Local anaesthetic blockade of neuronal nicotinic ACh receptor-channels in rat parasympathetic ganglion cells

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1 The effects of the local anaesthetics QX-222 and procaine on nicotinic acetylcholine (ACh)-evoked currents in cultured parasympathetic cardiac neurones of the rat were investigated by use of the whole-cell, perforated-patch, and outside-out recording configurations of the patch clamp method.

2 QX-222 and procaine, applied to the extracellular surface, reversibly inhibited the peak amplitude of the whole-cell nicotinic ACh-evoked current in a concentration-dependent manner, with half-maximal inhibitory concentrations (IC_{50}) of 28 μ M and 2.8 μ M, respectively, at -80 mV. In these neurones, the sustained inward current mediated by M_1 muscarinic receptor activation was unaltered by QX-222, and neither local anaesthetic affected the adenosine 5'-triphosphate (ATP)-evoked current.

3 QX-222 and procaine block of nicotinic ACh-evoked inward current was voltage-dependent and enhanced by hyperpolarization. An e-fold change in their dissociation equilibrium constants (K_d) resulted from a 62 mV and a 122 mV change in membrane potential, respectively.

4 Both local anaesthetics produce a concentration-dependent increase in the half-time of decay of the nicotinic ACh-evoked inward current.

5 Measurements of unitary currents in outside-out patches showed that QX-222 reversibly increased the mean burst duration and closed time and reduced the mean channel open time and open-state probability of the nicotinic ACh receptor-channel (AChR) in a concentration-dependent manner.

6 The K_d and voltage sensitivity of local anaesthetic block of the nicotinic AChR in rat intracardiac neurones suggests that the pore-forming region of this channel differs from that of the AChR in frog and rat skeletal muscle and from the neuronal $\alpha_4\beta_2$ ACh receptor-channel.

Keywords: Cardiac ganglia; parasympathetic neurones; nicotinic receptor; muscarinic receptor; acetylcholine; adenosine 5'-triphosphate; local anaesthetic; single channel current; patch-clamp

Introduction

Neuronal nicotinic acetylcholine receptor (AChR) channels are distributed throughout the central and peripheral nervous systems, and are structurally and pharmacologically distinct from the AChR channels at the motor endplate. Whereas the muscle-type nicotinic AChR is a pentameric structure composed of two ACh-binding subunits (α_1) and three structural subunits (β_1 , δ , and γ or ϵ), the neuronal nicotinic AChR appears to be comprised of only two subunit types, the ACh-binding, or α -subunit, and the β (or non- α) subunit. The stoichiometry for the neuronal AChR expressed in *Xenopus* oocytes has been defined as $2\alpha_3\beta_4$ (Anand *et al.*, 1991; Cooper *et al.*, 1991); however, it remains to be confirmed in neurones. At present, four α -subunits (α_2 , α_3 , α_4 , α_7) and two β -subunits (β_2 , β_4) have been demonstrated to be able to contribute to the formation of functional nicotinic AChRs (for review see Papke, 1993; Sargent, 1993). Muscle and neuronal AChRs exhibit differential sensitivity to receptor antagonists such as α -bungarotoxin and neuronal bungarotoxin (see Deneris *et al.*, 1991; Lukas & Bencherif, 1992). Among neuronal nicotinic AChRs composed of different subunit combinations, functional differences in agonist potency and block by neurotoxins have also been reported (Luetje *et al.*, 1990; Luetje & Patrick, 1991). The single channel properties of neuronal nicotinic AChRs are also dependent upon the subunit composition, the stage of development, and the host cell examined (for review see Deneris *et al.*, 1991; Papke, 1993). It would appear that AChRs are a heterogeneous family of neurotransmitter-activated channels with a diverse range of functional properties.

The membrane conductance change mediated by the activation of nicotinic AChRs can be modified by a variety of

drugs which appear to act by blocking within the open channel. Single AChR channel currents show characteristic kinetic alterations in the presence of local anaesthetics, and the voltage-dependence of cationic drug binding indicates that the binding site is within the membrane electric field (see review Colquhoun, 1986). The binding site of the muscle AChR channel for the open-channel blocker QX-222, a quaternary ammonium derivative of lignocaine, was recently probed using site-directed mutagenesis. QX-222 interacts with adjacent turns of the M2 helix of the muscle AChR (Leonard *et al.*, 1988; Charney *et al.*, 1990).

Anaesthetics have long been known to suppress the spike potential of neurones elicited by orthodromic stimulation, synaptic transmission, or exogenously applied acetylcholine in the central and peripheral nervous systems (Larrabee & Posternak, 1952; Galindo, 1969). Although local anaesthetics have been shown to depress synaptic transmission in rat sympathetic ganglia (Tabatabai & Booth, 1990), and to inhibit carbachol-evoked currents in bovine chromaffin cells (Charlesworth *et al.*, 1992), the precise mechanism of action of local anaesthetics on neuronal nicotinic AChRs remains unknown. Given that neuronal nicotinic AChRs are known to differ from muscle AChRs in their primary structure, ion permeation and pharmacological properties (Adams & Nutter, 1992; Sargent, 1993), investigation of the nature of the interaction of local anaesthetics with the neuronal nicotinic AChR may provide further insight into the functional properties of this ion channel.

In this study, we examine local anaesthetic block of ACh-evoked currents in cultured parasympathetic neurones from rat intracardiac ganglia. Mammalian intracardiac neurones possess both nicotinic and muscarinic ACh receptors (Hassall *et al.*, 1987; Seabrook *et al.*, 1990). In mammalian cultured cardiac neurones, ACh elicits a rapidly activating current

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mediated by nicotinic ACh-receptors (Fieber & Adams, 1991a; Adams & Nutter, 1992; Selyanko & Skok, 1992) and a slowly activating current mediated by muscarinic ACh-receptors (Allen & Burnstock, 1990a; Selyanko & Skok, 1992). Our results show that the local anaesthetics, procaine and QX-222, selectively inhibit with high efficacy the nicotinic ACh-evoked current in parasympathetic neurones via a rapid transient block of the open channel. A preliminary report of some of these results has been published (Nutter *et al.*, 1991).

Methods

Preparation

The isolation and culture of parasympathetic neurones from rat intracardiac ganglia have been described previously (Fieber & Adams, 1991a). Briefly, neonatal rats, 3 to 5 days old, were killed by decapitation. The heart and lungs were excised as a single unit and placed in physiological saline solution (PSS) containing (mM): NaCl 140, KCl 3, CaCl₂ 2.5, MgCl₂ 1.2, glucose 7.7 and histidine 10 (pH to 7.2 with NaOH). Atria were removed and incubated in PSS solution containing collagenase (1 mg ml⁻¹, Worthington-Biomedical) at 37°C for 1 h. Individual ganglia were then dissected from the ganglion plexus, transferred to a sterile culture dish containing high glucose culture medium (Dulbecco's Modified Eagle Media, 10% (v/v) foetal calf serum, 100 u ml⁻¹ penicillin and 0.1 mg ml⁻¹ streptomycin), triturated with a fine-bore Pasteur pipette and plated onto 18 mm glass cover-slips coated with laminin. The dissociated cells were incubated at 37°C in a 95% air, 5% CO₂ atmosphere for 48–72 h. Following this incubation period, the glass cover-slips could be transferred to a 0.5 ml recording chamber mounted on an inverted, phase contrast microscope (Nikon Diaphot, 400× magnification) and electrophysiological experiments conducted.

Electrical recordings and analysis

Acetylcholine-evoked currents in intracardiac neurones were studied under voltage clamp mode by standard whole-cell and outside-out recording configurations of the patch clamp technique (Hamill *et al.*, 1981). In some experiments electrical access to the cell was obtained by use of the perforated patch method (Horn & Marty, 1988), which prevents cell dialysis and the subsequent alterations in cell function due to loss of cytosolic components. Stock solutions of 25 mg ml⁻¹ nystatin (Sigma) and 25 mg ml⁻¹ pluronic (Molecular Probes), both in dimethylsulphoxide (DMSO), were prepared the day of the experiment and kept on ice, in the dark. Immediately prior to use, the pluronic and the nystatin stock solutions were diluted 500 fold in pipette solution and vortexed, yielding a final solution containing 50 µg ml⁻¹ of both pluronic and nystatin in 0.4% DMSO. In order to prevent nystatin-induced disruption of gigaseal formation, the tips of the electrodes were first filled with nystatin-free pipette solution and then backfilled with the nystatin-containing solution.

Immediately following gigaseal formation, the neurones were held at –70 mV and voltage pulses (20 ms) to –80 mV were applied at 1 Hz. Nystatin incorporation into the membrane resulted in the appearance of a slow capacitive transient and a decrease in the series resistance (*R_s*). Experiments were continued only if *R_s* was less than 10 MΩ within 10 min of seal formation. Rupture of the membrane under the pipette tip was indicated by a rapid change in the holding current or a depolarization of the cell, at which time the experiment was stopped.

Electrodes were pulled from thick-walled borosilicate glass (GF 150F; Clark Electromedical Inst., UK) using a Sutter Instruments P-87 micropipette puller, and coated with Sylgard (Dow Corning Corp.) to within 100 µm of the tip. Final

pipette resistance was 1–3 MΩ for normal and perforated patch whole-cell recordings and 4–6 MΩ for excised membrane patch recordings. Membrane currents evoked by agonist application were amplified and filtered (10 kHz) with a List patch clamp amplifier (L/M-EPC 7) and recorded on video tape using an analog to digital recorder adaptor (PCM 4, two channel mode; Medical Systems Corp., N.Y., U.S.A.). Membrane currents were continuously monitored on a digital oscilloscope (Nicolet 3091), and a chart recorder (Soltec 1242).

For analysis of ACh-evoked currents, data were played back from video-tape, filtered at 3 kHz (–3 dB, 4 pole low-pass Bessel filter; Frequency Devices 902), and digitized at 20 kHz (Tecmar Labmaster TL1-125 DMA interface). Whole-cell currents were viewed and analyzed using the Axotape programme (Axon Instruments Inc., CA, U.S.A.). Unitary current records were transferred to a computer (Northgate PC 80386/33 MHz) using a threshold detection device (AI 2020A event detector; Axon Instruments Inc.), and analyzed using pClamp programmes (Axon Instruments Inc.). To minimize attenuation of unitary current amplitude, data used for determining mean unitary current amplitude were filtered at 5 kHz.

Amplitude distributions of single channel openings were obtained by measuring the difference between the amplitude of each event and the baseline current. Each individual opening contributed one point to the amplitude distribution of the whole record. An idealized reconstruction of open-closed transitions was created using a detection threshold for channel opening set at 50% of the amplitude of a single channel opening, and a minimum resolvable time of 150 µs for single channel events. Frequency distribution histograms were constructed for open and closed state durations, and fitted using the minimum X² method.

To analyze bursts of openings, the critical gap length below which a closure was classified as a gap within a burst was calculated for each experimental condition according to a method described previously (Colquhoun & Sigworth, 1983; Clapham & Neher, 1984). Under control conditions the critical gap length was found to be 1 ms, and increased to 1.1 ms and 1.4 ms in the presence of 30 µM and 100 µM QX-222, respectively. Total conduction time within a burst was determined using the equation (cf. Colquhoun & Sakmann, 1985):

$$T' = T_t/N_t \quad (1)$$

where *T'* is the total conduction time within a burst, *T_t* is the total conduction time during time *t*, and *N_t* is the total number of bursts occurring during time *t*.

All numerical data are presented as mean ± standard error of the mean (s.e.mean).

Solutions

The control external solution for standard and perforated patch whole-cell recordings was normal PSS. The pipette solution for conventional whole-cell recordings consisted of (mM): CsCl₄ 140, MgATP 2, Cs₄BAPTA 2, HEPES 10 (pH to 7.2 with CsOH). The perforated patch pipette solution contained (mM): K₂SO₄ 75, KCl 55, MgSO₄ 5, HEPES 10 (pH to 7.2 with *N*-methyl-(+)-glucamine). The recording chamber was continuously perfused (2 ml min⁻¹) with the indicated solutions at room temperature (22–23°C). Acetylcholine- and adenosine 5'-triphosphate-mediated responses were evoked during whole-cell recordings by focal application of 100 µM AChCl or 300 µM MgATP (Sigma Chemical Co., St. Louis, MO, U.S.A.) in PSS via pressure ejection (10 ms, 15 psi; Picospritzer II, General Valve Corp., N.Y., U.S.A.) from an extracellular pipette. The pressure ejection pipette was positioned ≤ 50 µm from the neuronal soma to evoke maximal response to agonist under control conditions. The concentration of bath-applied drugs at the cell surface during pressure application of ACh or ATP was equal to or lower than that

of the bulk solution. Thus, the apparent dose-response curves for these drugs may be shifted towards higher antagonist concentrations. The external solution used for recording single channel currents contained (composition in mM): NaCl 136, CaCl₂ 2.5, MgCl₂ 1.2, glucose 7.7, histidine 5 (pH 7.2) and the intracellular pipette solution (mM): NaCl 130, Na₂BAPTA 2, Na₂ATP 2, HEPES 10 (pH 7.2 with NaOH). The osmotic activity of the solutions was monitored with a vapor pressure osmometer (Wescor 5500). In outside-out patch experiments, 1 μ M AChCl was bath-applied in the external solution, in the absence or presence of the local anaesthetic.

Procaine hydrochloride (Sigma Chemical Co.), the quaternary N-methyl lignocaine derivative QX-222 (Astra Pharmaceuticals; Westboro, MA, U.S.A.), and pirenzepine dihydrochloride (Research Biochemicals Inc., Natick; MA, U.S.A.) were bath applied at the indicated concentrations.

Results

Whole cell recordings of ACh-evoked currents

The effects of local anaesthetics on nicotinic and muscarinic ACh-currents were examined by the perforated-patch recording configuration to minimize the rundown of muscarinic ACh-induced current which occurs as a result of whole-cell dialysis. Figure 1 shows a typical series of inward currents elicited by pressure ejection of 100 μ M ACh, obtained from a single cell. Application of ACh to the soma of cardiac neurones, voltage-clamped at 0 mV, evoked a slowly activating inward current (Figure 1a) previously undetected in whole-cell experiments (Fieber & Adams, 1991a). At negative potentials, ACh activated a transient inward current, fol-

lowed by a steady-state inward current (Figure 1a). Bath application of QX-222 (100 μ M) reversibly reduced the amplitudes of both the transient and sustained currents, but did not significantly depress the amplitude of the slow activating inward current (Figure 1b). The amplitude of the QX-222-insensitive, slow activating inward current was reduced by bath application of the muscarinic (M₁) receptor antagonist, pirenzepine (100 nM; Figure 1c).

Under standard, dialyzed whole-cell configuration, exogenous application of ACh elicited a transient current with a voltage-dependent amplitude, but the sustained inward current and the slowly inactivating inward current observed under perforated-patch recording conditions were absent. The ACh-evoked transient current was inward at negative potentials and reversed at approximately 0 mV (Figure 2a). The ACh-evoked current was outward at positive potentials, and the *I-V* relationship displayed strong inward rectification. Currents evoked by brief application (10 ms) of ACh decayed to baseline within 3 s, and the rate of current decay was independent of membrane potential over the range of -100 to +60 mV.

Effects of QX-222 on whole-cell currents

Figure 2b shows inward currents evoked by ACh in the presence of 10 μ M QX-222. Externally applied QX-222 attenuated ACh-evoked current amplitude at negative membrane potentials, and produced a voltage-dependent decrease in the rate of current decay. The half-time of decay of the ACh-evoked current increased by $\sim 7\%$ in the presence of 10 μ M QX-222 at -40 mV and by $\sim 33\%$ at -100 mV. The whole-cell *I-V* relationships obtained in the absence and presence of QX-222 reveals a concentration-dependent reduc-

Figure 1 Whole-cell currents in rat parasympathetic neurones evoked by extracellular ACh (100 μ M) by use of the perforated patch recording technique. (a) Whole-cell currents evoked by ACh at the membrane potentials indicated (-90 to 0 mV). ACh-evoked currents obtained in the presence of bath applied 100 μ M QX-222 (b) or 100 nM pirenzepine (c). ACh-evoked whole-cell currents shown in (a), (b) and (c) were recorded from the same neurone.

Figure 2 Inhibition of ACh-evoked currents by QX-222 in rat isolated parasympathetic neurones. (a) Family of currents evoked by brief pulses (10 ms) of ACh focally applied to an isolated neurone held at the indicated membrane potentials. (b) ACh-evoked currents obtained from the same neurone in the presence of bath applied 10 μM QX-222. (c) I - V relation of ACh-evoked currents obtained in the absence (control) and presence of QX-222 at the concentrations indicated.

tion of current amplitude at negative potentials (Figure 2c), but no alteration of the reversal potential, nor any significant reduction of current amplitude at positive potentials. The effects of QX-222 on the ACh-induced current amplitude and decay rate, were rapidly reversed upon washout.

Whole-cell recordings of representative ACh-evoked currents obtained at -80 mV in the absence and presence of increasing concentrations of QX-222 are shown in Figure 3a. Raising the external QX-222 concentration reduced the ACh-evoked current amplitude, and increased the time course of decay in a concentration-dependent manner (Figure 3a). The half time of current decay increased from 0.40 ± 0.03 s (control) to 0.51 ± 0.05 s and 0.64 ± 0.05 s ($n = 5$ cells) in the presence of 30 μM and 100 μM QX-222, respectively. Figure 3b plots a normalized dose-response relationship obtained for the inhibition of ACh-evoked current by QX-222. The relationship was fit with a single-site adsorption isotherm with a half-maximal inhibition at 28 μM .

The influence of membrane potential on QX-222 inhibition of whole-cell ACh-evoked currents is shown in Figure 4. ACh-evoked current amplitude versus membrane potential, in the absence and presence of QX-222, is plotted in Figure 2c. Membrane hyperpolarization increased the inhibition of inward ACh-induced current amplitude: the change from -20 mV to -100 mV produced a 36% increase in inhibition of ACh-evoked current amplitude by 100 μM QX-222. The ratio (Δ) of the control ACh-evoked current amplitude to that obtained in the presence of 100 μM QX-222, was deter-

Figure 3 Concentration-dependence of QX-222 block of ACh-evoked currents. (a) ACh-evoked currents obtained in the absence (control) and presence of bath applied QX-222. Holding potential, -80 mV. (b) Relative amplitude of ACh-evoked currents (at -80 mV) normalized to control, as a function of QX-222 concentration. Data points represent the mean $\pm$ s.e. mean for five cells. The curve is a best fit of the data by a single-site adsorption isotherm with a half-maximal inhibition IC_{50} of 28 μM .

mined at different membrane potentials. A plot of $\Delta-1$ as a function of membrane potential (Figure 4), revealed an e-fold change for a 62 mV change in membrane potential. The relationship between $\Delta-1$ and the dissociation constant (K_d) for QX-222 binding to the ACh receptor-channel is approximated by the equation:

$$K_d \approx c_Q / \Delta - 1, \quad (2)$$

where c_Q is the concentration of QX-222 (Ascher *et al.*, 1979). This implies that hyperpolarization by 62 mV reduces the apparent K_d e-fold.

Figure 4 Voltage-dependence of QX-222 inhibition of ACh-activated currents in cardiac neurones. $\Delta-1$ values calculated from the current amplitudes obtained in the absence and presence of 100 μM QX-222, plotted as a function of membrane potential. Data points were fitted by linear regression.

Figure 5 Lack of effect of local anaesthetics on adenosine 5'-triphosphate (ATP)-evoked currents in rat parasympathetic neurones. Currents evoked by brief (10 ms), focal application of ATP to the soma held at -50 and -100 mV. ATP-evoked currents obtained from the same cell in the absence and presence of PSS containing either 100 μM QX-222 (a) or 10 μM procaine (b).

Figure 6 Inhibition of ACh-evoked currents by procaine in cultured parasympathetic neurones. (a) ACh-induced currents obtained in the absence (control) and presence of procaine. Holding potential, -80 mV. (b) $I-V$ relation of ACh-evoked currents obtained in the absence and presence of procaine. (c) Normalized current amplitude (at -80 mV) plotted as a function of procaine concentration. Points represent mean $\pm$ s.e. mean for 3 cells. The curve was fitted according to a one-site adsorption isotherm with an IC_{50} of 2.8 μM .

ATP evoked a transient inward current in rat parasympathetic neurones with a time course and I - V relationship similar to that obtained with ACh (Fieber & Adams, 1991b). Bath application of either 100 μ M QX-222 or 10 μ M procaine failed to inhibit the ATP-induced current in these neurones (Figure 5).

Effects of procaine on whole cell currents

Procaine inhibited ACh-evoked currents in parasympathetic neurones similarly to QX-222. Bath application of procaine reduced the amplitude of the transient inward current evoked by focal application of ACh to the soma voltage-clamped at negative membrane potentials (Figure 6a). The inhibition of ACh-evoked current amplitude by procaine was concentration-dependent and reversible upon washout (Figure 6b). The dissociation equilibrium constant, calculated from equation 2, was 3 μ M at -80 mV. Procaine inhibition of ACh-current amplitude also was voltage-dependent, changing e-fold per 122 mV change in membrane potential. Procaine did not alter the reversal potential of the ACh-evoked current, nor affect the current amplitude at positive potentials. The half time of current decay at -80 mV was increased from

0.41 ± 0.04 s (control) to 0.53 ± 0.05 s ($n = 3$ cells) in the presence of 30 μ M procaine.

The dose-response relationship for the inhibition of ACh-evoked currents by procaine, normalized to the control condition, is shown in Figure 6c. Half-maximal inhibition was calculated to be 2.8 μ M from the fit of a single-site adsorption isotherm to the data. Near complete block of the ACh-evoked current at -80 mV was obtained with 100 μ M procaine ($n = 3$).

Single-channel recordings

Single channel currents recorded from outside-out membrane patches in the absence and presence of QX-222, revealed complex gating kinetics. As described previously, unitary currents occurred in clusters separated by long shut periods (Mathie *et al.*, 1991). Within clusters, bursts of openings were separated by closures of intermediate length, whereas individual openings within a burst were separated by brief closings. Figure 7a shows unitary currents evoked by bath-application of 1 μ M ACh recorded from an outside-out patch held at -90 mV and -120 mV in symmetrical 140 mM NaCl solutions. The traces were recorded within a cluster of channel

Figure 7 Neuronal ACh receptor single channel activity in the absence and presence of QX-222. (a) ACh-induced unitary currents recorded from an excised outside-out membrane patch held at -90 mV and -120 mV in the absence and presence of QX-222. (b) Current amplitude histograms (0.1 pA bin width) obtained for five outside-out membrane patches held at -90 mV in 0, 30 and 100 μ M QX-222. Single AChR-channels were activated by 1 μ M ACh.

activity. The unitary I - V relationship was linear from -120 to -20 mV, and the mean single-channel slope conductance was 26 ± 3 pS ($n = 5$ cells).

The effects of QX-222 on the ACh-induced single channel currents were investigated in outside-out membrane patches exposed to bath applied QX-222 and $1 \mu\text{M}$ ACh. QX-222 reduced ACh-evoked single channel activity at negative potentials in a concentration-dependent manner, without a significant reduction of unitary current amplitude (Figure 7a). The unitary current amplitude at -90 mV was 2.2 ± 0.4 pA for control, 2.0 ± 0.4 pA in $30 \mu\text{M}$ QX-222, and 2.1 ± 0.5 pA in $100 \mu\text{M}$ QX-222 ($n = 5$; Figure 7b).

QX-222 inhibition of ACh-evoked currents is reflected in the altered single channel kinetics. Figure 8a shows representative bursts of ACh-induced channel openings obtained at -90 mV in the presence of 0, 30 and $100 \mu\text{M}$ QX-222. Open-time distributions for the combined data from five outside-out patches exposed to $1 \mu\text{M}$ ACh and the QX-222 concentrations indicated are shown in Figure 8b. The open-time distributions were best fit by the sum of two exponential functions. In the absence of QX-222, time constants of 0.30 ms and 1.28 ms were obtained whereas, in the presence of QX-222, a concentration-dependent shift of the distribu-

tion towards shorter open times was observed (Figure 8b; Table 1). Thus, bath application of QX-222 produced a significant decrease in the ACh receptor-channel apparent mean open time (t_o). At -90 mV, t_o for the ACh-activated channel was 0.83 ± 0.01 ms (control), and decreased to 0.73 ± 0.01 ms and 0.55 ± 0.02 ms ($n = 5$) in the presence of $30 \mu\text{M}$ and $100 \mu\text{M}$ QX-222, respectively.

QX-222 also increased the time that the ACh receptor-channel remained in a non-conducting state in a concentration-dependent manner. The mean non-conducting interval within a burst (t_c) increased from 0.35 ± 0.01 ms (control) to 0.48 ± 0.01 and 0.55 ± 0.03 ms by $30 \mu\text{M}$ and $100 \mu\text{M}$ QX-222, respectively (-90 mV, $n = 5$). This increased non-conducting time interval is also apparent from the closed time distributions obtained for these experiments, which were best fit with two exponentials. The time constant for the first exponential (τ_1), representing gaps within bursts, increased from 0.44 ms (control) to 0.60 ms and 0.80 ms in the presence of $30 \mu\text{M}$ and $100 \mu\text{M}$ QX-222, respectively. The time constant for the second exponential (τ_2), representing gaps between bursts, was also affected by QX-222, increasing from 10.64 ms (control) to 11.74 ms ($30 \mu\text{M}$ QX-222) and 19.15 ms ($100 \mu\text{M}$ QX-222). The open channel probability (P_o) at

Figure 8 Effects of QX-222 on the kinetics of single neuronal ACh receptor channels. (a) Burst of activity recorded from single channel in the absence (control) and presence of 30 and $100 \mu\text{M}$ QX-222 at -90 mV. (b) Distribution of open times (0.15 ms bin width) during a burst before and after addition of 30 and $100 \mu\text{M}$ QX-222 at -90 mV for combined data from 5 patches. (c) Open probability (hatched columns) and mean burst duration (solid columns) obtained from five patches during a 5 min exposure to 0, 30 and $100 \mu\text{M}$ bath applied QX-222. Single AChR channels were activated by $1 \mu\text{M}$ ACh.

Table 1 Kinetics of neuronal nicotinic acetylcholine receptors (AChR) in the absence and presence of QX-222

	Control	QX-222	
		30 μ M	100 μ M
<i>Open state</i>			
τ_r (ms)	0.30 $\pm$ 0.04	0.25 $\pm$ 0.05	0.28 $\pm$ 0.12
τ_s (ms)	1.28 $\pm$ 0.29	0.92 $\pm$ 0.17	0.66 $\pm$ 0.37
A_f/A_s	1.05	0.84	1.13
Open time (ms)	0.83 $\pm$ 0.01	0.73 $\pm$ 0.01	0.55 $\pm$ 0.02
<i>Closed state</i>			
τ_r (ms)	0.44 $\pm$ 0.05	0.60 $\pm$ 0.10	0.80 $\pm$ 0.10
τ_s (ms)	10.64 $\pm$ 2.12	11.74 $\pm$ 1.50	19.15 $\pm$ 5.04
A_f/A_s	0.72	0.26	0.35
Closed time (ms)	0.35 $\pm$ 0.01	0.48 $\pm$ 0.01	0.55 $\pm$ 0.03
<i>Burst</i>			
Critical gap (ms)	1.0	1.1	1.4
Burst length (ms)	3.5 $\pm$ 0.3	8.6 $\pm$ 1.0	9.4 $\pm$ 1.3
Open probability (%)	3.0 $\pm$ 0.7	2.3 $\pm$ 0.2	0.5 $\pm$ 0.1

Mean $\pm$ s.e.mean values were calculated for five outside-out patches ($> 10^3$ events/patch). Each membrane potential was activated by bath application of 1 μ M ACh in the absence and presence of 30 μ M and 100 μ M QX-222. Mean closed time represents the value obtained for gaps with duration greater than the minimum resolvable time and less than the critical gap time. A_f/A_s represents the ratio of the areas contributed by the components with the fast and slow time constants, respectively.

Table 2 Characteristics of local anaesthetic block of nicotinic acetylcholine receptor (AChR)-channel subtypes

Preparation	QX-222		Procaine		References
	K_d (μ M)	Voltage sensitivity (mV)	K_d (μ M)	Voltage sensitivity (mV)	
Rat neuronal	31	62	3	122	Present study
Rat $\alpha_4\beta_2$	> 2000	26			Charnet <i>et al.</i> (1992)
Rat muscle	20	64			Neher (1983)
Frog muscle (junctional)	9	50			Ruff (1982)
			20	50	Adams (1977)
			42	86	Gage <i>et al.</i> , (1983)
Frog muscle (extra-junctional)	80	32			Neher & Steinbach (1978)

– 90 mV decreased from $3.0 \pm 0.7\%$ (control) to $2.3 \pm 0.2\%$ and $0.5 \pm 0.1\%$ in membrane patches exposed to 30 μ M and 100 μ M QX-222, respectively ($n = 5$, Figure 8c). These changes in gating kinetics and P_o of the AChR channel produced by QX-222 were reversible upon washout.

QX-222 also produced a concentration-dependent increase in ACh receptor-channel burst duration. Burst duration increased from 3.5 ± 0.3 ms (control) to 8.6 ± 1.0 ms and 9.4 ± 1.3 ms in solutions containing 30 μ M and 100 μ M QX-222, respectively ($n = 5$, Figure 8c). While burst duration increased in the presence of QX-222, conduction time within a burst decreased from 2.3 ms (control) to 2.2 ms (100 μ M QX-222) in the same patches. The equilibrium dissociation constant (K_d) may be estimated from the burst duration (T) and total conduction time (T') (Neher, 1983)

$$K_d = c_0 T' / (T - T') \quad (3)$$

where c_0 is the concentration of QX-222 and $(T - T')$ is the total blocked time. Here an apparent K_d of 31 μ M was calculated for QX-222 at – 90 mV from the experimental values of 9.4 ms and 2.2 ms for T' and T , respectively.

Discussion

In rat intracardiac neurones, the local anaesthetics QX-222 and procaine inhibit nicotinic ACh-evoked current in a concentration- and voltage-dependent manner, without affecting muscarinic ACh-evoked currents. QX-222 inhibits the ACh-evoked current via a rapid transient block of the open channel, decreasing the ACh receptor-channel open probability. The sensitivity of the neuronal nicotinic ACh-

evoked currents to local anaesthetics suggests a possible role for these channels in local anaesthetic inhibition of ganglionic transmission.

The pirenzepine-sensitive, slowly-activating ACh-evoked current observed using the perforated-patch recording configuration was unaffected by the quaternary amine QX-222. This current appears to be identical with the M_1 -activated current described in cultured intracardiac neurones of the guinea-pig (Allen & Burnstock, 1990a). In contrast, QX-222 reversibly inhibited the ACh-evoked transient inward current and steady-state current. These currents were, however, insensitive to the muscarinic receptor antagonists, pirenzepine and atropine (data not shown) and thus constitute a nicotinic AChR-mediated current. An ATP-induced current has also been described in parasympathetic neurones of mammalian intracardiac ganglia (Allen & Burnstock, 1990b; Fieber & Adams, 1991b). However, ATP-evoked currents were unaffected by bath application of either 100 μ M QX-222 or 10 μ M procaine (Figure 5), concentrations 3 fold higher than that required to produce half-maximal inhibition of the nicotinic ACh-evoked currents.

QX-222 reversibly decreased the amplitude of the transient ACh-evoked current of rat parasympathetic neurones in a concentration-dependent manner similar to that described for the motor endplates of frog (Beam, 1976a,b; Neher & Steinbach, 1978; Ruff, 1982) and rat (Neher, 1983). A comparison of the K_d and voltage-dependence of QX-222 and procaine block of several nicotinic ACh receptor-channel subtypes is presented in Table 2. QX-222 inhibition of transient ACh-evoked currents was potentiated by membrane hyperpolarization. The K_d for QX-222 block of ACh-induced whole-cell current changed e-fold with a 62 mV change in membrane

potential. This voltage sensitivity suggests that QX-222 binds at a site within the electric field, presumably the pore-forming region, of the ACh receptor-channel in parasympathetic neurones. The magnitude of voltage sensitivity of the rat neuronal and muscle ACh receptor-channels to QX-222 inhibition correspond to a binding site approximately 40% into the membrane field of these channels (Woodhull, 1973) compared to 75% for frog muscle and 92% for rat $\alpha_4\beta_2$ neuronal receptors, respectively (Neher & Steinbach, 1978; Charnet *et al.*, 1992).

Procaine also produced a reversible, concentration-dependent block of the nicotinic ACh-evoked current in rat cardiac neurones. The K_d for procaine block occurred at a concentration of 3 μM (Table 2). Procaine block of the AChR channel was also voltage-dependent; however, this voltage-dependence was less than that observed for QX-222. An e-fold change in K_d was produced by a 122 mV change in membrane potential, indicating a binding site $\sim 20\%$ into the membrane field. These values differ from those calculated for the ACh receptor-channel at the frog endplate (Gage *et al.*, 1983), where the voltage-dependence was 86 mV per e-fold change in K_d suggesting the binding site was $\sim 30\%$ into the membrane field.

The effects of QX-222 on the kinetics of the ACh receptor-channels of rat intracardiac neurones are similar to those reported for muscle ACh receptor-channel in frog (Neher & Steinbach, 1978) and rat (Neher, 1983) (see Table 1). QX-222 binding to the ACh receptor-channel decreased t_o and total conduction time within a burst, and increased both t_c and mean burst duration. Furthermore, QX-222 increased the duration of gaps between bursts, and decreased the open channel probability.

Several models have been proposed to explain the kinetics of local anaesthetic block of nicotinic AChR channels. The sequential kinetic model of channel blockade (Adams, 1976; Ruff, 1977; Neher & Steinbach, 1978) accurately predicts the decrease in nicotinic AChR channel mean open time and increase in burst duration observed in the present study. However, this model fails to account for the concentration-dependent increase in the intra- and inter-burst gaps and the decrease in intra-burst conduction time and open probability observed in the presence of QX-222. Neher (1983) similarly found that AChR kinetic data obtained in the presence of high concentrations of QX-222 was inconsistent with predictions of the sequential block model and therefore proposed an alternative model in which a channel could enter multiple blocked states. If one of these blocked states were more long-lived than the closed state(s), then P_o would be expected to decrease, as found in the present study. The observed increase in inter-burst gaps may be due to the channel entering a long-lived blocked state, and not a closed state.

An apparent K_d of 31 μM was calculated for QX-222 at -90 mV from the changes in the ratio of total conduction time and total blocked time within a burst (see equation 3). The apparent K_d indicates that the affinity of QX-222 for the ACh receptor-channel in rat parasympathetic cardiac neurones is slightly less than that for the ACh receptor-channel in rat cultured muscle cells (Neher, 1983) and frog skeletal muscle (Ruff, 1982), but more than that observed for ACh receptor-channels of frog muscle extrajunctional membranes (Neher & Steinbach, 1978), and rat $\alpha_4\beta_2$ neuronal ACh re-

ceptor-channel expressed in *Xenopus* oocytes (Charnet *et al.*, 1992) at -90 mV.

The K_d of 3 μM for procaine block at -80 mV was significantly less than that determined for ACh receptor-channels at the frog endplate (Adams, 1977; Gage *et al.*, 1983). Thus, procaine, unlike QX-222, has a greater affinity for ACh receptor-channels in rat intracardiac neurones than in frog muscle. This difference is not surprising, given that procaine block of ACh receptor-channels has been shown to occur via a mechanism different from that of quaternary local anaesthetics (Gage *et al.*, 1983). Whereas quaternary local anaesthetics block the ACh-evoked current exclusively from the extracellular side of the membrane (Horn *et al.*, 1980), procaine is also able to act within the lipid environment.

The membrane spanning (M2) region of the ACh receptor-channel protein is proposed to line the ion channel pore (see Dani, 1989), and it has been suggested that the QX-222 binding site lies within the pore of the channel. This suggestion is substantiated by the finding that mutations of specific amino acids in this transmembrane region affect QX-222 binding to the receptor (Charnet *et al.*, 1990). Therefore, the difference between the affinity of QX-222 for rat neuronal and muscle junctional AChR channels may suggest a difference in the primary structure of these channels. Furthermore, the higher affinity of QX-222 for the ACh receptor-channel of rat intracardiac neurones compared to rat $\alpha_4\beta_2$ ACh receptor-channel, together with the difference in the estimated binding site of QX-222 within these channels, suggests a difference in their primary structure. These results also suggest that the ACh receptor-channel of rat parasympathetic neurones is not the $\alpha_4\beta_2$ receptor-channel studied by Charnet *et al.* (1992).

The effects of local anaesthetics on nerve conduction and synaptic transmission at the motor endplate are well documented, but, their action on ganglionic transmission is poorly understood. Tabatabai & Booth (1990) demonstrated that local anaesthetics can completely block synaptic transmission in sympathetic ganglia, acting at presynaptic and postsynaptic sites. One mechanism by which local anaesthetics inhibit synaptic transmission is via a decrease and eventual elimination of excitatory postsynaptic potentials (e.p.s.ps). The present study suggests that local anaesthetic block of ACh receptor-channels may be responsible for the attenuation of e.p.s.ps at cholinergic synapses.

In conclusion, the local anaesthetics, procaine and QX-222, have been shown to block selectively nicotinic ACh receptor-channels of rat parasympathetic cardiac neurones. The apparent K_d s and voltage-sensitivities calculated for QX-222 and procaine binding to these channels are significantly different from those obtained in other preparations, and suggest that their M2 region differs from that of frog and rat skeletal muscle and neuronal $\alpha_4\beta_2$ ACh receptor-channels. Local anaesthetics do not affect muscarinic or ATP-evoked currents in cardiac ganglia, indicating that the mechanism by which local anaesthetics affect ganglionic (vagal) transmission is via a block of nicotinic ACh receptor-channels.

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