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Intravenous anaesthetics inhibit nicotinic acetylcholine receptor-mediated currents and Ca^{2+} transients in rat intracardiac ganglion neurons

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1 The effects of intravenous (i.v.) anaesthetics on nicotinic acetylcholine receptor (nAChR)-induced transients in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and membrane currents were investigated in neonatal rat intracardiac neurons.

2 In fura-2-loaded neurons, nAChR activation evoked a transient increase in $[Ca^{2+}]_I$, which was inhibited reversibly and selectively by clinically relevant concentrations of thiopental. The half-maximal concentration for thiopental inhibition of nAChR-induced $[Ca^{2+}]_i$ transients was 28 μM, close to the estimated clinical EC_{50} (clinically relevant (half-maximal) effective concentration) of thiopental.

3 In fura-2-loaded neurons, voltage clamped at −60 mV to eliminate any contribution of voltage-gated Ca^{2+} channels, thiopental (25 μM) simultaneously inhibited nAChR-induced increases in $[Ca^{2+}]_i$ and peak current amplitudes. Thiopental inhibited nAChR-induced peak current amplitudes in dialysed whole-cell recordings by ~ 40% at −120, −80 and −40 mV holding potential, indicating that the inhibition is voltage independent.

4 The barbiturate, pentobarbital and the dissociative anaesthetic, ketamine, used at clinical EC_{50} were also shown to inhibit nAChR-induced increases in $[Ca^{2+}]_i$ by ~40%.

5 Thiopental (25 μM) did not inhibit caffeine-, muscarine- or ATP-evoked increases in $[Ca^{2+}]_i$, indicating that inhibition of Ca^{2+} release from internal stores *via* either ryanodine receptor or inositol-1,4,5-trisphosphate receptor channels is unlikely.

6 Depolarization-activated Ca^{2+} channel currents were unaffected in the presence of thiopental (25 μM), pentobarbital (50 μM) and ketamine (10 μM).

7 In conclusion, i.v. anaesthetics inhibit nAChR-induced currents and $[Ca^{2+}]_i$ transients in intracardiac neurons by binding to nAChRs and thereby may contribute to changes in heart rate and cardiac output under clinical conditions.

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Abbreviations: BAPTA, 1,2-*bis*(2-aminophenoxy)ethane-*N*,*N*,*N*′, *N*′-tetraacetic acid; CICR, calcium-induced calcium release; clinical EC_{50}, clinically relevant (half-maximal) effective concentration; IC_{50}, half-maximal inhibitory concentration; IP_3, inositol-1,4,5-trisphosphate; i.v., intravenous; mAChR, muscarinic acetylcholine receptor; nAChR, nicotinic acetylcholine receptor; P2, purinergic receptor; PSS, physiological salt solution; RyR, ryanodine receptor; SR, sarcoplasmic reticulum

Introduction

Intravenous (i.v.) anaesthetics are known to affect cardiac parameters such as heart rate and cardiac output under clinical conditions and in chronically instrumented animals (Blake & Korner, 1981; Inoue & Arndt, 1982; Akine *et al*., 2001). The underlying mechanisms are complex and could involve anaesthetic effects on the myocardium, central and peripheral neurons. Interestingly, excitatory neurotransmission in sympathetic ganglia is blocked by i.v. anaesthetics and therefore cannot explain the increases in heart rate observed during i.v. anaesthesia (Nicoll, 1978; Mahmoodi *et al*., 1980). This effect, however, could in part be due to anaesthetic inhibition of parasympathetic neurons that are involved in the regulation of cardiac function (Inoue & Konig, 1988). For example, it has recently been shown that ketamine inhibits nicotinic excitation in cardiac preganglionic parasympathetic neurons of the nucleus ambiguus of the brainstem (Irnaten *et al*., 2002). Postganglionic intracardiac neurons have also been shown to modulate heart rate in a nicotine-dependent manner, indicating an involvement of nicotinic acetylcholine receptor (nAChR) channels (Bibevski *et al*., 2000; Ji *et al*., 2002). These findings strongly suggest an involvement of nAChRs on

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intracardiac neurons in the modulation of heart rate during anaesthesia, but i.v. anaesthetic effects on nAChR channels in these neurons have not been studied to date.

On a molecular level, clinically relevant concentrations of i.v. anaesthetics have been shown to target glutamate receptor channels and a superfamily of ligand-gated ion channels including nicotinic ACh, $GABA_A$, glycine and $5\text{-}HT_3$ receptor channels (Franks & Lieb, 1994; Krasowski & Harrison, 1999; Yamakura *et al*., 2001). In particular, barbiturates and ketamine have also been shown to inhibit several subtypes of neuronal nAChR channels (Andoh *et al*., 1997; Downie *et al*., 2000; Flood & Krasowski, 2000; Yamakura *et al*., 2000; Coates & Flood, 2001; Coates *et al*., 2001; Kamiya *et al*., 2001), although the $\alpha 7$ homomer appears less sensitive (Tassonyi *et al*., 2002). Most of these studies, however, have been carried out in expression systems. This is a drawback because nAChR channels expressed in *Xenopus* oocytes and mammalian cell lines are not necessarily functionally similar to native nAChR channels in ganglionic neurons (Lewis *et al*., 1997). In particular, the effects of anaesthetics on the electrophysiological properties of ligand-gated ion channels in native neurons can differ from those in expression systems, as demonstrated for $GABA_A$ channels in the absence of the (native) channel modulating GABA-receptor-associated protein (Everitt *et al*., 2004). Therefore, to relate studies of anaesthetic effects on ion channels in expression systems to physiological function, it is important to carry out studies in functionally relevant native cells.

While the $\alpha 3$ and either the $\beta 2$ or $\beta 4$ subunits contribute to the composition of nAChRs in all rat intracardiac neurons, $\alpha 7$ subunits are also present in a majority of these neurons (Poth *et al*., 1997; Cuevas & Berg, 1998), which can form a highly Ca^{2+}-permeable homomeric channel when expressed in *Xenopus* oocytes (Bertrand *et al*., 1993; Tassonyi *et al*., 2002). Electrophysiological recordings have shown that neuronal nAChR channels in rat intracardiac ganglia have a higher Ca^{2+} permeability than muscle nAChRs found at the neuromuscular junction (Fieber & Adams, 1991; Adams & Nutter, 1992), and it has recently been shown that nAChR activation in these neurons is followed by an increase in cytoplasmic Ca^{2+} levels concomitant with an influx of Na^+ and Ca^{2+} ions and a transient membrane depolarization (Beker *et al*., 2003). These findings suggest that nAChR channels are likely to play a significant role in the intracellular Ca^{2+} homeostasis of intracardiac neurons.

Given that Ca^{2+} homeostasis is crucial for the regulation of various neuronal functions including membrane excitability, neurotransmitter release and gene transcription (reviewed in Berridge, 1998), it is of interest to determine if anaesthetics alter Ca^{2+} signalling. In addition to nAChR channels, the activation of voltage-dependent Ca^{2+} channels, muscarinic ACh receptors (mAChRs) and purinergic (P2) receptors increase $[Ca^{2+}]_i$ in rat intracardiac neurons (Liu *et al*., 2000; Beker *et al*., 2003). Although voltage-gated Ca^{2+} channels and purinergic P2X receptor channels mediate Ca^{2+} entry, these channels appear to be relatively insensitive to i.v. anaesthetics (Franks & Lieb, 1994; Andoh *et al*., 1997; Hirota *et al*., 2002). The activation of mAChR and P2Y receptors has been shown to initiate Ca^{2+} release from inositol-1,4,5-trisphosphate (IP_3)-sensitive Ca^{2+} stores (Liu *et al*., 2000; Beker *et al*., 2003), whereas ryanodine receptor (RyR) channels have been shown to amplify nAChR-induced Ca^{2+} transients *via* Ca^{2+}-induced Ca^{2+} release (CICR) in these neurons (Beker *et al*., 2003). However, little is known about the effects of these anaesthetics on intracellular Ca^{2+} release channels in neurons. In rat papillary muscles, thiopental may inhibit ryanodine-induced Ca^{2+} release from the sarcoplasmic reticulum (SR) (Komai & Rusy, 1994), whereas in rat smooth aortic muscle, thiopental has been shown to induce Ca^{2+} release from the SR (Mousa *et al*., 2000).

The present study investigates the effects of thiopental, pentobarbital and ketamine on parasympathetic neurons of intrinsic cardiac ganglia. Given that modulation of nAChRs in autonomic neurons may contribute to cardiovascular side effects of i.v. anaesthetics, it was of interest to determine if i.v. anaesthetics affect nAChR-activated membrane currents and cytoplasmic Ca^{2+} levels in rat intracardiac ganglion neurons. A preliminary report of some of these results has been published previously in abstract form (Weber *et al*., 2002).

Methods

Preparation

Parasympathetic neurons from rat intracardiac ganglia were isolated and placed in tissue culture as described previously (Fieber & Adams, 1991). Briefly, Wistar rats (3–8 days old) were killed by cervical dislocation in accordance with guidelines of Animal Experimentation Ethics Committees of the University of Queensland and the University of Heidelberg. The heart was excised and transferred into dissection solution and atria were removed, placed into dissection solution containing collagenase ($0.8\,mg\,ml^{-1}$) and incubated for 1 h at 37°C. After the enzymatic treatment, clusters of ganglia were dissected, transferred to a culture media containing sterile culture dish and triturated using a Pasteur pipette with a narrow, fire-polished opening. The isolated neurons were plated on laminin-coated glass cover slips and incubated at 37°C for 24–48 h under a 95% air, 5% CO_2 atmosphere. Experimental data presented were obtained from >100 cells dissociated from intracardiac ganglia dissected from 43 neonatal rats.

Absorption spectra measurements

Experiments were performed to determine whether thiopental interferes with the absorption characteristics of the ratiometric Ca^{2+}-sensitive fluorescence indicator, fura-2. Absorption spectra of standard Ca^{2+}-EGTA solutions containing either 0 (control) or 25 μM thiopental, and 0 or 10 μM fura-2 pentapotassium salt were recorded with a UV/vis spectrophotometer (Beckman DU 640, Beckman Instruments Inc., Fullerton, CA, U.S.A.) with a wavelength range from 190 to 1100 nm, a spectral excitation bandwidth of $\leqslant$1.8 nm and a silicon diode for the detection of the transmitted light.

Thiopental (25 μM) slightly increased the absorbance of solutions both in the absence and presence of 10 μM fura-2, indicating the presence of a minor unspecific (not dye-related) thiopental effect. To eliminate the nonspecific effect of thiopental on Ca^{2+}-EGTA solutions in general (in the absence of the dye), the absorbance of the dye-free solutions was subtracted from that of dye-containing solutions. The resulting absorbance curves were virtually identical for solutions in

the absence and presence of thiopental, over the complete wavelength spectra of relevance for fura-2 excitation, indicating that thiopental did not interfere with the absorption characteristics of fura-2.

Microfluorimetric measurements

Intracellular $[Ca^{2+}]$ in response to the application of ACh or caffeine was determined in fura-2-loaded rat intracardiac neurons using single-cell ratiometric photometry. Neurons were incubated for 1 h at room temperature in fura-2-loading solution. Subsequently, they were washed in physiological salt solution (PSS) and a recovery period of $\sim$30 min before experiments was used. Isolated neurons were selected for the experiments to minimize synaptic contacts and the activation of nearby cells. Fura-2-loaded cells were illuminated with light from a 75 W xenon arc lamp, which was split by an optical chopper (OC-4000 Optical Chopper, Photon Technology International (PTI), South Brunswick, NJ, U.S.A.) and passed alternately through 340 and 380 nm band-pass filters. A 510 nm band-pass emission filter was used and a variable aperture set around the cell image. The emitted light was collected by a Hamamatsu R 928 photomultiplier tube, the output of which was digitized using a PTI interface and sampled at 5 Hz using Felix 1.1 software (PTI) run on a 133 MHz computer. Some experiments were carried out on a ratiometric imaging system based on an Olympus IX 70 microscope with UV optics, a Polychrome II monochromator (Till Photonics, Gräfeling, Germany) alternating between 340 and 380 nm illumination, a Hamamatsu C3077 CCD camera with a C2400-80 intensifier head to collect the fluorescence images, a Meteor II frame grabber board (Matrox, Dorval, Quebec, Canada) and Simple PCI 5.0 (Compix Inc., Imaging Systems, Cranberry Township, PA, U.S.A.) software run on a 1600 MHz computer.

Changes in intracellular $[Ca^{2+}]$ ($\Delta[Ca^{2+}]_i$) were obtained measuring the ratio of the intensity of the emitted 510 nm fluorescence $R(F_{340}/F_{380})$ when the cell was illuminated with 340 nm light (F_{340}) to that when illuminated with 380 nm light (F_{380}) and converting this ratio to approximate Ca^{2+} concentrations using the equation:

$$[Ca^{2+}]_i = K_d \times \frac{R - R_{min}}{R_{max} - R} \times \frac{S_{f2}}{S_{b2}} \quad (1)$$

where R_{min} and R_{max} are the F_{340}/F_{380} ratios of the Ca^{2+}-free and Ca^{2+}-saturated fura-2 sample, respectively, S_{f2} is F_{380} of the Ca^{2+}-free fura-2 sample and S_{b2} is F_{380} of the Ca^{2+}-bound sample. An 11-step *in vitro* calibration procedure during which $[Ca^{2+}]$ was increased from approximately 0 to saturation of the dye at $>$ mM $[Ca^{2+}]$ was used to determine the numerical values for the constants in this equation using fura-2 pentapotassium salt and standard Ca^{2+}-EGTA solutions (Grynkiewicz *et al*., 1985). Under the experimental conditions of the PTI set-up, the dissociation constant K_d was determined as 178.9 nM.

A concentration–response curve with thiopental as antagonist for nAChR activation was obtained by measuring the peak increase in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) at each antagonist concentration and the experimental data points were fitted using the equation:

$$\Delta[Ca^{2+}]_i/\Delta[Ca^{2+}]_{i(max)} = 1/[1 + ([A]/IC_{50})^n] \quad (2)$$

where $\Delta[Ca^{2+}]_i/\Delta[Ca^{2+}]_{i(max)}$ represents the relative peak increase in $\Delta[Ca^{2+}]_i$, [A] is the antagonist concentration, IC_{50} is the concentration giving half-maximal inhibition and n is the Hill coefficient.

To exclude any thiopental quenching of the ratiometric fura-2 signal, standard calibration procedures were carried out on the ratiometric imaging set-up both in the absence (control: $R_{min} = 0.738 \pm 0.003$, $R_{max} = 1.411 \pm 0.026$, $K_d = 196 \pm 7$ nM ($n = 8$)) and presence of 25 μM thiopental ($R_{min} = 0.735 \pm 0.002$, $R_{max} = 1.410 \pm 0.017$, $K_d = 190 \pm 4$ nM ($n = 8$)). None of these values was significantly different from those obtained under the control condition ($P < 0.3$ for all values), indicating that 25 μM thiopental does not interfere with the spectral characteristics of fura-2.

Electrophysiological recordings

Membrane currents were measured with the whole-cell recording method of the patch-clamp technique, using either the conventional, dialysed (Hamill *et al*., 1981) or the perforated patch (Horn & Marty, 1988) recording configuration. For perforated patch recordings, amphotericin B-containing solutions were used (Beker *et al*., 2003). The pipette was first tip-filled with an antibiotic-free solution to prevent disruption of seal formation and then backfilled with the amphotericin B-containing solution. No systematic differences were observed between dialysed and perforated patch recording configurations. Pipettes were pulled from thin-walled borosilicate glass (150TF; Harvard Apparatus Ltd, Edenbridge, Kent, U.K.) using a Sutter Instruments P-87 pipette puller, fire polished and had resistances of $\sim$2.5 MΩ.

Filled patch pipettes were mounted on a pipette holder connected with the head stage of a patch-clamp amplifier (EPC-7, List-Medical, Darmstadt, Germany or RK300, Bio-Logic, Claix, France). Voltage protocols were applied using Clampex software (Version 8.0, Axon Instruments Inc., Union City, CA, U.S.A.). Calcium channel currents were elicited by step depolarization from -100 mV to -20 mV at 10 s intervals and a –P/4 pulse protocol was employed to subtract leak and transient capacitive currents. Series resistance compensation was routinely used in recording of Ca^{2+} channel currents and the access resistance was usually <2 MΩ following compensation. Currents were filtered at 5 kHz and sampled at 20 kHz using the Digidata 1200 interface (Axon Instruments Inc.). Agonist-evoked currents were filtered at 200 Hz, digitized at 1 kHz and stored on the hard disc of a Pentium PC for further analysis.

Solutions and drugs

Solutions for absorption measurements and for the calibration procedure of the fura-2 signal were standard Ca^{2+}-EGTA solutions containing 10 mM EGTA, 100 mM KCl, 10 mM K-MOPS and either 10 mM $CaCl_2$ (Ca^{2+}-EGTA solution) or no added Ca^{2+} (EGTA solution), respectively (Grynkiewicz *et al*., 1985). The free $[Ca^{2+}]$ of the solution was <0.1 mM in the Ca^{2+}-EGTA solution and approximately zero in the EGTA solution. The dissection solution contained (mM): 140 NaCl, 3 KCl, 2.5 $CaCl_2$, 0.6 $MgCl_2$, 7.7 glucose and 10 histidine (pH adjusted to 7.2 with NaOH). The culture media was high glucose Dulbecco's modified Eagle's media, with 10% (v v^{-1}) foetal calf serum, 100 U ml^{-1} penicillin and

0.1 mg ml^{-1} streptomycin. The fura-2-loading solution was based on PSS containing (mM): 140 NaCl, 3 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 7.7 glucose and 10 HEPES (pH adjusted to 7.2 with NaOH) to which pluronic-127 and fura-2 AM ester from a stock solution (1 mM fura-2/AM in DMSO) was added to obtain a final concentration of 0.02% pluronic-127 and 5 μM fura-2 AM. During experiments, rat intracardiac neurons were continuously superfused with $\sim$2 ml PSS/min. Racemic mixtures of thiopental, pentobarbital or ketamine were added to the extracellular solution, stirred thoroughly and bath applied prior to agonist application. Perfusion volume during application of the anaesthetics ($<$5 min) was approximately 10-fold the chamber volume (1 ml) to ensure that the desired anaesthetic concentrations were obtained before agonist application. Estimated clinically relevant anaesthetic concentrations were taken from the literature (Franks & Lieb, 1994; Krasowski & Harrison, 1999; Yamakura *et al*., 2001) and are free aqueous concentrations corrected for protein binding. Thiopental concentrations are nominal values not adjusted for the 9% sodium carbonate content of the powder. Agonists were applied to intracardiac neurons by pressure ejection (3–8 p.s.i.; Picospritzer II, General Valve Corp., Fairfield, NJ, U.S.A.) from a micropipette (3–5 μm diameter) positioned 50–100 μm from the cell soma. Nicotinic ACh receptor activation was obtained by focal application of maximally effective ACh concentrations (300–500 μM), with maximally effective concentrations of the muscarinic receptor antagonist atropine ($\geqslant$100 nM) present both in the bath solution and extracellular pipette solution. In unclamped cells and perforated patch-clamp experiments, 500 μM ACh or 10 mM caffeine were applied for 1.2 s and a delay of at least 5 min between agonist applications was maintained. In dialysed patch-clamp experiments, 300 μM ACh was applied for 0.1 s and a delay of $\geqslant$70 s between ACh applications was maintained to minimize receptor desensitization. Agonists were applied several times before the superfusion of the anaesthetic and experiments were continued only if stable responses to agonist application were obtained. The pipette solution for perforated patch experiments contained (mM): 75 K_2SO_4, 55 KCl, 5 $MgSO_4$ and 10 HEPES, titrated with *N*-methyl-D-glucamine to pH 7.2. A stock solution of 60 mg ml^{-1} amphotericin B in DMSO was prepared daily and diluted in pipette solution, providing a final concentration of 240 μg ml^{-1} amphotericin B in 0.4% DMSO, which was kept on ice and protected from light. The pipette solution for dialysed patch experiments contained (mM): 140 CsCl, 2 Mg_2ATP, 5 Cs_4BAPTA, 10 HEPES titrated with CsOH to pH 7.2 and that used to record Ca^{2+} channel currents was supplemented with 0.2 mM NaGTP to minimize current rundown. Whole-cell Ca^{2+} channel currents were recorded using Ba^{2+} as a charge carrier with the bath solution containing 140 TEACl, 4 $BaCl_2$, 10 D-glucose and 10 HEPES-TEAOH (pH 7.2). 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, adenosine 5-triphosphate, amphotericin B, atropine sulphate, α-bungarotoxin, DMSO, ($\pm$) muscarine chloride, (Sigma Chemical Co., St Louis, MO, U.S.A.), ketamine hydrochloride (ICN Biomedicals Inc., Aurora, OH, U.S.A.), pentobarbitone sodium (Rhone Merieux Australia Ltd, Pinkenba, Queensland, Australia), thiopentone sodium (Jurox Pty. Ltd, Rutherford, New South Wales, Australia), caffeine (Fluka Chemie, Buchs, Switzerland), collagenase (type II; Worthington, Biochemical Corp., Lakewood, NJ, U.S.A.), BAPTA (1,2-*bis*(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid) tetracesium salt, fura-2/AM, fura-2 pentapotassium salt and pluronic-127 (Molecular Probes, Eugene, OR, U.S.A.).

Data analysis

Peak (F_{340}/F_{380}) and resting (F_{340}/F_{380}) values of ratiometric fluorescence transients were determined by averaging F_{340}/F_{380} values obtained from a 1.5 s period during the peak response and a 5 s period prior to the agonist application, respectively. Peak and resting F_{340}/F_{380} ratios were then transformed to peak and resting $[Ca^{2+}]_i$, using Eq. (1), and peak increases in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) were calculated by subtracting resting $[Ca^{2+}]_i$ from peak $[Ca^{2+}]_i$. ACh-evoked peak current amplitudes and $\Delta[Ca^{2+}]_i$ in the presence of the anaesthetic were averaged and compared to the mean control response obtained in the absence of the anaesthetic in each individual cell. The mean control responses were determined by averaging responses obtained before and after superfusion with the anaesthetic to take into account cell rundown or incomplete recovery during washout that may occur during the experiment. Data are expressed as the mean $\pm$ s.e.m. (n = number of cells) and were analysed statistically using Student's (paired, two-tailed) t-test with the level of significance being taken as $P<0.05$ (*), 0.01 (**) or 0.001 (***). Levels of significance were conservatively adjusted using the Bonferroni method when currents were tested for differences at several holding potentials.

Results

In fura-2-loaded intracardiac neurons, the mean resting $[Ca^{2+}]_i$ was 46 $\pm$ 4 nM in the absence and 44 $\pm$ 4 nM (n = 30) in the presence of thiopental, pentobarbital and ketamine, respectively. The lack of effect of i.v. anaesthetics on resting Ca^{2+} levels indicates that, in unstimulated neurons, $[Ca^{2+}]_i$ and thus transmembrane concentration gradients for Ca^{2+} ions were unchanged by the i.v. anaesthetics.

Clinically relevant concentrations of thiopental inhibit nAChR-mediated increases in $[Ca^{2+}]_i$ in rat intracardiac neurons

In fura-2-loaded rat intracardiac neurons, focal application of 500 μM ACh to the cell soma in the presence of the muscarinic ACh receptor antagonist, atropine (100 nM) in the bath solution, evoked a rapid, transient increase in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) of 70 $\pm$ 5 nM above resting $[Ca^{2+}]_i$ (n = 21). The ACh-mediated transient increase in $[Ca^{2+}]_i$ was not appreciably changed ($<$10%) in the presence of the 100 nM α-bungarotoxin, a selective antagonist of α7 nAChRs (Cuevas & Berg, 1998).

In the presence of the barbiturate, thiopental (25 μM = clinical EC_{50} (clinically relevant (half-maximal) effective concentration); Franks & Lieb, 1994; Krasowski & Harrison, 1999; Yamakura *et al*., 2001), however, the increase in $[Ca^{2+}]_i$ evoked by focal application of 500 μM ACh in the presence of atropine was inhibited by 42% from 66 $\pm$ 14 nM to 38 $\pm$ 10 nM ($P<0.01$; n = 6) as shown in Figure 1a. A concentration–

response curve was obtained by applying ACh in the presence of atropine and varying concentrations of thiopental. Half-maximal inhibition of $\Delta[Ca^{2+}]_i$ by thiopental occurred at 28 μM, a value comparable to the clinical EC_{50} of thiopental, with a Hill coefficient of 1.24, as shown in Figure 1b.

Thiopental simultaneously inhibits nAChR-induced currents and increases in $[Ca^{2+}]_i$ in voltage-clamped rat intracardiac neurons

To exclude any contribution of a secondary influx of Ca^{2+} ions to the increase in $[Ca^{2+}]_i$ due to the involvement of voltage-dependent Ca^{2+} channels activated by nAChR-induced depolarization of the cell membrane, the influx of Ca^{2+} mediated by nAChR activation was studied under voltage-clamp conditions. Using the perforated patch recording configuration, fura-2-loaded neurons were held at −60 mV and application of 500 μM ACh evoked peak current amplitudes and increases in peak $[Ca^{2+}]_i$ of -602 ± 195 pA and 88 ± 19 nM ($n = 4$), respectively, in the presence of atropine. Bath application of 25 μM thiopental reduced the peak current amplitude by 61% to -237 ± 84 pA ($P < 0.05$; $n = 4$) and the $[Ca^{2+}]_i$ response by 53% to 41 ± 10 nM ($P < 0.05$) as shown in Figure 2. This result shows that a clinically relevant concentration of thiopental inhibits nAChR-induced peak current amplitude and simultaneous increases in $[Ca^{2+}]_i$, without any contribution of voltage-gated Ca^{2+} channels.

Further experiments were carried out using the dialysed patch recording configuration in which the pipette solution contained BAPTA to surmount any intracellular Ca^{2+} buffers. The ACh-evoked currents exhibited strong inward rectification and reversed close to 0 mV as described previously (Fieber & Adams, 1991; Adams & Nutter, 1992). As shown in Figure 3a and b, bath application of 25 μM thiopental reversibly

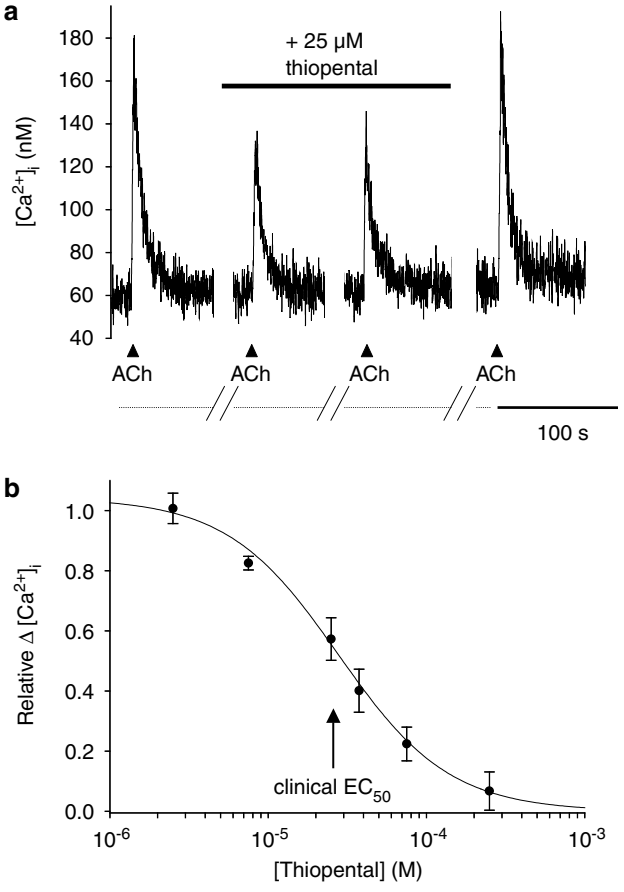

Figure 1 Clinically relevant concentrations of thiopental inhibit nAChR-mediated $[Ca^{2+}]_i$ transients in rat intracardiac neurons. (a) Representative $[Ca^{2+}]_i$ transients in response to focal application of 500 μM ACh obtained in the absence (control) and presence of 25 μM thiopental in the bath solution. The bath solution also contained 100 nM atropine to inhibit mAChRs. (b) Concentration–response relationship for inhibition of ACh-induced $[Ca^{2+}]_i$ transients by thiopental in the presence of 100 nM atropine. Average $[Ca^{2+}]_i$ responses to ACh in the presence of thiopental were normalized to average responses obtained with agonist applications before and after superfusion with thiopental in the same cells. Data points represent the mean normalized $[Ca^{2+}]_i$ response ± s.e.m. from two to seven cells. The curve of best fit to the data had an $IC_{50} = 28$ μM and Hill coefficient of 1.24.

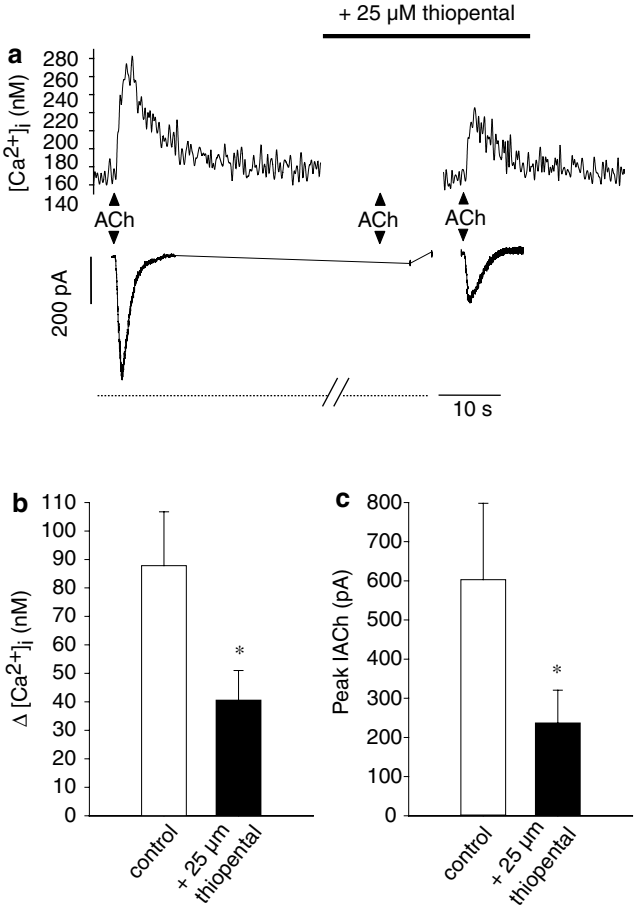

Figure 2 Inhibition of nAChR-induced $[Ca^{2+}]_i$ transients and membrane currents by thiopental. (a) Fura-2-loaded rat intracardiac neurons were voltage clamped at −60 mV using the perforated patch whole-cell recording configuration. Representative traces of transient increases in $[Ca^{2+}]_i$ and whole-cell inward currents recorded simultaneously in response to 500 μM ACh in the absence and presence of 25 μM thiopental bath applied. The bath solution also contained 100 nM atropine. (b and c) Bar graphs of the changes in ACh-induced $[Ca^{2+}]_i$ increases ($\Delta[Ca^{2+}]_i$) and membrane currents (I_{ACh}) by thiopental together with $\geqslant 100$ nM atropine bath applied. Bath application of 25 μM thiopental significantly reduced ACh-induced increases in $[Ca^{2+}]_i$ ($P < 0.05$, $n = 4$) and peak inward current amplitude ($P < 0.05$, $n = 4$).

inhibited nAChR-induced peak current amplitudes at negative holding potentials. Thiopental significantly inhibited peak current amplitudes at −120 mV by 38% from −1077±122 pA to −671±105 pA, at −80 mV by 38% from −732±81 pA to −454±70 pA and at −40 mV by 35% from −314±32 pA to −205±30 pA ($n=11$, $P<0.0033$ for each holding potential). A linear regression of the relative peak current amplitudes and the holding potentials revealed that the slope was not significantly different from zero ($P<0.3$), indicating that thiopental inhibition of nAChR-induced currents was voltage independent over the range of −40 to −120 mV (see Figure 3c).

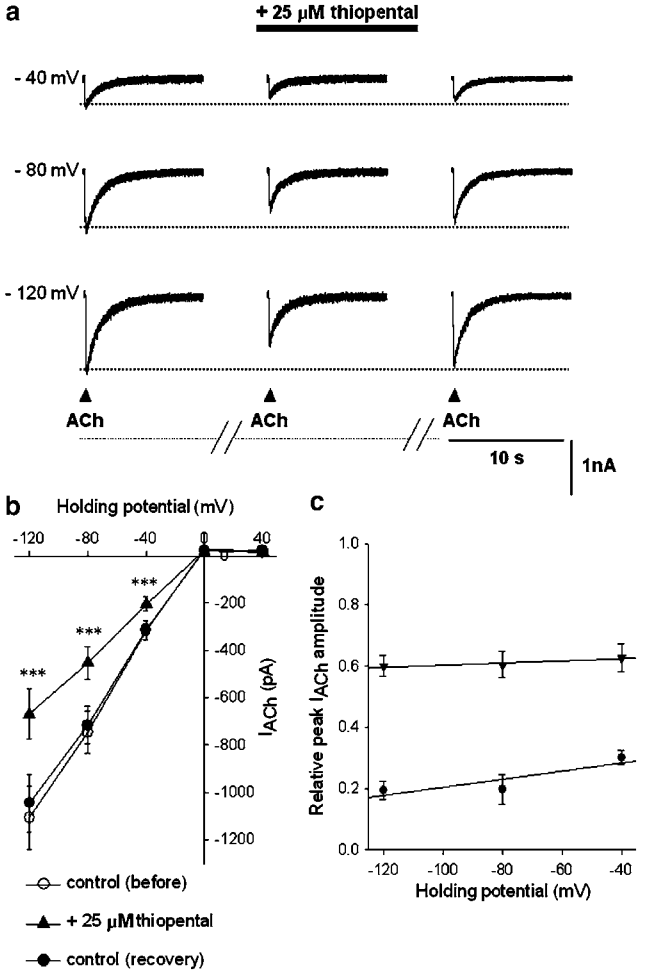

Figure 3 Voltage-independent inhibition of nAChR-mediated membrane currents by thiopental in voltage-clamped rat intracardiac neurons. (a) Whole-cell currents evoked by 300 μM ACh in the presence of 100 nM atropine at various membrane potentials in the absence (control) and presence of 25 μM thiopental, as indicated by the horizontal bar. The dashed horizontal lines indicate control responses obtained by averaging the peak current amplitudes before and after superfusion with thiopental. (b) Current–voltage relationship for peak current (I_{ACh}) amplitude evoked by 300 ACh μM in the presence 100 nM atropine. Data points represent mean peak current amplitudes±s.e.m. of 11 cells, before, during and after superfusion with 25 μM thiopental. (c) Inhibition of ACh-induced peak current (I_{ACh}) amplitudes by thiopental and ketamine as a function of membrane potential in the presence of 100 nM atropine. Data points represent normalized mean peak current amplitudes±s.e.m. in the presence of 25 μM thiopental (triangles, $n=11$) and 10 μM ketamine (circles, $n=3$). Responses were normalized to average responses obtained before and after the superfusion with an anaesthetic in each individual cell. A linear regression of the relative peak current amplitudes and the holding potentials indicated that the slope was not significantly different from zero ($P<0.3$).

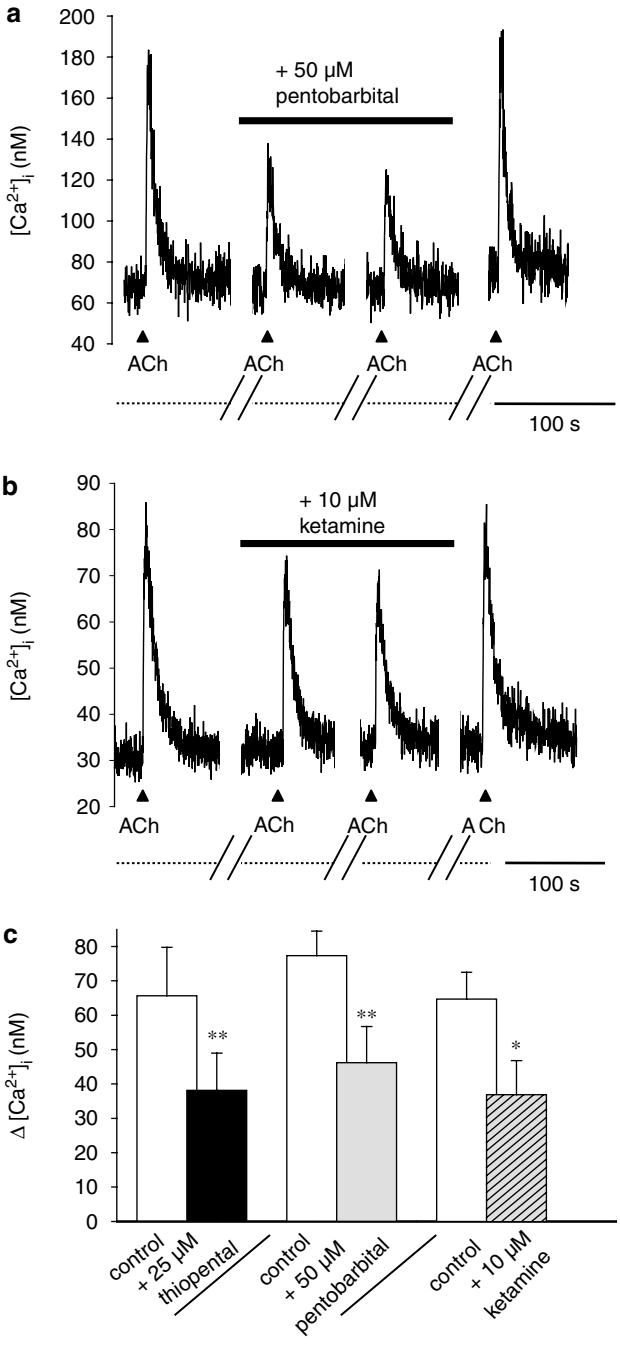

Figure 4 Clinically relevant concentrations of thiopental, ketamine and pentobarbital inhibit nAChR-mediated $[Ca^{2+}]_i$ transients. (a) Representative $[Ca^{2+}]_i$ transients in rat intracardiac neurons in response to focal application of 500 μM ACh obtained in the absence (control) and presence of 50 μM pentobarbital in the bath solution. The bath solution also contained 100 nM atropine to inhibit mAChRs. (b) Representative $[Ca^{2+}]_i$ responses to 500 μM ACh focally applied in the presence of 100 nM atropine obtained in the absence (control) and presence of 10 μM ketamine in the bath solution. (c) Bar graph of $\Delta[Ca^{2+}]_i$ in response to ACh (control), ACh + 25 μM thiopental ($n=6$), 50 μM pentobarbital ($n=8$) and 10 μM ketamine ($n=7$). Atropine (100 nM) was present in all bath solutions to inhibit mAChRs.

Similarly, the inhibition of nAChR-mediated peak current amplitude by 10 μM ketamine was relatively voltage independent at membrane potentials between −40 and −120 mV ($n=3$) consistent with that reported previously for nAChRs in PC12 cells (Furuya *et al*., 1999).

Clinically relevant concentrations of pentobarbital and ketamine also inhibit nAChR-mediated increases in $[Ca^{2+}]_i$ in rat intracardiac neurons

Bath application of the barbiturate, pentobarbital (50 μM = clinical EC_{50}; Franks & Lieb, 1994; Krasowski & Harrison, 1999; Yamakura *et al*., 2001), reversibly inhibited increases in $[Ca^{2+}]_i$ evoked by focal application of 500 μM ACh by 40% from 77±7 to 46±11 nM ($n=8$; $P<0.01$) as shown in Figure 4a. Similarly, the dissociative anaesthetic, ketamine (10 μM = clinical EC_{50}; Krasowski & Harrison, 1999; Yamakura *et al*., 2001), also reversibly inhibited ACh-induced increases in $[Ca^{2+}]_i$ by 43% from 65±8 to 37±10 nM ($n=7$; $P<0.05$) as shown in Figure 4b. Bath application of pentobarbital and ketamine produced a concentration-dependent inhibition of the peak amplitude of ACh-induced increases in $[Ca^{2+}]_i$. A summary of the relative changes in ACh-induced increases in $[Ca^{2+}]_i$ obtained in the presence of the i.v. anaesthetics is presented in Figure 4c.

Ca^{2+} release from caffeine-sensitive Ca^{2+} stores is not inhibited by clinically relevant concentrations of thiopental

Recently, we have shown that Ca^{2+} influx upon nAChR activation leads to CICR from intracellular, ryanodine-sensitive Ca^{2+} stores in rat intracardiac neurons (Beker *et al*., 2003). The secondary Ca^{2+} release from intracellular Ca^{2+} stores *via* RyR channels thus amplifies the nAChR-induced increase in $[Ca^{2+}]_i$. Given that thiopental has been suggested to inhibit ryanodine-induced Ca^{2+} release from the SR of papillary muscle cells (Komai & Rusy, 1994), the effect of thiopental on RyR channels was examined in rat intracardiac neurons. Caffeine, which is known to activate RyR channels while inhibiting IP_3 receptor channels (Ehrlich *et al*., 1994), was used to examine the effects of thiopental on Ca^{2+} release from ryanodine-sensitive intracellular stores. As shown in Figure 5, application of 10 mM caffeine, which is likely to induce maximal Ca^{2+} release from ryanodine-sensitive stores, evoked a transient increase in $[Ca^{2+}]_i$ of 92±14 nM ($n=9$), which was not significantly different from that obtained in the presence of 25 μM thiopental (90±14 nM; 98% of control, $n=9$; $P<0.3$). Increasing the duration of exposure to thiopental to 30 min had no appreciable effect on the amplitude of the caffeine-induced $[Ca^{2+}]_i$ transient. These data indicate that the clinical EC_{50} of thiopental does not inhibit RyR-mediated Ca^{2+} transients in rat intracardiac neurons.

Intravenous anaesthetics at clinical EC_{50} do not inhibit depolarization-activated Ca^{2+} channel currents or muscarine- and ATP-evoked increases in $[Ca^{2+}]_i$ in rat intracardiac neurons

In order to identify the primary molecular target(s) of the action of i.v. anaesthetics in rat intracardiac neurons, a series of experiments were carried out to evaluate the effect of i.v. anaesthetics on other cell-surface receptors and ion channels, which may mediate an increase in $[Ca^{2+}]_i$ in these neurons. The activation of voltage-gated Ca^{2+} channels was examined using Ba^{2+} as the charge carrier and representative traces of Ba^{2+} currents evoked upon step depolarization from −100 to −20 mV in the absence (control) and presence of the i.v. anaesthetics are shown in Figure 6a. Peak Ba^{2+} current amplitude was not significantly changed during ⩾10 min bath application of thiopental (25 μM, $n=4$), pentobarbital (50 μM, $n=5$) and ketamine (10 μM, $n=7$). However, raising the concentrations of either thiopental (100 μM) or pentobarbital (200 μM) four-fold reduced peak Ba^{2+} current amplitude by 19.6±3% ($n=4$) and 19.9±0.01% ($n=3$), respectively.

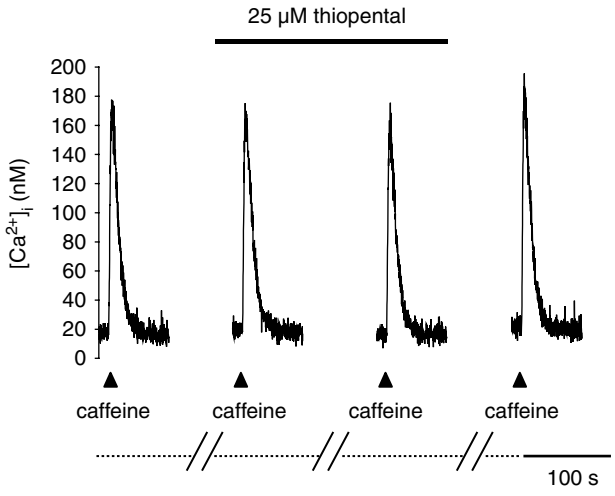

Figure 5 Caffeine-induced $[Ca^{2+}]_i$ transients in rat intracardiac neurons are not inhibited by thiopental. Representative $[Ca^{2+}]_i$ transients evoked upon activation of ryanodine receptors by application of 10 mM caffeine in the absence (control) and presence of 25 μM thiopental, as indicated by the horizontal bar.

The activation of mAChRs and purinergic (P2X and P2Y) receptors have also been shown previously to elicit a transient increase in $[Ca^{2+}]_i$ in rat intracardiac neurons (Liu *et al*., 2000; Beker *et al*., 2003). Bath application of thiopental (25 μM), pentobarbital (50 μM) and ketamine (10 μM) for up to 20 min duration, however, failed to inhibit increases in $[Ca^{2+}]_i$ evoked by focal application of either 100 μM muscarine (Figure 6b) or 100 μM ATP (data not shown). Similarly, the transient increase in $[Ca^{2+}]_i$ evoked upon activation of mAChRs by ACh in the presence of mecamylamine (10 μM) was unchanged in the presence of 25 μM thiopental ($n=3$). These data indicate that the mobilization of Ca^{2+} in rat intracardiac neurons *via* Ca^{2+} entry through voltage-gated Ca^{2+} channels and P2X receptor channels and release from intracellular IP_3-sensitive Ca^{2+} stores (mAChRs and P2Y receptors) appears to be resistant to clinical EC_{50} of the i.v. anaesthetics.

Discussion

The present study shows that activation of nAChR channels in fura-2-loaded rat intracardiac neurons induces an inward current and a transient increase in $[Ca^{2+}]_i$, which can be inhibited by clinically relevant concentrations of i.v. anaes-

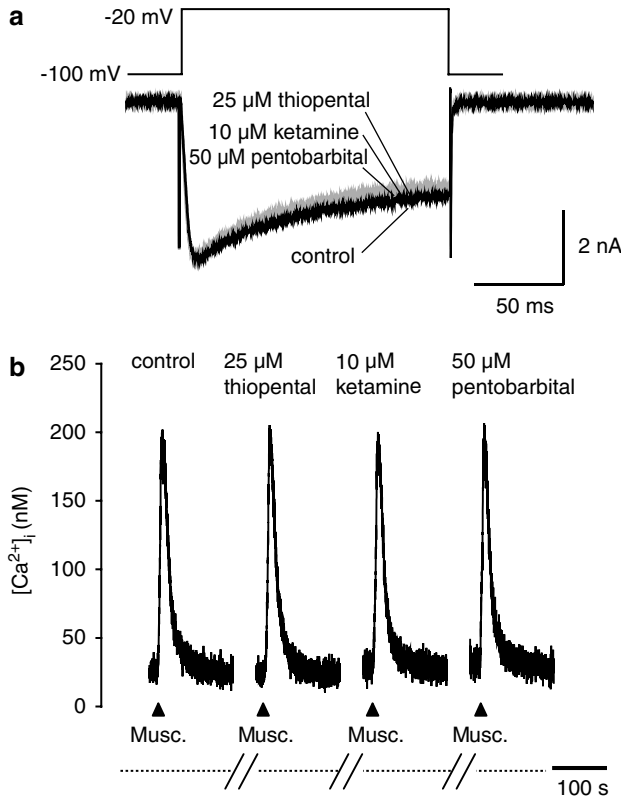

Figure 6 Clinically relevant concentrations of i.v. anaesthetics do not inhibit voltage-dependent Ca^{2+} channel currents or muscarinic ACh receptor-mediated $[Ca^{2+}]_i$ increases in rat intracardiac neurons. (a) Superimposed whole-cell Ba^{2+} currents obtained in response to step depolarization from −100 to −20 mV in the absence (control, black trace) and presence of 25 μM thiopental, 50 μM pentobarbital and 10 μM ketamine (grey traces). (b) Representative $[Ca^{2+}]_i$ transients in response to 100 μM muscarine focally applied in the absence (control) and presence of 25 μM thiopental, 10 μM ketamine and 50 μM pentobarbital in the bath solution.

thetics. Thiopental (25 μM) inhibited nAChR-induced increases in $[Ca^{2+}]_i$ by 42% and a similar inhibition was observed with pentobarbital (50 μM; 40% inhibition) and with the dissociative anaesthetic, ketamine (10 μM; 43% inhibition). These results are consistent with previously reported studies using oocyte expression systems in which i.v. anaesthetics, including barbiturates and ketamine, have been shown to inhibit neuronal nAChR-mediated currents (see Krasowski & Harrison, 1999; Yamakura *et al*., 2001). On a molecular and cellular level, this corresponds with the clinical observation that no specific chemical group is required to produce the effects that occur during general anaesthesia (see Franks & Lieb, 1994). Resting $[Ca^{2+}]_i$ was similar in the absence and the presence of the i.v. anaesthetics. This indicates that concentration gradients for Ca^{2+} were unaffected, and therefore changes in the driving force for Ca^{2+} cannot account for the inhibition of ACh-induced $[Ca^{2+}]_i$ transients observed in the presence of the i.v. anaesthetics. Taken together, these results clearly show that clinically relevant concentrations of i.v. anaesthetics can modulate intracellular Ca^{2+} signals in response to nAChR activation and thus possibly modify various cellular, Ca^{2+}-dependent functions, ranging from neuronal excitability to neurotransmitter secretion in intracardiac ganglia. Bath application of the i.v. anaesthetics prior to ACh application may simulate the situation occurring during the maintenance of general anaesthesia, whereby the receptor and anaesthetic are at equilibrium (Downie *et al*., 2000). The concentration–response curve for the thiopental inhibition of nAChR-induced increases in $[Ca^{2+}]_i$ had a Hill coefficient of 1.24 and a half-maximal inhibition, IC_{50} of 28 μM, which is close to the clinical EC_{50} of thiopental, indicating that the nAChR may represent an important molecular target of clinically relevant concentrations of thiopental. Thus, the data presented strongly suggest that similar effects occur at intracardiac postganglionic neurons receiving nicotinic (vagal) stimulation in the presence of these i.v. anaesthetics during anaesthesia.

Under voltage-clamp conditions using the perforated patch recording configuration, ACh-induced transient increases in $[Ca^{2+}]_i$ concomitant with rapid inward currents were inhibited in the presence of 25 μM thiopental. These data demonstrate that (i) thiopental inhibition of nAChR-induced Ca^{2+} transients in rat intracardiac neurons occurs without any contribution of voltage-gated Ca^{2+} channels and is similar to that observed in unclamped cells and (ii) a common mechanism underlies the simultaneous inhibition of nAChR-induced inward currents and $[Ca^{2+}]_i$ transients by an i.v. anaesthetic, that is, is block of Ca^{2+} influx through nAChRs. Inhibition of ACh-evoked inward currents by thiopental has also been described in PC12 cells (Andoh *et al*., 1997) and for nAChRs exogenously expressed in *Xenopus* oocytes (Downie *et al*., 2000; Coates *et al*., 2001).

Under dialysed patch recording conditions, thiopental inhibited nAChR-mediated currents in rat intracardiac neurons in a voltage-independent manner at membrane potentials from −120 to −40 mV, consistent with that reported previously for thiopental inhibition of nAChRs in PC12 cells (Andoh *et al*., 1997) and human α7 homomeric nAChR channels expressed in *Xenopus* oocytes (Coates *et al*., 2001). The persistence of thiopental block of nAChR-mediated currents in dialysed patch experiments in which the pipette solution dictates the cytoplasmic composition indicates that the mechanism underlying the inhibition is not dependent on diffusible intracellular second messenger pathways. In contrast, the involvement of diffusible intracellular second messengers in mediating the inhibition of receptor channels by anaesthetics in *Xenopus* oocytes cannot be entirely eliminated (Downie *et al*., 2000). In particular, the pipette solution containing the rapid Ca^{2+} chelator, BAPTA, is likely to (i) surmount any endogenous intracellular Ca^{2+} buffer, (ii) to ensure a stable resting $[Ca^{2+}]_i$ and (iii) to substantially suppress CICR. Therefore, it is unlikely that the inhibition of nAChR-induced currents observed in the presence of thiopental is due to a reduced driving force for Ca^{2+} caused either by reduced intracellular Ca^{2+} buffer capacities or increased CICR responses from internal stores. Furthermore, the voltage-independent block of nAChR-induced currents by ketamine is consistent with that reported previously for nAChRs in PC12 cells (Furuya *et al*., 1999). Thus, the inhibition of nAChR-mediated currents in rat intracardiac neurons by these anaesthetic agents is most likely due to a direct action on nAChR channels as proposed previously in studies using oocyte expression systems (Downie *et al*., 2000; Coates *et al*., 2001). Given that the proportion of the ACh-induced whole-cell current carried by Ca^{2+} through rat

neuronal α7 nAChRs has been reported to be <10% (Fucile *et al*., 2003) and that α-bungarotoxin did not appreciably change nAChR-induced Ca^{2+} responses, then it is unlikely that i.v. anaesthetic inhibition of α7 homomers alone contributes significantly to the inhibition of the transient increase in $[Ca^{2+}]_i$ observed in rat intracardiac neurons.

Given that CICR *via* the activation of RyRs has been shown to contribute to nAChR-induced $[Ca^{2+}]_i$ transients in rat intracardiac neurons (Beker *et al*., 2003), the effects of thiopental on RyR-induced CICR were examined upon activation of RyRs with caffeine. Clinically relevant concentrations of thiopental had no effect on caffeine-induced increases in $[Ca^{2+}]_i$, indicating that thiopental is unlikely to inhibit CICR from internal stores by inhibiting RyR channels. It remains to be determined whether thiopental potentiation of Ca^{2+} release from ryanodine-sensitive Ca^{2+} stores may occur, as reported previously, for high concentrations of volatile anaesthetics on Ca^{2+} release from the SR in skeletal muscle (Kunst *et al*., 1999).

Further evidence for the selectivity of i.v. anaesthetic inhibition of nAChRs was obtained from the lack of effect of the i.v anaesthetics at clinically relevant concentrations on depolarization-activated Ba^{2+} currents and muscarine- and ATP-evoked $[Ca^{2+}]_i$ transients. The voltage-dependent Ca^{2+} channels, which are predominantly N-type in rat intracardiac neurons (Xu & Adams, 1992), were unaffected by clinical EC_{50} of thiopental, pentobarbital and ketamine. Furthermore, the increase in $[Ca^{2+}]_i$ that occurs upon the activation of either mAChRs or purinergic (P2X and P2Y) receptors is insensitive to the i.v. anaesthetics compared to nAChR-mediated $[Ca^{2+}]_i$ transients in these neurons. Recombinant muscarinic receptor (M1–M3)-mediated $[Ca^{2+}]$ responses have been shown to be relatively insensitive to i.v. anaesthetics (Hirota *et al*., 2002) and the differential effects of thiopental on neuronal nAChRs and P2X receptors in PC12 cells have also been reported previously (Andoh *et al*., 1997).

In conclusion, we have shown that clinically relevant concentrations of i.v. anaesthetics modulate Ca^{2+} homeostasis in intracardiac neurons by inhibiting ACh-induced currents and $[Ca^{2+}]_i$ transients. We suggest that the inhibition of ACh-induced $[Ca^{2+}]_i$ transients by thiopental is due to a direct interaction with nAChR channels to inhibit Ca^{2+} influx and not a consequence of block of voltage-dependent Ca^{2+} channels, mAChRs, P2 purinoceptors or inhibition of Ca^{2+} release following RyR activation. Given that ACh is the primary neurotransmitter mediating parasympathetic (vagal) regulation of the heart and the presence of functional nAChR channels in intracardiac postganglionic neurons, clinically relevant concentrations of i.v. anaesthetics may modulate cardiac parameters during anaesthesia by binding to postsynaptic nAChRs and depressing ganglionic transmission. The observed reductions in ACh-induced current amplitude by the i.v. anaesthetics would also depress excitatory postsynaptic potential amplitude and therefore affect synaptic transmission in cardiac ganglia. A direct and selective effect of i.v. anaesthetics on nAChR channels in mammalian intrinsic cardiac neurons has not previously been taken into account when interpreting changes in heart rate and cardiac output during anaesthesia. Given the presence of presynaptic nAChRs in peripheral ganglia (see MacDermott *et al*., 1999), it will be of interest to determine if i.v. anaesthetics similarly target nAChRs in preganglionic parasympathetic nerve terminals.

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