutions with [Ca\(^{2+}\)] in a range between 0 and 1 μM allowed to determine the dissociation constant, K\(_d\) = 473 nM. The minimum ratio (R\(_{\text{min}}\)) was 0.11 and the maximum ratio (R\(_{\text{max}}\)) was 2.12 and the ratio of the fluorescence intensity of the Ca\(^{2+}\)-free and Ca\(^{2+}\)-bound fura-2 samples at 380 nm ([S\(_a\)/S\(_d\)]) = 5.64. To calibrate the fluorescence signal, solutions containing 10 mM EGTA, 100 mM KCl, 10 mM K-MOPS, 1 μM fura 2 pentapotassium salt, and either 10 mM CaCl\(_2\) or no added Ca\(^{2+}\), respectively, were made to carry out the calibration procedure.

**Electrophysiological recordings**

Membrane currents were monitored using the whole cell recording configuration of the patch-clamp technique. Electrical access to the cell interior was obtained using the perforated-patch whole cell configuration (Horn and Marty 1988). The perforated-patch configuration allows electrical access to the cell interior without the loss of cytoplasmic components, which are important in maintaining functional responses in these cells (Cuevas et al. 1997). A stock solution of 60 mg/ml amphotericin B in DMSO was prepared on the day of the experiment and diluted in pipette solution to yield a final concentration of 240 μg/ml amphotericin B in 0.4% DMSO. The pipette tip was first filled with antibiotic-free solution to prevent any disruption of seal formation and then backfilled with the amphotericin B-containing solution. Pipettes were pulled from thin-walled boro-silicate glass (Harvard Apparatus, Edenbridge, UK) using a Sutter Instruments P-87 pipette puller and following fire polishing had resistances of ~1 MΩ. Access resistances using the perforated-patch configuration were ≤4 MΩ after series resistance compensation, which was typically ≥80%.

Filled patch pipettes were mounted on the head stage of a patch-clamp amplifier (EPC-7, List-Medical, Darmstadt, Germany). Voltage protocols were applied using Clampex software (Version 7.0, Axon Instruments, Union City, CA). Signals were filtered at 200 Hz then digitized at 1 kHz (Digidata 1200A interface, Axon Instruments) and stored on the hard disc of a Pentium computer for viewing and analysis.

**Solutions and drugs**

Rat intracardiac neurons were superfused with physiological salt solution (PSS) containing (in mM) 140 NaCl, 3 KCl, 2.5 CaCl\(_2\), 1.2 MgCl\(_2\), 7.7 glucose, and 10 HEPES-NaOH, pH 7.2 or Ca\(^{2+}\)-free PSS containing 1 mM EGTA (<10 nM free Ca\(^{2+}\)). In these neurons, the nicotinic ACh-induced current amplitude was maximal in the presence of 2.5 mM extracellular Ca\(^{2+}\) (Fieber and Adams 1991). Bath solutions containing drugs used in a series of experiments were prepared daily. The pipette solution for perforated patch experiments contained (in mM): 75 K\(_2\)SO\(_4\), 55 KCl, 5 Mg SO\(_4\), and 10 HEPES, titrated with N-methyl-d-glucamine to pH 7.2. Amphotericin B-containing solutions were prepared daily and kept on ice and light protected. The osmolarity of all solutions (290–310 mmol/kg) was monitored with a vapor pressure osmometer (Wescor 5500, Logan, UT). Agonists were applied to cells by brief pressure ejection (≤10 psi; Picospritzer II, General Valve, Fairfield, NJ) from an extracellular micropipette (3–5 μm diam) positioned 50–100 μm from the cell soma to evoke maximal responses to agonists. Maximally effective agonist concentrations (≥300 μM) determined from ACh dose-response relations for [Ca\(^{2+}\)] increases, were used for cholinergic receptor activation. To minimize receptor desensitization, a delay of ≥100 s between agonist applications was maintained. All experiments were carried out at room temperature (22°C).

All chemical reagents used were of analytical grade. The following drugs were used: acetylcholine chloride, amphotericin B, atropine.
sulfate, caffeine, DMSO, mecamylamine hydrochloride, (±) muscarine chloride (Sigma Chemical, St. Louis, MO), cyclopiazonic acid (CPA), ryanodine, U-73122 and U-73343 (Calbiochem, La Jolla, CA), pirenzepine dihydrochloride (Research Biochemicals International, Natick, MA), fura-2/AM, fura-2 pentapotassium salt, and pluronic-L7 (Molecular Probes, Eugene, OR).

**Data analysis**

Data are expressed as the means ± SE and n values refer to the number of cells. Data were analyzed statistically using Student’s paired t-test with the level of significance being taken as $P < 0.05$. A Student’s unpaired t-test was carried out for the CPA experiments. The control condition was the response of the same cell to the agonist prior to changes in the superfusion solution.

**RESULTS**

**Activation of n- and mACHRs increases cytoplasmic Ca$^{2+}$ levels in rat intracardiac neurons**

Focal application of a maximally effective dose of ACh (300 μM) to the soma membrane evoked a rapid, transient increase in [Ca$^{2+}$], after activation of cholinergic receptors in fura-2-loaded rat intracardiac neurons. The mean $\Delta[Ca^{2+}]$, evoked by 300 μM ACh was 103 ± 9 nM from a resting [Ca$^{2+}$], of 94 ± 9 nM (n = 49) as shown in Fig. 1. ACh-evoked [Ca$^{2+}$], responses remained stable with constant amplitude on repeated agonist application at intervals of ≥100 s. The relative contribution of nicotinic and muscarinic ACh receptor activation to the increase in [Ca$^{2+}$], in response to stimulation by ACh is shown in Fig. 1A. Mecamylamine (3 μM), a selective antagonist of ganglionic nAChRs (Fieber and Adams 1991), significantly reduced the ACh-induced [Ca$^{2+}$], response to 66 ± 5% of control (n = 4). A similar inhibition of the ACh-induced [Ca$^{2+}$], response (reduced to 60 ± 6%, n = 8) was observed on bath application of 1 μM atropine, a selective antagonist of mAChRs. Bath application of mecamylamine (3 μM) and atropine (1 μM) together reduced the ACh-induced [Ca$^{2+}$], response to 19 ± 10% of control (n = 3) but did not inhibit the [Ca$^{2+}$], increase completely. Figure 1B shows the relative $\Delta[Ca^{2+}]$, in response to ACh activation of n- and mAChRs, respectively.

**Sources of Ca$^{2+}$ mobilized by n- and mAChR activation in rat intracardiac neurons**

The relative contributions of extra- and intracellular Ca$^{2+}$ to ACh-induced [Ca$^{2+}$], responses mediated by n- and mAChR activation were investigated in isolated rat intracardiac neurons. Increases in [Ca$^{2+}$], evoked by ACh persisted in Ca$^{2+}$-free external solutions containing 1 mM EGTA, but peak amplitudes were reduced to 58 ± 8% (n = 4) compared with that observed in PSS (2.5 mM Ca$^{2+}$; Fig. 2). The component of the [Ca$^{2+}$], response dependent on extracellular Ca$^{2+}$ may be due to Ca$^{2+}$ influx across the plasma membrane and/or Ca$^{2+}$-induced Ca$^{2+}$ release from intracellular Ca$^{2+}$ stores. The [Ca$^{2+}$], response obtained on selective activation of mAChRs by ACh (300 μM) + mecamylamine (3 μM) in the presence of Ca$^{2+}$-free external solution (47 ± 13%, n = 7) was not significantly different from the [Ca$^{2+}$], response obtained with ACh alone in Ca$^{2+}$-free external solution. This mobilization of Ca$^{2+}$ activated by mAChRs independent of extracellular Ca$^{2+}$ is in contrast to that observed with activation of nAChRs, which did not evoke a substantial [Ca$^{2+}$], response (5 ± 3%, n = 8) in the presence of Ca$^{2+}$-free external solutions.

Increases in [Ca$^{2+}$], evoked in response to focal application of 300 μM muscarine in the presence of external Ca$^{2+}$ (122 ± 31 nM, n = 4) were almost completely abolished after bath application of 100 nM pirenzepine (8 ± 4 nM, n = 4), a relatively selective antagonist for M1 receptor (see Caulfield and Birdsell 1998). This suggests that the M1 mAChR subtype most likely contributes to the increase in [Ca$^{2+}$], in response to mAChR activation.

To characterize the signal transduction pathway for muscarinic receptor-mediated mobilization of intracellular Ca$^{2+}$, the PLC inhibitor, U-73122, was examined on muscarine-induced [Ca$^{2+}$], transients in rat intracardiac neurons. Bath application of 5 μM U-73122 reversibly inhibited the amplitude of [Ca$^{2+}$], transients induced by either 100 μM muscarine or 100 μM

FIG. 1. Activation of nicotinic and muscarinic ACh receptors increases [Ca$^{2+}$], in rat intracardiac neurons. A: representative traces of ACh-induced increases in [Ca$^{2+}$], in response to ACh activating muscarinic and nicotinic receptors. Fura-2-loaded cells were continuously perfused with physiological salt solution (PSS) or PSS containing 3 μM mecamylamine, 1 μM atropine, or mecamylamine + atropine as indicated by horizontal bars. ACh (300 μM) was focally applied for 1 s at indicated times. Peak and baseline values for [Ca$^{2+}$], are indicated by the broken lines. B: bar graph of relative changes in ACh-induced [Ca$^{2+}$], increases ($\Delta[Ca^{2+}]$), obtained in the absence (control) and presence of mecamylamine (n = 4), atropine (n = 8), and mecamylamine + atropine (n = 3). All values were significantly different to the control response ($P < 0.05$).
hand, ryanodine, in micromolar concentrations is an inhibitor of caffeine-induced Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores in rat autonomic neurons (Smith and Adams 1999; Thayer et al. 1988). Focal application of caffeine (10 mM) evoked a transient [Ca\(^{2+}\)], increase (114 ± 19 nM,  \(n = 4\)) that was inhibited by bath application of 10 \(\mu M\) ryanodine (14 ± 5% of control,  \(n = 4\),  \(P < 0.005\)) as shown in Fig. 3. However, the known interaction between fura-2 and caffeine yields to a redshift in the excitation peak and a reduced dynamic range of the dye. The absolute increase in [Ca\(^{2+}\)], due to caffeine is therefore underestimated, whereas the relative caffeine-induced changes in [Ca\(^{2+}\)], in the presence of ryanodine are unaffected (Muschol et al. 1999; Uttenweiler et al. 1995). These data are consistent with the presence of intracellular ryanodine-sensitive Ca\(^{2+}\) stores, which may mediate CICR in rat intracardiac neurons.

The ACh-evoked increase in [Ca\(^{2+}\)], obtained in the presence of atropine (1 \(\mu M\)) was significantly reduced on bath application of 10 \(\mu M\) ryanodine (50 ± 2%,  \(n = 4\),  \(P < 0.005\))

\[\text{nAChR activation induces Ca}^{2+}\text{ release from internal Ca}^{2+}\text{ stores}\]

While inhibiting IP\(_3\) receptors, caffeine is an activator of ryanodine receptors (Ehrlich et al. 1994), thus depleting ryanodine-sensitive Ca\(^{2+}\) stores (Pozzan et al. 1994). On the other

**FIG. 2.** Sources of Ca\(^{2+}\) mobilized by nicotinic and muscarinic ACh receptor (nAChR and mAChR) activation in rat intracardiac neurons. **A:** representative [Ca\(^{2+}\)], responses to focal application of 300 \(\mu M\) ACh obtained in the presence (control) and absence of extracellular Ca\(^{2+}\) (Ca\(^{2+}\)-free PSS). The bath solution contained either 3 \(\mu M\) mecamylamine or 1 \(\mu M\) atropine to inhibit n- and mAChRs, respectively, as indicated by horizontal bars. **B:** bar graph of relative ACh-induced [Ca\(^{2+}\)], increases (\(\Delta[Ca^{2+}]\)) obtained in control PSS (2.5 mM Ca\(^{2+}\)) and in Ca\(^{2+}\)-free PSS, and Ca\(^{2+}\)-free PSS containing either mecamylamine or atropine. All values were significantly different to control (\(P < 0.05\)). The response to ACh obtained in the absence of external Ca\(^{2+}\) (Ca\(^{2+}\)-free PSS) was significantly different to that obtained in Ca\(^{2+}\)-free PSS + atropine (\(n = 8\),  \(P < 0.005\)).

**FIG. 3.** Ryanodine-sensitive Ca\(^{2+}\) stores contribute to nAChR-mediated increase in [Ca\(^{2+}\)]. **A:** representative [Ca\(^{2+}\)], increases to nAChR activation obtained in response to ACh (300 \(\mu M\)) + atropine (1 \(\mu M\)) were significantly reduced by bath application of 10 \(\mu M\) ryanodine. Caffeine (10 mM)-induced increases in [Ca\(^{2+}\)], are attenuated in the presence of 10 \(\mu M\) ryanodine. **B:** bar graph of relative \(\Delta[Ca^{2+}]\), in response to ACh + atropine, ACh + atropine and ryanodine (\(n = 4\),  \(P < 0.05\)). Caffeine-induced [Ca\(^{2+}\)], responses were inhibited by ryanodine (\(n = 4\),  \(P < 0.005\)).
as shown in Fig. 3, A and B. In contrast, ryanodine (10 μM) did not affect the nAChR-mediated [Ca\(^{2+}\)] response obtained in response to focal application of ACh + mecamylamine (86 ± 8% of control, n = 6). This suggests that the release of Ca\(^{2+}\) from ryanodine-sensitive Ca\(^{2+}\) stores contributes to the [Ca\(^{2+}\)] response evoked by n- but not mAChR activation.

**Effect of inhibition of Ca\(^{2+}\) reuptake by internal stores on nAChR-mediated [Ca\(^{2+}\)] responses**

To further elucidate the nAChR-dependent activation of internal Ca\(^{2+}\) stores, we tested cyclopiazonic acid (CPA), a sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) inhibitor that has been reported to deplete intracellular Ca\(^{2+}\) stores sensitive to IP\(_3\) in HL-60 cells (Demaurex et al. 1992) and to ryanodine in guinea pig myenteric neurons (Kimball et al. 1996). Elevation of cytoplasmic Ca\(^{2+}\) levels with nAChR activation and the involvement of intracellular Ca\(^{2+}\) stores in this response was investigated in rat intracardiac neurons during bath application of 10 μM CPA. Figure 4 shows a representative [Ca\(^{2+}\)] response obtained on activation of nAChRs by focal application of ACh (300 μM) prior to and during exposure to 10 μM CPA in the presence of atropine (1 μM). Bath application of CPA raised the resting [Ca\(^{2+}\)], by 142 ± 31 nM (n = 6). The amplitude of the first ACh-induced [Ca\(^{2+}\)], response obtained after 12-min exposure to CPA was already significantly reduced as shown by a paired t-test (reduced to 78 ± 6% of control, P < 0.05). This difference is also statistically significant when the ACh-induced [Ca\(^{2+}\)], response after 12 min in the presence of CPA is compared with a time-matched control group of cells not exposed to CPA (P < 0.01, unpaired Student’s t-test). In the maintained presence of CPA, subsequent applications of ACh-evoked [Ca\(^{2+}\)], increases were progressively smaller in amplitude (Fig. 4A).

This continuing decline of ACh-evoked [Ca\(^{2+}\)], increases was accompanied by a slow recovery of the Ca\(^{2+}\) concentration, indicating that the slightly reduced Ca\(^{2+}\) gradient cannot be solely responsible for the decline of the ACh-induced [Ca\(^{2+}\)], transients. Under control conditions, in the absence of CPA, repeated application of ACh did not significantly change the amplitude of the [Ca\(^{2+}\)], response (Fig. 4B). These data support the suggestion that intracellular Ca\(^{2+}\) stores sensitive to CPA are involved in [Ca\(^{2+}\)], signaling mediated by nAChR activation. However, the [Ca\(^{2+}\)], response was not completely abolished after repeated application of ACh in the presence of CPA. The [Ca\(^{2+}\)], increase after nAChR activation may be mainly due to two components: Ca\(^{2+}\) influx across the plasma membrane and Ca\(^{2+}\) release from internal stores.

**ACh-evoked [Ca\(^{2+}\)], responses and membrane currents in voltage-clamped rat intracardiac neurons**

To eliminate depolarization-induced changes in [Ca\(^{2+}\)], measurements of [Ca\(^{2+}\)], and membrane currents were made simultaneously in fura-2 loaded cells under voltage-clamp conditions. Brief application of 300 μM ACh to the cell soma held at −60 mV evoked a transient inward current (−647 ± 62 pA, n = 7) in one cell followed by a slow outward current of +20 pA (see also following text). The ACh-evoked inward current is consistent with activation of nAChR channels, which are cation selective as previously described in rat intracardiac neurons (Fieber and Adams 1991). The [Ca\(^{2+}\)], response measured simultaneously, was not significantly different to that observed under unclamped conditions. Bath application of mecamylamine (3 μM) substantially reduced the ACh-induced [Ca\(^{2+}\)], increase (57 ± 8%, n = 7, P < 0.005) and evoked a biphasic response that consisted of an inward current (−154 ± 15 pA, n = 7, P < 0.001) followed by a slow outward current (26 ± 6 pA, n = 7) as described in Fig. 5B. Focal application of muscarine (5 μM) also evoked a biphasic response in a subpopulation of rat intracardiac neurons similar to that observed in intracardiac neurons of guinea pig (Allen and Burnstock 1990) and dog (Xi-Moy et al. 1993). The [Ca\(^{2+}\)], response was unchanged in the presence of a Ca\(^{2+}\)-free extracellular solution whereas the inward current was reduced (−68 ± 16 pA, n = 7) and the outward current amplitude was substantially increased (97 ± 18 pA, n = 7). The residual inward current was abolished in the presence of 10 μM mecamylamine (n = 3). Under voltage-clamp conditions, the [Ca\(^{2+}\)], response to mAChR activation was similar in the...
absence and presence of extracellular Ca\(^{2+}\) and was accompanied by a slow outward current. Taken together, these data suggest that mAChR activation mobilizes the release of Ca\(^{2+}\) from intracellular Ca\(^{2+}\) stores, independent of Ca\(^{2+}\) entry across the plasma membrane, and activates an outward current.

[Ca\(^{2+}\)\(_i\)] and membrane current responses evoked on nAChR activation in voltage-clamped neurons

Activation of nAChRs in mammalian autonomic neurons evokes an inward current and an increase in [Ca\(^{2+}\)\(_i\)] (Fieber and Adams 1991; Rogers and Dani 1995; Trouslard et al. 1993). To eliminate any contribution of depolarization-activated Ca\(^{2+}\) channels to the ACh-induced increase in [Ca\(^{2+}\)\(_i\)], the mobilization of Ca\(^{2+}\) by nAChR activation was studied in voltage-clamped neurons in the presence of atropine. Atropine (1 \(\mu\)M) attenuated the [Ca\(^{2+}\)\(_i\)] increase to 68 \pm 7% of control \((n = 4, P < 0.05)\) but did not significantly affect the inward current amplitude (Fig. 6). In the absence of external Ca\(^{2+}\), the [Ca\(^{2+}\)\(_i\)] response (12 \pm 4%, \(n = 4, P < 0.005\)) and the inward current amplitude were substantially reduced (32 \pm 10% of control, \(n = 4, P = 0.05\)) as shown in Fig. 6, A and B. The ACh-induced outward current observed in the presence of mecamylamine (3 \(\mu\)M) was abolished in the presence of 1 \(\mu\)M atropine. Under voltage-clamp conditions, the [Ca\(^{2+}\)\(_i\)] response to nAChR activation observed in the absence and presence of extracellular Ca\(^{2+}\) was similar to that observed in unclamped neurons. The [Ca\(^{2+}\)\(_i\)] response measured simultaneously was not significantly different to that observed under nonclamped conditions indicating that Ca\(^{2+}\) influx through open voltage-dependent Ca\(^{2+}\) channels does not, under these conditions, appreciably contribute to the [Ca\(^{2+}\)\(_i\)] response evoked by nAChR activation.
DISCUSSION

The present findings demonstrate that in fura-2-loaded neurons from rat intrinsic cardiac ganglia, activation of cholinergic receptors causes an increase in $[Ca^{2+}]_i$, due to the mobilization of $Ca^{2+}$ from extra- and intracellular sources. An increase in $[Ca^{2+}]_i$ in response to focal application of ACh was reduced by approximately half by either of the selective n- or mAChR antagonists, mecamylamine or atropine, respectively. Simultaneous application of mecamylamine and atropine inhibited the $Ca^{2+}$ response by >80%. Therefore in individual rat intracardiac neurons, both nicotinic and muscarinic AChRs contribute to the $[Ca^{2+}]_i$ increase observed in response to ACh similar to that observed in carotid body type 1 cells (Dasso et al. 1997). The remaining small ACh-induced $[Ca^{2+}]_i$ response observed in the presence of mecamylamine and atropine may be due to incomplete block of nAChRs with a low sensitivity to mecamylamine, such as the homomeric α7 receptor channel.

Under physiological conditions, the primary neurotransmitter, ACh, is released from synaptic boutons on the cell body of intracardiac neurons (Klemm et al. 1997), which may be expected to lead to spatially organized exposure to ACh and not uniformly exposed to neurotransmitter as occurs under our experimental conditions. Hence, the physiological response to ACh in vivo may be different to that observed under experimental conditions depending on the distribution of n- and mAChR with respect to synaptic boutons.

Previous studies in rat intracardiac neurons have shown that the activation of nAChRs, a nonselective cation channel, induces a rapid inward current primarily due to the electrochemical gradients for Na+ and $Ca^{2+}$ influx with a concurrent depolarization (Fieber and Adams 1991; Nutter and Adams 1995). Our experiments on voltage-clamped, fura-2-loaded rat intracardiac neurons show that the fraction of the inward current carried by $Ca^{2+}$ causes a rise in global $[Ca^{2+}]_i$. This result confirms previous reports of a substantial $Ca^{2+}$ influx in rat parasympathetic (Adams and Nutter 1992) and sympathetic (Rogers and Dani 1995; Trouslard et al. 1993) neurons on activation of nAChR channels. Under physiological conditions, however, the increase in $[Ca^{2+}]_i$ after nAChR activation is most likely to be due to $Ca^{2+}$ influx through both nAChR channels and voltage-dependent $Ca^{2+}$ channels that are likely to open on membrane depolarization during an action potential.

Ryanodine-sensitive $Ca^{2+}$ stores have been shown to mediate CICR, which amplifies and sharpens the $[Ca^{2+}]_i$ signal in vertebrate neurons (Hernandez-Cruz et al. 1997; Verkhratsky and Shmigol 1996). There is evidence for the involvement of intracellular ryanodine receptors in the regulation of $Ca^{2+}$ homeostasis in mammalian autonomic neurons. Ryanodine reduced nAChR-mediated increases in $[Ca^{2+}]_i$ by 50%, suggesting that part of the $[Ca^{2+}]_i$ increase evoked by nAChR activation is due to $Ca^{2+}$ release from ryanodine-sensitive $Ca^{2+}$ stores. Further evidence for the presence of CICR in rat intracardiac neurons, was the reduction to 14% by ryanodine of the increase in $[Ca^{2+}]_i$ evoked by focal application of caffeine, which is known to activate ryanodine receptors and inhibit IP3 receptors (Ehrlich et al. 1994). The presence of ryanodine-sensitive $Ca^{2+}$ stores and CICR in rat intracardiac neurons reflects the importance of the intracellular second-messenger $Ca^{2+}$ and its regulation.

Cyclopiazonic acid blocks uptake of cytoplasmic $Ca^{2+}$ into intracellular $Ca^{2+}$ stores mediated by $Ca^{2+}$-ATPases (Pozzan et al. 1994), leading to depletion of intracellular $Ca^{2+}$ stores and increased resting $[Ca^{2+}]_i$ levels in rat intracardiac neurons. The persistent increase in $[Ca^{2+}]_i$ after depleting the intracellular $Ca^{2+}$ stores shows that $Ca^{2+}$ entry through $Ca^{2+}$ permeable nAChR and/or voltage-dependent $Ca^{2+}$ channels, and the amount of the transient $Ca^{2+}$ increase is sufficient to cause significant increases in global $[Ca^{2+}]_i$. In this context, the activation of nAChRs in hippocampal astrocytes ( Sharma and Vijayaraghavan 2001), sympathetic nerve varicosities (Brain et al. 2001) and chick ciliary ganglion cells (Shoop et al. 2001) has recently been shown to induce CICR after $Ca^{2+}$ influx through nAChRs. Furthermore, in adult rat sympathetic neurons, it has been shown that increases in $[Ca^{2+}]_i$ induced by the nonselective cholinergic agonist, carbachol, were not dependent on intracellular $Ca^{2+}$ pools (Foucart et al. 1995). How-
ever, in the present study on rat intracardiac neurons, ACh-induced \([Ca^{2+}]\) increases observed in the presence of mecamylamine are independent of extracellular \(Ca^{2+}\), suggesting that \(Ca^{2+}\) mobilization by mACHRs activation is largely due to \(Ca^{2+}\) release from intracellular \(Ca^{2+}\) stores. The PLC inhibitor U-73122 significantly reduced \([Ca^{2+}]\), responses to muscarine suggesting that \([Ca^{2+}]\), increases are mediated by PLC activation and \(Ca^{2+}\) release from IP_3-sensitive \(Ca^{2+}\) stores. M1 and M3 receptor subtypes are coupled to an intracellular second-messenger pathway leading to the production of IP_3 and subsequent release of \(Ca^{2+}\) from intracellular IP_3-sensitive \(Ca^{2+}\) stores. Although ACh and muscarine have different relative affinities for mACHR subtypes, the inhibition of muscarine-induced \([Ca^{2+}]\), increases by the M1 receptor antagonist, pirenzepine, suggests that the M1 receptor mediates a substantial increase in ACh-induced \(Ca^{2+}\) response when nAChRs are blocked. Under voltage-clamp conditions, the \([Ca^{2+}]\), response mediated by mACHR activation is often accompanied by an outward current that is likely due to the activation of \(Ca^{2+}\)-dependent \(K^+\) channels. The remaining small inward current observed in the presence of mecamylamine might be due to incomplete block or the presence of nAChR channels with low sensitivity to mecamylamine. In adult rat sympathetic neurons, the contributions of the intracellular second-messenger pathways to the regulation of \([Ca^{2+}]\), are reported to be more complex with at least two mACHR subtypes expressed which mediate opposite effects on \([Ca^{2+}]\), homeostasis (Foucart et al. 1995).

In conclusion, we have shown that n- and mACHR activation in rat intracardiac neurons may differentially mobilize extra- and intracellular \(Ca^{2+}\) via two distinct signaling pathways. A schematic diagram of the signal transduction pathways mediated by n- and mACHR activation is shown in Fig. 7. nACHR activation leads to an ionicotropic response that involves \(Ca^{2+}\) influx across the plasma membrane and the resulting \(Ca^{2+}\) transient is potentiated by CICR via \(Ca^{2+}\) release from ryanodine-sensitive intracellular stores. In contrast, mACHR activation leads to a metabotropic response that involves the generation of IP_3 and subsequent \(Ca^{2+}\) release from IP_3-sensitive intracellular stores. The presence of CICR and IP_3-mediated \(Ca^{2+}\) release mechanisms facilitate the coupling of electrical signals to \([Ca^{2+}]\), levels and contribute to cholinergic transmission in intracardiac ganglia. Furthermore, the nature and spatial distribution of ACh receptors and intracellular \(Ca^{2+}\) signals may play a significant role in the differentiated regulation of \(Ca^{2+}\)-dependent mechanisms, in particular, in response to synaptic and nonsynaptic input received from adja-

**DISCUSSIONS**

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**REFERENCES**


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