Muscarinic and nicotinic ACh receptor activation
differentially mobilize Ca$^{2+}$ in rat intracardiac neurons

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ABSTRACT

The origin of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{\text{i}}\)]) transients stimulated by nicotinic (nAChR) and muscarinic (mAChR) receptor activation was investigated in fura-2 loaded neonatal rat intracardiac neurons. ACh evoked [Ca\(^{2+}\)\(_{\text{i}}\)] increases which were reduced to ~60% of control in the presence of either atropine (1 \(\mu\)M) or mecamylamine (3 \(\mu\)M) and to < 20% in the presence of both antagonists. Removal of external Ca\(^{2+}\) reduced ACh-induced responses to 58% of control, which was unchanged in the presence of mecamylamine but reduced to 5% of control by atropine. The nAChR-induced [Ca\(^{2+}\)\(_{\text{i}}\)], response was reduced to 50% by 10 \(\mu\)M ryanodine whereas the mAChR-induced response was unaffected by ryanodine suggesting that Ca\(^{2+}\) release from ryanodine-sensitive Ca\(^{2+}\) stores may only contribute to the nAChR-induced [Ca\(^{2+}\)\(_{\text{i}}\)] responses. Perforated patch whole-cell recording at –60 mV shows that the rise in [Ca\(^{2+}\)\(_{\text{i}}\)], is concomitant with slow outward currents upon mAChR activation and with rapid inward currents following nAChR activation. In conclusion, different signaling pathways mediate the rise in [Ca\(^{2+}\)\(_{\text{i}}\)], and membrane currents evoked by ACh binding to nicotinic and muscarinic receptors in rat intracardiac neurons.

Keywords: parasympathetic ganglia, intracellular Ca\(^{2+}\), membrane current, Ca\(^{2+}\)-induced Ca\(^{2+}\) release, ryanodine.
**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\(^{2+}\)]\(_i\)) following 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 \(\alpha_3\) subunit in combination with \(\beta_2\) and/or \(\beta_4\) and \(\alpha_5\) subunits (Poth et al. 1997). These neurons also express the \(\alpha_7\) subunit, which can form a functional homomeric pentamer that may correspond to the \(\alpha\)-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) which 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 Birdsall 1998). Interestingly, in adult rat sympathetic neurons it has been suggested that increases in [Ca$^{2+}$], evoked by the non-selective cholinergic agonist carbachol are not dependent on intracellular stores (Foucart et al. 1995). Activation of the M1 receptor has been shown to inhibit muscarine-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 non-selective 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 and phosphorylation of protein kinase A-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 nicotinic and muscarinic ACh receptor 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 and cell culture

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 (mM): 140 NaCl, 3 KCl, 2.5 CaCl₂, 0.6 MgCl₂, 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 Corp., Freehold, NJ).

Following enzymatic treatment, clusters of ganglia were dissected, transferred to a sterile
culture dish containing high glucose culture media (Dulbecco’s Modified Eagle Media), 10%
(v/v) fetal calf serum, 100 U/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% air, 5% CO₂ atmosphere.

Microfluorimetric measurements

Measurement of \([\text{Ca}^{2+}]_i\) 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% w/v
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 x oil immersion objective
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 fibre 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 ryanodine 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-optics. The fluorescence emission was detected by a photomultiplier via a detection pinhole of variable size and position (Uttenweiler et al. 1995).

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

$$[\text{Ca}^{2+}]_i = K_d \times \frac{R - R_{\text{min}}}{R_{\text{max}} - R} \times \frac{S_f}{S_b}$$

The ratio of the fluorescence intensities ($R = F_{340}/F_{380}$) 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 $\text{Ca}^{2+}$-EGTA solutions (Grynkiewicz et al. 1985). Measurements of solutions with $[\text{Ca}^{2+}]$ in a range between 0 and $\geq 1 \mu M$ allowed to determine the dissociation constant, $K_d = 473 \text{ nM}$. 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 ($S_2/S_0$) = 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 borosilicate glass (Harvard Apparatus Ltd., 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 following 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 Inc., Union City, CA). Signals were filtered at 200 Hz then digitised at 1 kHz (Digidata 1200A interface, Axon Instruments Inc.) 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 (mM): 140 NaCl, 3 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 7.7 glucose and 10 HEPES-NaOH, pH 7.2 or Ca²⁺-free PSS containing 1mM EGTA (<10 nM free Ca²⁺). In these neurons, the nicotinic ACh-induced current amplitude was maximal in the presence of 2.5 mM extracellular Ca²⁺ (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 K₂SO₄, 55 KCl, 5 Mg SO₄ 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 vapour pressure osmometer (Wescor 5500, Logan, UT). Agonists were applied to cells by brief pressure ejection (≤ 10 psi; Picospritzer II, General Valve Corp., Fairfield, NJ) from an extracellular micropipette (3 - 5 µm diameter) 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 [Ca²⁺]ₐ increases, were used for cholinergic receptor activation. To minimise 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 Co., St. Louis, MO), cyclopiazonic acid, ryanodine, U-73122 and U-73343 (Calbiochem Corp., La Jolla, CA), pirenzepine dihydrochloride (Research Biochemicals International, Natick, MA), fura 2/AM, fura-2 pentapotassium salt, and pluronic-127 (Molecular Probes, Eugene, OR).
Data Analysis

Data are expressed as the mean ± S.E.M. and n values refer to the number of cells. Data were analysed 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 nicotinic and muscarinic ACh receptors 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\(^{2+}\)]\(_i\), following activation of cholinergic receptors in fura-2-loaded rat intracardiac neurons. The mean $\Delta$[Ca\(^{2+}\)]\(_i\) evoked by 300 μM ACh was 103 ± 9 nM from a resting [Ca\(^{2+}\)]\(_i\) of 94 ± 9 nM (n = 49) as shown in Fig. 1. ACh-evoked [Ca\(^{2+}\)]\(_i\) responses remained stable with constant amplitude upon repeated agonist application at intervals of ≥ 100 s. The relative contribution of nicotinic and muscarinic ACh receptor activation to the increase in [Ca\(^{2+}\)]\(_i\) 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\(^{2+}\)]\(_i\) response to 66 ± 5% of control (n = 4). A similar inhibition of the ACh-induced [Ca\(^{2+}\)]\(_i\) response (reduced to 60 ± 6%, n = 8) was observed upon 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\(^{2+}\)]\(_i\) response to 19 ± 10% of control (n = 3), but did not inhibit the [Ca\(^{2+}\)]\(_i\) increase completely. Figure 1B shows the relative $\Delta$[Ca\(^{2+}\)]\(_i\) in response to ACh activation of nAChRs and mAChRs, respectively.
Sources of Ca\textsuperscript{2+} mobilized by nAChR and mAChR activation in rat intracardiac neurons

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

Increases in [Ca\textsuperscript{2+}]\textsubscript{i}, evoked in response to focal application of 300 µM muscarine in the presence of external Ca\textsuperscript{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 Birdsall, 1998). This suggests that the M1 mAChR subtype most likely contributes to the increase in [Ca\textsuperscript{2+}]\textsubscript{i}, in response to mAChR activation.

In order to characterize the signal transduction pathway for muscarinic receptor-mediated mobilization of intracellular Ca\textsuperscript{2+}, the PLC inhibitor, U-73122, was examined on muscarine-induced [Ca\textsuperscript{2+}]\textsubscript{i} transients in rat intracardiac neurons. Bath application of 5 µM U-73122 reversibly inhibited the amplitude of [Ca\textsuperscript{2+}]\textsubscript{i}, transients induced by either 100 µM
muscarine or 100 µM ACh in the presence of mecamylamine. The ACh-induced [Ca^{2+}]_i increases were reduced by 82 ± 4% (n = 5) after 5 min exposure to U-73122 in all cells examined whereas the inactive analogue of U-73122, U-73343 (5 µM), did not significantly reduce the mAChR-mediated [Ca^{2+}]_i responses (n =2). Taken together these results suggest that the G protein-coupled muscarinic receptors in rat intracardiac neurons activate PLC to stimulate IP_3 production and release Ca^{2+} from intracellular stores.

[Figure 2 near here]

**Nicotinic ACh receptor activation induces Ca^{2+} release from internal Ca^{2+} 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 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+}]_i increase (114 ± 19 nM, n = 4) which was inhibited by bath application of 10 µM ryanodine (14 ± 5 % of control, n = 4, P < 0.0005) 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+}]_i due to caffeine is therefore underestimated whereas the relative caffeine-induced changes in [Ca^{2+}]_i 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+}]_i obtained in the presence of atropine (1 µM) was significantly reduced upon bath application of 10 µM ryanodine (50 ± 2%, n = 4, P < 0.0005)
as shown in Fig. 3A,B. In contrast, ryanodine (10 µM) did not affect the mAChR-mediated 
[Ca\textsuperscript{2+}], increase obtained in response to focal application of ACh + mecamylamine (86 ± 8% of control, n = 6). This suggests that the release of Ca\textsuperscript{2+} from ryanodine-sensitive Ca\textsuperscript{2+} stores contributes to the [Ca\textsuperscript{2+}], response evoked by nAChR but not mAChR activation.

[Figure 3 near here]

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

To further elucidate the nAChR-dependent activation of internal Ca\textsuperscript{2+} stores, we tested cyclopiazonic acid (CPA), a sarcoplasmic/endoplasmic reticulum Ca\textsuperscript{2+}- ATPase (SERCA) inhibitor that has been reported to deplete intracellular Ca\textsuperscript{2+} stores sensitive to IP\textsubscript{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\textsuperscript{2+} levels with nAChR activation and the involvement of intracellular Ca\textsuperscript{2+} stores in this response was investigated in rat intracardiac neurons during bath application of 10 µM CPA. Figure 4 shows a representative [Ca\textsuperscript{2+}], response obtained upon 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\textsuperscript{2+}], by 142 ± 31 nM (n = 6). The amplitude of the first ACh-induced [Ca\textsuperscript{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\textsuperscript{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\textsuperscript{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 following nAChR activation may be mainly due to two components: Ca\(^{2+}\) influx across the plasma membrane and Ca\(^{2+}\) release from internal stores.

[Figure 4 near here]

**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 below). 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 which consisted of an
inward current ($-154 \pm 15$ pA, n = 7, $P < 0.001$) followed by a slow outward current ($26 \pm 6$ 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 $[Ca^{2+}]_i$ response was unchanged in the presence of a Ca$^{2+}$-free extracellular solution whereas the inward current was reduced ($-68 \pm 16$ pA, n = 7) and the outward current amplitude was substantially increased ($97 \pm 18$ pA, n = 7). The residual inward current was abolished in the presence of 10 µM mecamylamine (n = 3). Under voltage clamp conditions, the $[Ca^{2+}]_i$ 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.

[Figure 5 near here]

**[Ca$^{2+}]_i$ and membrane current responses evoked upon 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 µM) 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, $P <$
and the inward current amplitude were substantially reduced (32 ± 10% of control, n = 4, \( P = 0.05 \)) as shown in Fig. 6A,B. The ACh-induced outward current observed in the presence of mecamylamine (3 \( \mu \text{M} \)) was abolished in the presence of 1 \( \mu \text{M} \) atropine. Under voltage clamp conditions, the \([\text{Ca}^{2+}]_i\), response to nAChR activation observed in the absence and presence of extracellular \( \text{Ca}^{2+} \) was similar to that observed in unclamped neurons. The \([\text{Ca}^{2+}]_i\), response measured simultaneously was not significantly different to that observed under non-clamped conditions indicating that \( \text{Ca}^{2+} \) influx through open voltage-dependent \( \text{Ca}^{2+} \) channels does not, under these conditions, appreciably contribute to the \([\text{Ca}^{2+}]_i\), 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 \([\text{Ca}^{2+}]_i\), due to the mobilization of \( \text{Ca}^{2+} \) from extra- and intra-cellular sources. An increase in \([\text{Ca}^{2+}]_i\), in response to focal application of ACh was reduced by approximately half by either of the selective nAChRs or mAChRs antagonists, mecamylamine or atropine, respectively. Simultaneous application of mecamylamine and atropine inhibited the \( \text{Ca}^{2+} \) response by > 80%. Therefore, in individual rat intracardiac neurons, both nicotinic and muscarinic AChRs contribute to the \([\text{Ca}^{2+}]_i\), 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 \([\text{Ca}^{2+}]_i\), 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 \( \alpha 7 \) 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 organised 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 nAChR and mAChR, with respect to synaptic boutons.

Previous studies in rat intracardiac neurons have shown that the activation of nAChRs, a non-selective 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\(^{2+}\)]. 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 upon activation of nAChR channels. Under physiological conditions, however, the increase in [Ca\(^{2+}\)] 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 upon membrane depolarization during an action potential.

Ryanodine-sensitive Ca\(^{2+}\) stores have been shown to mediate CICR which amplifies and sharpens the [Ca\(^{2+}\)], 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\(^{2+}\)], by 50% suggesting that part of the [Ca\(^{2+}\)], 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\(^{2+}\)], 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 Ca²⁺ stores and CICR in rat intracardiac neurons reflects the importance of the intracellular second messenger Ca²⁺ and its regulation.

Cyclopiazonic acid blocks uptake of cytoplasmic Ca²⁺ into intracellular Ca²⁺ stores mediated by Ca²⁺-ATPases (Pozzan et al. 1994) leading to depletion of intracellular Ca²⁺ stores and increased resting [Ca²⁺], levels in rat intracardiac neurons. The persistent increase in [Ca²⁺], after depleting the intracellular Ca²⁺ stores shows that Ca²⁺ entry through Ca²⁺ permeable nAChR and/or voltage-dependent Ca²⁺ channels, and the amount of the transient Ca²⁺ increase is sufficient to cause significant increases in global [Ca²⁺]. 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 following Ca²⁺ influx through nAChRs. Furthermore, in adult rat sympathetic neurons it has been shown that increases in [Ca²⁺], induced by the non-selective cholinergic agonist, carbachol, were not dependent on intracellular Ca²⁺ pools (Foucart et al. 1995). However, in the present study on rat intracardiac neurons, ACh-induced [Ca²⁺], increases observed in the presence of mecamylamine are independent of extracellular Ca²⁺ suggesting that Ca²⁺ mobilization by mAChRs activation is largely due to Ca²⁺ release from intracellular Ca²⁺ stores. The PLC inhibitor U-73122 significantly reduced [Ca²⁺], responses to muscarine suggesting that [Ca²⁺], increases are mediated by PLC activation and Ca²⁺ release from IP₃-sensitive Ca²⁺ stores. M1 and M3 receptor subtypes are coupled to an intracellular second messenger pathway leading to the production of IP₃ and subsequent release of Ca²⁺ from intracellular IP₃-sensitive Ca²⁺ stores. Although ACh and muscarine have different relative affinities for mAChR subtypes, the inhibition of muscarine-induced [Ca²⁺], increases by the M1 receptor antagonist, pirenzepine, suggests that the M1 receptor mediates a substantial increase in ACh-induced
Ca\textsuperscript{2+} response when nAChRs are blocked. Under voltage-clamp conditions, the [Ca\textsuperscript{2+}]\textsubscript{i} response mediated by mAChR activation is often accompanied by an outward current which is likely due to the activation of Ca\textsuperscript{2+}-dependent K\textsuperscript{+} 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\textsuperscript{2+}]\textsubscript{i} are reported to be more complex with at least two mAChR subtypes expressed which mediate opposite effects on [Ca\textsuperscript{2+}]\textsubscript{i} homoeostasis (Foucart et al. 1995).

In conclusion, we have shown that nAChR and mAChR activation in rat intracardiac neurons may differentially mobilize extra- and intra-cellular Ca\textsuperscript{2+} via two distinct signaling pathways. A schematic diagram of the signal transduction pathways mediated by nAChR and mAChR activation is shown in Fig. 7. Nicotinic AChR activation leads to an ionotropic response that involves Ca\textsuperscript{2+} influx across the plasma membrane and the resulting Ca\textsuperscript{2+} transient is potentiated by CICR via Ca\textsuperscript{2+} release from ryanodine-sensitive intracellular stores. In contrast, mAChR activation leads to a metabotropic response that involves the generation of IP\textsubscript{3} and subsequent Ca\textsuperscript{2+} release from IP\textsubscript{3}-sensitive intracellular stores. The presence of CICR and IP\textsubscript{3}-mediated Ca\textsuperscript{2+} release mechanisms facilitate the coupling of electrical signals to [Ca\textsuperscript{2+}]\textsubscript{i} levels and contribute to cholinergic transmission in intracardiac ganglia. Furthermore, the nature and spatial distribution of ACh receptors and intracellular Ca\textsuperscript{2+} signals may play a significant role in the differentiated regulation of Ca\textsuperscript{2+}-dependent mechanisms, in particular, in response to synaptic and non-synaptic input received from adjacent cells.
ACKNOWLEDGMENTS

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FIG. 1. Activation of nicotinic and muscarinic ACh receptors increases [Ca\(^{2+}\)]\(_i\) in rat intracardiac neurons. 

**A**: representative traces of ACh-induced increases in [Ca\(^{2+}\)]\(_i\) in response to ACh activating muscarinic and nicotinic receptors. Fura-2 loaded cells were continuously perfused with 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\(^{2+}\)]\(_i\) are indicated by the broken lines. 

**B**: bar graph of relative changes in ACh-induced [Ca\(^{2+}\)]\(_i\) increases (Δ[Ca\(^{2+}\)]\(_i\)) 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).

FIG. 2. Sources of Ca\(^{2+}\) mobilized by nAChR and mAChR activation in rat intracardiac neurons. 

**A**: representative [Ca\(^{2+}\)]\(_i\) responses to focal application of 300 µM ACh obtained in the presence (control) and absence of extracellular Ca\(^{2+}\) (Ca\(^{2+}\)-free PSS). The bath solution contained either 3 µM mecamylamine or 1 µM atropine to inhibit nAChRs and mAChRs, respectively, as indicated by horizontal bars. 

**B**: bar graph of relative ACh-induced [Ca\(^{2+}\)]\(_i\) increases (Δ[Ca\(^{2+}\)]\(_i\)) 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\(^{2+}\)]\(_i\).

**A**: representative [Ca\(^{2+}\)]\(_i\) increases to nAChR activation obtained in response to ACh (300
µM) + atropine (1 µM) were significantly reduced by bath application of 10 µM ryanodine. Caffeine (10 mM) -induced increases in [Ca²⁺], are attenuated in the presence of 10 µM ryanodine. B: bar graph of relative Δ[Ca²⁺], in response to ACh + atropine, ACh + atropine and ryanodine (n = 4, P < 0.05). Caffeine-induced [Ca²⁺], responses were inhibited by ryanodine (n = 4, P < 0.005).

FIG. 4. Nicotinic ACh receptor activation induces Ca²⁺ release from intracellular Ca²⁺ stores. A: representative trace of the increase the resting [Ca²⁺], upon bath application of 10 µM cyclopiazonic acid (CPA, black bar). In the maintained presence of CPA, subsequent [Ca²⁺], responses induced by nAChR activation (300 µM ACh + 1 µM atropine) became progressively smaller but were not abolished. B: comparison of the relative peak Δ[Ca²⁺], response evoked by repetitive application of ACh (300 µM) + atropine (1 µM) as a function of time in the absence (control, open symbols; n = 8) and in the presence of CPA (filled symbols; n = 6).

FIG. 5. [Ca²⁺], and membrane current responses to mAChR activation in voltage-clamped rat intracardiac neurons. A: representative traces of [Ca²⁺], in response to activation of muscarinic receptors by ACh in PSS (2.5 mM Ca²⁺) and Ca²⁺-free external solutions containing mecamylamine (3 µM), as indicated by horizontal bars. B: bar graph of the relative changes in ACh-induced membrane current amplitude. ACh evoked a transient inward current in PSS (control). In the presence of mecamylamine (3 µM), ACh-evoked a biphasic response whereby the amplitude of the inward current was reduced and a small, slow outward current was observed which increased in amplitude upon the removal of external Ca²⁺ (n = 7).
FIG. 6. [Ca$^{2+}$], and membrane current responses to nAChR activation in voltage-clamped rat intracardiac neurons. A: simultaneous measurements of [Ca$^{2+}$], and membrane currents in response to activation of nicotinic receptors by ACh in PSS (2.5 mM Ca$^{2+}$) and Ca$^{2+}$-free PSS containing atropine (1 µM), as indicated by horizontal bars. Representative ACh-evoked currents obtained at a holding potential of −60 mV. B: bar graph of the relative changes in ACh-induced [Ca$^{2+}$], increases (□) and peak current amplitude (■). Bath application of 1 µM atropine in the presence of external Ca$^{2+}$ reduced ACh-induced increases in [Ca$^{2+}$], (n = 4, $P < 0.05$) but not peak inward current amplitude. In the absence of external Ca$^{2+}$ (nominally Ca$^{2+}$-free PSS), the increase in [Ca$^{2+}$], as well as the inward current amplitude were significantly reduced (n = 4, $P < 0.05$).

FIG. 7. Schematic diagram of the cholinergic receptors and intracellular Ca$^{2+}$ stores mediating Ca$^{2+}$ entry and release in rat intracardiac neurons. Agonist binding to nAChRs and mAChRs may mobilize Ca$^{2+}$ by stimulating either Ca$^{2+}$ entry or Ca$^{2+}$ release from intracellular stores, respectively. Activation of nAChRs stimulates Ca$^{2+}$ influx via the open nAChR channel and promotes CICR from ryanodine-sensitive Ca$^{2+}$ stores. Activation of mAChRs coupled by a G-protein to phospholipase C (PLC) hydrolysis of phosphotidylinositol-4,5-bisphosphate (PIP$_2$) yielding diacylglycerol (DAG) and IP$_3$. IP$_3$ serves as mediator of Ca$^{2+}$ release from a compartment of the endoplasmatic reticulum (ER). Elevated cytoplasmic [Ca$^{2+}$] activates Ca$^{2+}$-dependent K$^+$ channels (K$_{Ca}$) before being extruded across the plasma membrane and sequestered into the mitochondria or into the ER compartments via a Ca$^{2+}$-ATPase pump.
Figure 1
**Figure 2**

**A**

- Ca\(^{2+}\) - free
- 3 \(\mu\)M Mecamylamine
- 1 \(\mu\)M Atropine

ACh

100 nM

50 s

**B**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Relative (\Delta [\text{Ca}^{2+}]_i)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>1.0</td>
</tr>
<tr>
<td>Ca(^{2+}) - free</td>
<td>0.6</td>
</tr>
<tr>
<td>Ca(^{2+}) - free + Mecamylamine</td>
<td>0.4</td>
</tr>
<tr>
<td>Ca(^{2+}) - free + Atropine</td>
<td>0.2</td>
</tr>
</tbody>
</table>

* p < 0.05
** p < 0.01

Figure 2
Figure 3

A

10 µM Ryanodine

1 µM Atropine

ACh ACh

Caffeine Caffeine

100 nM

B

relative Δ[Ca^{2+}]

0.0 0.2 0.4 0.6 0.8 1.0

Acetylcholine + Atropine + Ryanodine Caffeine + Ryanodine

*** ***
Figure 4

A

10 μM CPA

10 μM Atropine

ACh

100 nM

Time (min)

B

Δ [Ca^{2+}]_i

relative

control

+ CPA

10 μM CPA

0.4 0.5 0.6 0.7 0.8 0.9 1.0 1.1

0 12 14 16 18

Time (min)

Figure 4
Figure 5

(A) ACh amplitude in pA

(B) Peak $I_{ACh}$ amplitude in pA
Figure 6

A. 1 µM Atropine in Ca²⁺-free solution.

B. Graph showing relative peak IACh amplitude and relative Δ[Ca²⁺]i.
Figure 7