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Multiple inhibitory actions of lidocaine on *Torpedo* nicotinic acetylcholine receptors transplanted to *Xenopus* oocytes

Armando Alberola-Die,* Juan Martinez-Pinna,* José Manuel González-Ros,† Isabel Ivorra* and Andrés Morales*

*División de Fisiología, Dpto. de Fisiología, Genética y Microbiología, Universidad de Alicante, Alicante, Spain

†Instituto de Biología Molecular y Celular, Universidad Miguel Hernández, Elche, Spain

Abstract

Lidocaine is a local anaesthetic that blocks sodium channels, but also inhibits several ligand-gated ion-channels. The aim of this work was to unravel the mechanisms by which lidocaine blocks *Torpedo* nicotinic receptors transplanted to *Xenopus* oocytes. Acetylcholine-elicited currents were reversibly blocked by lidocaine, in a concentration dependent manner. At doses lower than the IC_{50}, lidocaine blocked nicotinic receptors only at negative potentials, indicating an open-channel blockade; the binding site within the channel was at about 30% of the way through the electrical field across the membrane. In the presence of higher lidocaine doses, nicotinic receptors were blocked both at positive and negative potentials, acetylcholine dose–response curve shifted to the right and lidocaine pre-application, before its co-application with acetylcholine, enhanced the current inhibition, indicating all together that lidocaine also blocked resting receptors; besides, it increased the current decay rate. When lidocaine, at low doses, was co-applied with 2-(triethylammonio)-*N*-(2,6-dimethylphenyl) acetamide bromide, edrophonium or 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide, which are quaternary-ammonium molecules that also blocked nicotinic receptors, there was an additive inhibitory effect, indicating that these molecules bound to different sites within the channel pore. These results prove that lidocaine blocks nicotinic receptors by several independent mechanisms and evidence the diverse and complex modulation of this receptor by structurally related molecules.

Keywords: lidocaine, local anaesthetics, microtransplantation, nicotinic acetylcholine receptors, QX-314, *Xenopus* oocytes.

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Nicotinic acetylcholine (ACh) receptors (nAChRs) belong to the 'Cys-loop' superfamily of ligand-gated ion channels and are widely expressed in the central and peripheral nervous systems (Albuquerque *et al.* 2009; Taly *et al.* 2009). In humans, nAChR dysfunctions have been implicated in a variety of severe pathologies, including certain types of epilepsy, myasthenic syndromes, schizophrenia and Parkinson's and Alzheimer's diseases (Gotti and Clementi 2004; Sacco *et al.* 2004; Dani and Bertrand 2007; Steinlein and Bertrand 2010). Thus, the study of the mechanisms by which different drugs modulate nAChR function is of great interest not only for expanding our knowledge on the function of this receptor protein but also for developing new therapeutic molecules which might be useful for the treatment of nAChR-involved pathologies.

A variety of compounds, including the quaternary-ammonium cholinesterase inhibitors 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW284c51), decamethonium or edrophonium (Olivera-Bravo *et al.* 2005, 2007), and the local anaesthetics (LAs) 2-(triethylammonio)-*N*-(2,6-dimethylphenyl) acetamide bromide (QX-314), 2-(trimethylammonio)-*N*-(2,6-dimethylphenyl) acetamide chloride (QX-222), tetracaine, proadifen, lidocaine, adiphenine or procaine (Steinbach 1968; Adams 1977; Neher and Steinbach 1978; Koblin and Lester 1979; Pascual and Karlin 1998;

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Address correspondence and reprints requests to Dr Andrés Morales, División de Fisiología, Dpto. de Fisiología, Genética y Microbiología, Universidad de Alicante, Apdo. 99, E-03080 Alicante, Spain.
E-mail: andres.morales@ua.es

Abbreviations used: ACh, acetylcholine; ANR, normal Ringer solution with atropine; BW284c51, 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide; CD_{ti}, percentage of current decay at the specified time; I_{ACh}, ACh-elicited current; LA, local anaesthetic; nAChR, nicotinic acetylcholine receptor; QChEI, quaternary-ammonium cholinesterase inhibitor; QX-222, 2-(trimethylammonio)-*N*-(2,6-dimethylphenyl) acetamide chloride; QX-314, 2-(triethylammonio)-*N*-(2,6-dimethylphenyl) acetamide bromide.

Gentry and Lukas 2001; Spitzmaul *et al.* 2009; Wang *et al.* 2010) have shown significant effects on muscle-type nAChRs. Besides muscle-type nAChRs, lidocaine blocks several ligand-gated ion channel, including glycine and GABA receptors (Hara and Sata 2007), 5-hydroxytryptamine-3 (Ueta *et al.* 2007) and several subtypes of neuronal nAChRs, with different potencies (Gentry and Lukas 2001; Papke *et al.* 2001). In fact, it has been proposed that the behavioral effects of lidocaine and other LAs such as restlessness, euphoria, disorientation and muscle twitching could be due, at least partially, to the modulating actions of these compounds on nAChRs (Butterworth and Strichartz 1990; Arias 1999). These observations indicate that nAChRs are relevant targets for lidocaine, but the precise mechanisms by which lidocaine modulates nAChRs function are yet poorly understood.

As other tertiary-amines, lidocaine is an uncharged molecule that can easily cross the cell membrane, with a diffusion coefficient in lipid bilayers close to 0.5×10^{-5} cm^2/s (Hille 1977). However, this molecule becomes partially protonated at physiological pH, because of its pK_a of 7.8 (Liu *et al.* 2003). Given that lidocaine is found in both charged and uncharged states in physiological conditions, the mechanisms underlying nAChR blockade by lidocaine could be heterogeneous, as noted in batrachotoxin-activated cardiac and skeletal muscle sodium channels (Zamponi *et al.* 1993). The permanently charged derivatives of lidocaine, QX-314 and QX-222, act on nAChRs mainly as non-competitive inhibitors, causing an open-channel blockade (Neher and Steinbach 1978; Pascual and Karlin 1998), although at higher doses they can also interact with closed states of the nAChR, promoting desensitisation (Neher 1983), like procaine (Adams 1977), which is a tertiary-amine LA. As lidocaine easily crosses the cell membrane, it can interact with nAChRs sites, unattainable by polar molecules, such as its derivatives QX-314 or QX-222 and, consequently, it might cause different modulation of nAChRs. Therefore, the aim of this work was to unravel the mechanisms underlying the effects of lidocaine on purified *Torpedo marmorata* muscle-type nAChRs transplanted to *Xenopus* oocytes (Morales *et al.* 1995). This experimental approach allowed us to test the effects of lidocaine on the function of fully processed and correctly assembled nAChRs. Preliminary results have been published elsewhere (Alberola-Die *et al.* 2010).

Methods

Purification and reconstitution of nAChRs

nAChRs from *Torpedo marmorata* electroplax were purified by affinity chromatography in the presence of asolectin lipids and with cholate as a detergent, as previously described (Morales *et al.* 1995).

Oocyte preparation and microinjection

Adult female *Xenopus laevis* (purchased from Harlan Interfauna Ibérica S.L., Barcelona, Spain) were immersed in cold 0.17% ethyl 3-aminobenzoate methanesulfonate for 20 min and a piece of ovary was drawn out aseptically. Animal handling was carried out in accordance with the guidelines for the care and use of experimental animals adopted by the E.U. Stage V and VI oocytes were isolated and their surrounding layers removed manually. Cells were kept at 15–16°C in a modified Barth's solution (88 mM NaCl, 1 mM KCl, 2.40 mM $NaHCO_3$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 10 mM HEPES (pH 7.4), 100 U/mL penicillin and 0.1 mg/mL streptomycin) until used. Oocytes were microinjected with 100 nL of an aliquot of reconstituted nAChRs.

Electrophysiological recordings

Membrane current recordings were performed at 21–25°C, 16–72 h after injection. The recording methodology has been described in detail elsewhere (Morales *et al.* 1995; Olivera-Bravo *et al.* 2007). Briefly, oocytes were placed in a 150 µL recording chamber and continuously superfused with normal frog Ringer's solution (NR: 115 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.0) supplemented with 0.5 µM atropine sulphate (normal Ringer with atropine, ANR) to block eventual muscarinic responses (Kusano *et al.* 1982). The membrane potential was held at −60 mV, unless otherwise stated. ACh and other tested drugs were diluted in ANR solution and oocytes superfused at a flow rate of 13–17 ml/min. The pH of ANR containing lidocaine was routinely checked before its application.

Experimental design

The inhibitory effects of lidocaine on the currents elicited by different concentrations of ACh (I_{ACh}) were determined by measuring I_{ACh} evoked by 10 µM ACh alone or together with different lidocaine doses (hereafter we will use the term 'dose' instead of 'concentration' referred to lidocaine because it is present in two molecular forms and hence, there are two different lidocaine concentrations, corresponding to the charged and the uncharged forms). I_{ACh}s elicited in the presence of lidocaine were normalised to the I_{ACh} evoked by ACh alone and data were fitted to sigmoid curve using the Origin 6.1 software (OriginLab Corp., Northampton, MA, USA).

For competition assays, ACh concentration–I_{ACh} amplitude curves were obtained by exposing injected oocytes to increasing ACh concentrations, either alone or together with 70 µM lidocaine. I_{ACh}s obtained in the presence and absence of lidocaine were normalised to the I_{ACh} evoked by 1 mM ACh alone and fitted to a sigmoid curve. To reduce nAChR desensitisation, the interval between consecutive ACh applications was, at least, 7 min for low ACh concentrations and 30 min for the highest concentrations. To assess the blockade of resting nAChRs by lidocaine, the I_{ACh} was elicited by 10 µM or 1 mM ACh alone or co-applied with 70 µM lidocaine either directly or after a 12 s pre-application.

The voltage dependence of the I_{ACh} blockade was studied as previously described (Olivera-Bravo *et al.* 2007). More detailed information concerning data analysis is given as Data S1.

Statistical procedures

Unless otherwise specified, values given are the mean ± SEM; '*n*' indicates the number of oocytes and '*N*' is the number of donors from which data were obtained. When comparing two-group means of normally distributed values, the Student's *t*-test was used;

otherwise, Mann–Whitney rank-sum test was applied. Among-group differences were determined by the Kruskal–Wallis analysis of variance on ranks; the comparison of groups was made using the Dunn's test. A significance level of $p < 0.05$ was considered for all cases.

Drugs

ACh, atropine sulphate, lidocaine, QX-314, edrophonium, BW284c51, ethyl 3-aminobenzoate methanesulfonate, penicillin and streptomycin were from Sigma (St Louis, MO, USA). HEPES was obtained from Acros Organics (New Jersey, NJ, USA). Reagents of general use were purchased from Scharlau Chemie SA (Barcelona, Spain). All solutions were made in ANR just before each application unless otherwise stated.

Results

Dose-dependent effects of lidocaine on I_{ACh} and their reversibility

Either in oocytes bearing nAChRs or uninjected cells, with the membrane potential clamped at −60 mV, application of lidocaine did not appreciably modify the cell membrane conductance, even at concentrations as high as 10 mM. However, in oocytes that had incorporated nAChRs, co-application of 10 μM ACh with lidocaine, at concentrations ranging from 100 nM to 10 mM, inhibited I_{ACh} in a dose-dependent manner (Fig. 1). The half-inhibitory lidocaine concentration (IC_{50}), obtained by fitting the data to the Hill equation was 73 μM (range 62–83 μM; $n = 4$, $N = 2$), and the Hill coefficient (n_H) was 1.3 ± 0.1 (Fig. 1b).

The effect of lidocaine on I_{ACh} current decay was markedly dependent on the dose, as shown in Fig. 2. Thus, 20 μM lidocaine did not change the rate of I_{ACh} decay (CD_{ti} values at 2 and 20 s were: 27 ± 3% and 90 ± 1%, $n = 25$, $N = 11$, for 100 μM ACh alone vs. 30 ± 2% and 92 ± 1%, $n = 30$, $N = 12$, for 100 μM ACh plus 20 μM lidocaine, respectively; Fig. 2ai; $p > 0.05$, t-test; see Equation 3 in Data S1); however, 70 μM lidocaine significantly accelerated I_{ACh} decay (CD_{ti} values at 2 and 20 s were: 31 ± 3% and 92 ± 1%, $n = 39$, $N = 15$, for 100 μM ACh alone vs. 48 ± 2% and 95 ± 1%, $n = 64$; $N = 24$, for 100 μM ACh plus 70 μM lidocaine, respectively; Fig. 2aii; $p < 0.05$, t-test). The increase in the rate of I_{ACh} decay elicited by lidocaine was fully reverted 7 min after rinsing the LA (post-control, dashed line of Fig. 2aii). Moreover, 70 μM, but not 20 μM, lidocaine reduced the time lapsed from I_{ACh} onset to the I_{ACh} peak (time-to-peak in Fig. 2bi and bii) and this effect also vanished 7 min after lidocaine washout (Fig. 2bii). Noticeably, the maximum slope of the I_{ACh} rising phase decreased significantly when 10 μM ACh was co-applied with 70 μM lidocaine (−0.131 ± 0.018 nA/ms, $n = 16$, versus −0.085 ± 0.014 nA/ms, same cells, for ACh alone and ACh co-applied with lidocaine, respectively; $p < 0.001$, paired t-test); this indicating that lidocaine was blocking very fast the channels as they opened.

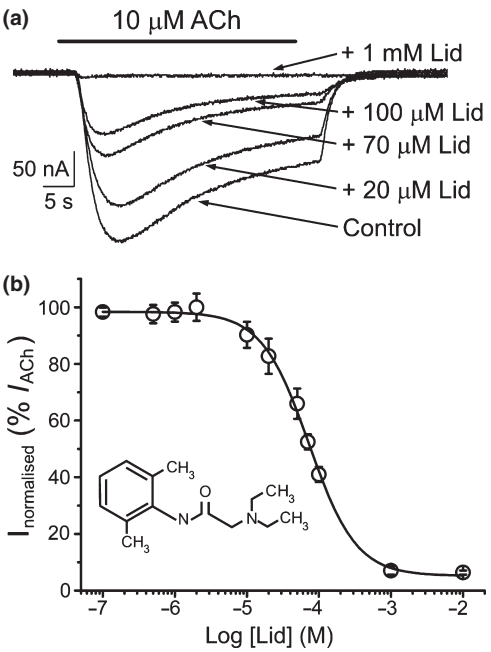

Fig. 1 Lidocaine effect on ACh-induced currents (I_{ACh}). (a) Superimposed currents elicited in one nAChR-bearing oocyte by application, for 32 s, of 10 μM ACh either alone (Control) or together with lidocaine (Lid), at the indicated concentrations. I_{ACh}s were obtained at intervals of, at least, 7 min. In this and subsequent figures, unless otherwise stated, the holding potential was −60 mV, downward deflections denote inward currents and the bar above the records corresponds to the timing of drug application. (b) Lidocaine dose–I_{ACh} inhibition relationship. Amplitude of the I_{ACh}s evoked in the presence of lidocaine was normalised to the I_{ACh} elicited by ACh alone and plotted as a function of the logarithm of the lidocaine dose. Data are the average of four oocytes ($N = 2$, hereafter 'N' indicate the number of donors). Solid line is a sigmoid curve fitted to the data and error bars are SEM. Inset shows the chemical structure of lidocaine.

With regard to reversibility, nAChR blockade by lidocaine outlasted the drug application. So, as shown in Fig. 3ai, 20 s after lidocaine (70 μM) removal, the percentage of I_{ACh} recovery was 61.4 ± 3.1 ($n = 8$, $N = 2$; Fig. 3b), suggesting that lidocaine remains longer bound to the nAChR. Seven minutes after lidocaine withdrawal (Fig. 3aii), the percentage of I_{ACh} recovery was 87.0 ± 2.5 ($n = 26$, $N = 13$) (Fig. 3b).

Competition assays

To study the pharmacological profile of nAChR inhibition, ACh was applied at different concentrations (1, 3, 10, 100 μM and 1 mM) either alone (Fig. 4ai) or co-applied with 70 μM lidocaine (Fig. 4aii). Fig. 4b shows the relationship between ACh-concentration and I_{ACh} amplitude in absence (solid symbols) and presence (open symbols) of lidocaine. The dose–response curves were fitted to a sigmoid curve with the Hill equation. The estimated EC_{50} for I_{ACh}s elicited by ACh alone was 34 μM and the n_H 1.7 ± 0.1 (range 19–

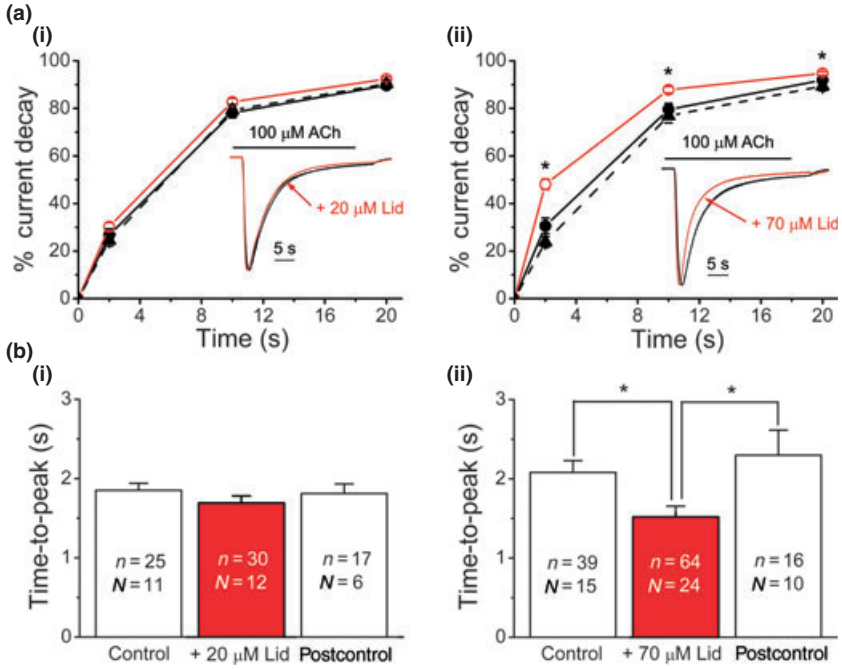

Fig. 2 Lidocaine effects on I_{ACh} decay and time-to-peak. (ai, aii) I_{ACh} recordings (insets) and I_{ACh} decay plots (CD_{ti} values from Equation 3 of Data S1) obtained by application of 100 μM ACh alone (black recording; filled black circles and solid black lines in ai, aii) or plus lidocaine either at 20 (red recording; open red circles and solid red line in ai) or 70 μM (red recording; open red circles and solid red line in aii) and by re-applying 100 μM ACh alone 7 min after lidocaine washout (black recording; filled black triangles and dashed black lines in ai, aii). Note that amplitude of I_{ACh} recordings have been scaled to the same size to better showing differences in I_{ACh} decay. Asterisks indicate significant differences ($p < 0.05$, *t*-test) with the control group. (bi, bii) Column graph of the I_{ACh} time-to-peak when applying ACh alone (Control in bi, bii) or with lidocaine, either 20 (+ 20 μM Lid in bi) or 70 μM (+ 70 μM Lid in bii). Asterisks indicate significant differences ($p < 0.05$, *t*-test) among the indicated groups. The number of oocytes (*n*) and donors (*N*) for panels a and b are the same, and are shown in each column of panels bi and bii.

50 μM; $n = 7$, $N = 7$), resembling those previously reported (Morales *et al.* 1995). When co-applying those ACh concentrations with 70 μM lidocaine, I_{ACh} decreased, even with almost saturating ACh concentrations, indicating a non-competitive action. Furthermore, the dose–response curve shifted to the right (Fig. 4b), increasing the EC_{50} to 99 μM and decreasing the slope to 1.0 ± 0.1 (range 35–267 μM; $n = 7$, $N = 7$). Besides, to avoid any bias due to desensitisation, the n_H was estimated from double-logarithmic plots (inset of Fig. 4b), considering only low ACh concentrations. The n_H values obtained this way, from the curves in the absence and presence of 70 μM lidocaine, were 1.6 ± 0.1 and 1.2 ± 0.1, respectively ($n = 7$, $N = 7$), confirming a reduction in the cooperativity index by lidocaine.

nAChR blockade by lidocaine was not merely a non-competitive antagonism, because the percentage of I_{ACh} inhibition was dependent on ACh concentration. So, 70 μM lidocaine co-applied with 10 μM ACh blocked roughly half the control I_{ACh} (Fig. 5ai and 5b), because this dose is very close to the IC_{50} (Fig. 1). However, when it was co-applied with 1 mM ACh, the percentage of blockade decreased (Fig. 5b, open circles), suggesting an apparent competitive mechanism of inhibition, most likely mediated by the binding of lidocaine to the nAChR in the closed state. To confirm this hypothesis, we compared the I_{ACh} blockade elicited by direct co-application of ACh (10 μM and 1 mM) plus 70 μM lidocaine with that obtained by pre-applying 70 μM lidocaine for 12 s before the co-applications of ACh plus lidocaine (Fig. 5). Pre-application of lidocaine before its co-application with 10 μM ACh increased moderately the percentage of I_{ACh} inhibition (52.2 ± 0.9%, $n = 34$, $N = 17$, for direct co-application, versus 67.1 ± 1.1%, $n = 6$, $N = 6$, for pre-application followed by co-application; Fig. 5ai, aii and b). However, the enhancement of I_{ACh} inhibition by lidocaine pre-application was stronger when it was co-applied with 1 mM ACh (43.4 ± 2.2%, $n = 12$, $N = 7$ for direct co-application against 89.2 ± 1.6%, $n = 7$, $N = 7$, when lidocaine was pre- and co-applied with ACh; Fig. 5aiii, aiv and b).

Voltage-dependence of nAChR blockade by lidocaine

To determine whether I_{ACh} inhibition by lidocaine is voltage-dependent, membrane currents were measured at different membrane potentials (see Fig. 6a), in the absence or presence

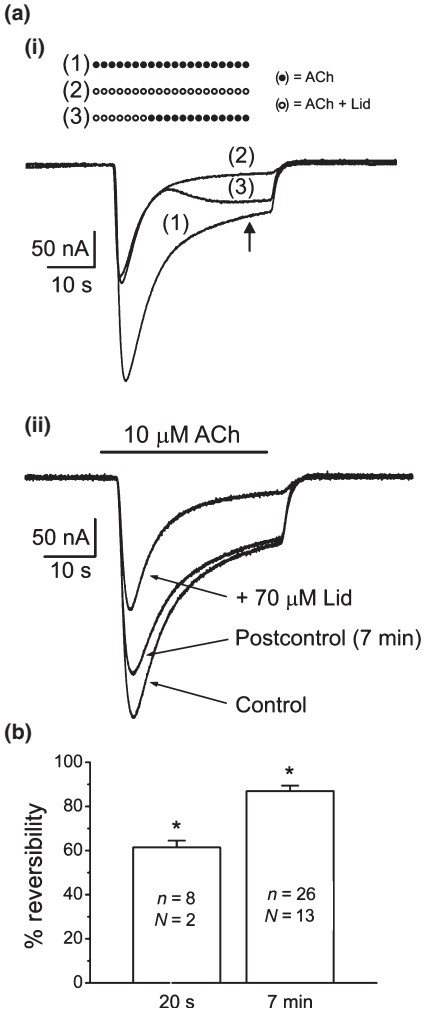

Fig. 3 Reversibility of I_{ACh} blockade by lidocaine. (ai) I_{ACh}s elicited, in the same oocyte, by 10 μM ACh alone (●; 1) or co-applied with 70 μM lidocaine (○, 2). Mixed symbols (3) indicate the change from ANR containing 10 μM ACh plus 70 μM lidocaine (○), to ANR containing 10 μM ACh alone (●). (aii) Superimposed currents obtained by superfusing one oocyte with 10 μM ACh alone (Control) or plus 70 μM lidocaine (+ 70 μM Lid). Seven min after lidocaine withdrawal (Postcontrol), I_{ACh} did not reach the control amplitude. (b) Column graph showing the percentages of I_{ACh} recovery after 20 s or 7 min of lidocaine removal. Asterisks indicate significant differences ($p < 0.05$, *t*-test) respect to control response.

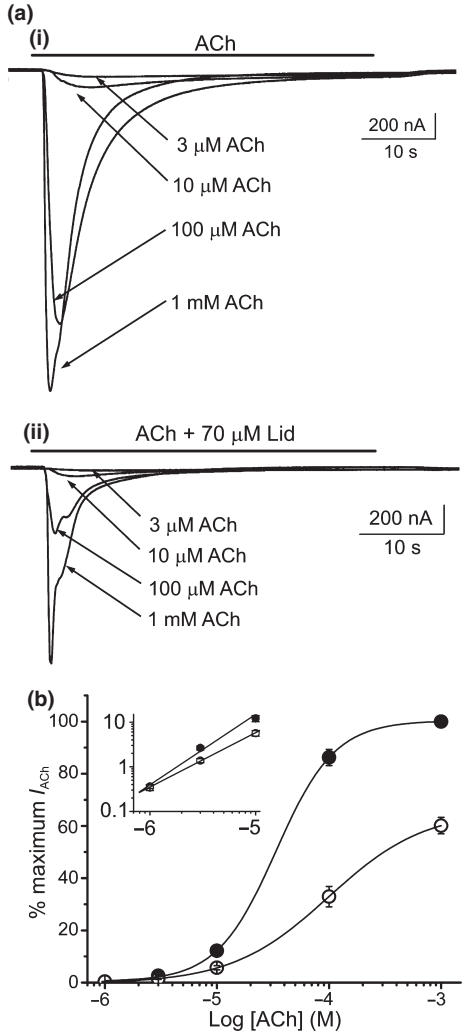

Fig. 4 Lidocaine effects on the ACh concentration–I_{ACh} amplitude relationship. (ai, aii) Superimposed recordings obtained by applying sequentially to the same cell, 3, 10, 100 μM, and 1 mM ACh either alone (ai) or co-applied with 70 μM lidocaine (aii). (b) Averaged ACh concentration–I_{ACh} amplitude curves, evoked by ACh either alone (filled circles; $n = 7$, $N = 7$) or plus 70 μM lidocaine (open circles; $n = 7$, $N = 7$). Data were normalized to the maximal I_{ACh} elicited by ACh alone and fitted to the Hill equation (continuous lines). The inset shows double-logarithmic plots of the corresponding ACh concentration–I_{ACh} amplitude relationships for low ACh concentrations.

of 10 μM ACh applied either alone or together with diverse lidocaine doses (0.7, 7, 20, 70 and 200 μM). Fig. 6b shows the *i/v* curves of net I_{ACh}s elicited by ACh either alone or co-applied with the different lidocaine concentrations tested and normalised to the control I_{ACh} at −60 mV.

The *i/v* relationships for I_{ACh}s obtained in the presence of ACh alone (Fig. 6b, closed circles) showed a reversal potential close to 0 mV and the characteristic inward rectification of this receptor (Morales *et al.* 1995). The I_{ACh} reversal potential was not affected by lidocaine (Fig. 6b), indicating that the channel permeability was unaffected. Lidocaine, at concentrations lower than the IC_{50} (except for 0.7 μM) decreased I_{ACh}, although only at negative potentials (Fig. 6b). By contrast, at 70 μM, or higher concentrations, lidocaine blocked I_{ACh} in a voltage-independent way (Fig. 6b and c). In fact, roughly 40% of I_{ACh} blockade caused by 70 μM lidocaine was voltage-independent and, therefore, present at any membrane potential, and this percentage increased up to ≈75% with 200 μM lidocaine (Fig. 6c). To discard the possibility that lidocaine could be blocking

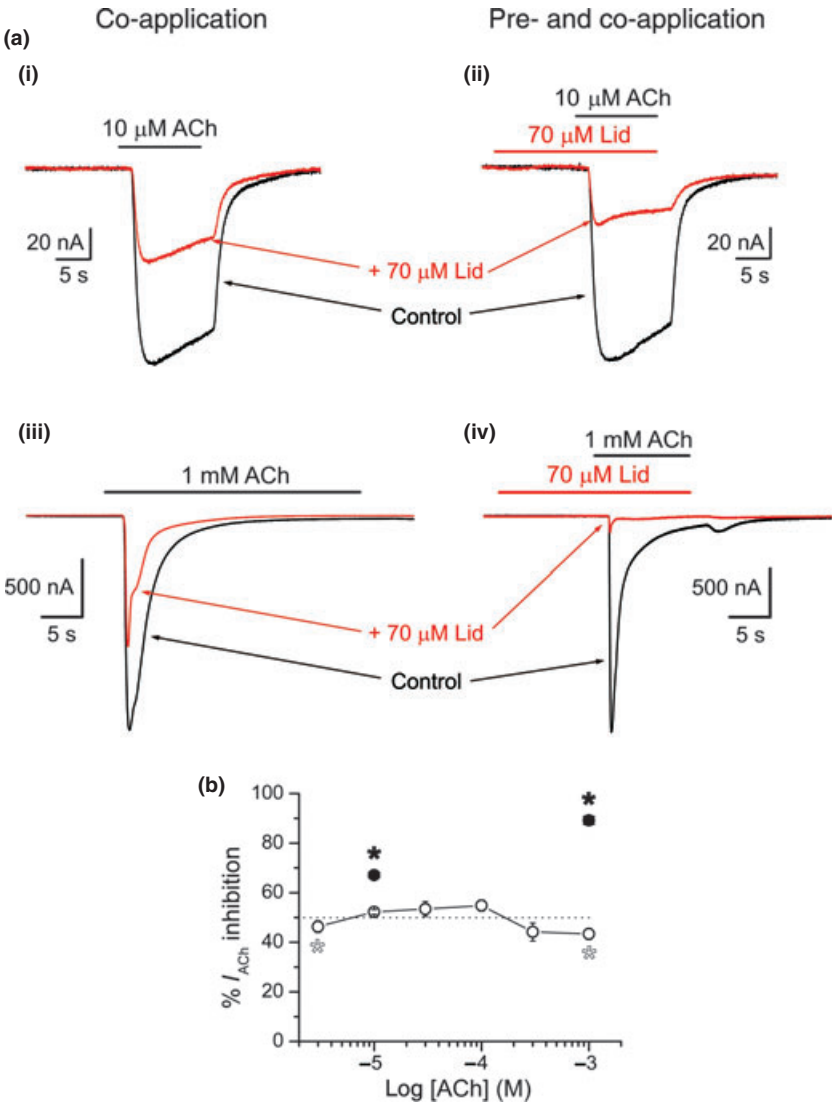

Fig. 5 Effect of lidocaine pre-application on nAChR blockade. (ai, aiii) I_{ACh}s elicited by applying 10 μM (ai) or 1 mM (aiii) ACh either alone (Control, black records), or co-applied with 70 μM lidocaine (+ 70 μM Lid, red records). (aii, aiv) I_{ACh}s obtained by superfusing 10 μM (aii) or 1 mM (aiv) ACh alone (Control, black records), or plus 70 μM lidocaine after pre-applying lidocaine, at the same concentration, for 12 s (+ 70 μM Lid, red recordings). (b) Plot showing the percentage of I_{ACh} inhibition at different ACh concentrations when ACh was directly co-applied with 70 μM lidocaine (open circles and solid line; n = 10–34, N = 3–17), or they were co-applied after pre-application of 70 μM lidocaine for 12 s (filled circles; n = 6–7, N = 6–7). The dashed line corresponds to 50% inhibition. Open asterisks indicate significant differences between the percentage of I_{ACh} blockade elicited by 10 μM ACh and 70 μM lidocaine co-application and those obtained when co-applying lidocaine (70 μM) with other ACh concentrations. Solid asterisks denote significant differences in the percentage of I_{ACh} blockade between direct lidocaine and ACh co-application and pre-application of lidocaine before its co-application with ACh. The slight decrease in I_{ACh} blockade by lidocaine observed at 3 μM ACh might not be genuine but due to the small size of I_{ACh} at this agonist concentration.

the outward-going I_{ACh} by open-channel blockade intracellularly, a few oocytes were injected with 3–5 nL of 0.1 M lidocaine. Although the injected lidocaine could reach an intracellular concentration around 300 μM, it had no effect on the evoked I_{ACh}. By contrast, focal extracellular application of a similar drop of lidocaine caused an evident blockade of nAChRs, indicating that lidocaine was mainly acting from the extracellular side (see Figure SI-1 of the Appendix S1).

The apparent inhibition constant (K_i) for the different voltages tested was determined by plotting the relative amplitude of the I_{ACh} left by lidocaine at each membrane potential versus the corresponding lidocaine concentrations, and fitting the values to Equation 5 (see Appendix S1; Fig. 6d). Then, the obtained K_i values were used in the Woodhull equation (Woodhull 1973; Equation 6 of the Appendix S1) to estimate the fraction of the voltage field sensed by lidocaine at its binding site (δ). When plotting the K_i values against the membrane potential in a semi-logarithmic scale, a linear relationship was obtained (correlation coefficient of 0.947, p-value < 0.0001; Fig. 6e). The δ value for lidocaine, calculated from the slope of the fitted line, was 0.26, assuming that it had only one charged group (the protonated tertiary-amine group), which indicates that it binds to the external third of the channel span.

Additive inhibitory effects of lidocaine with QX-314, edrophonium and BW284c51

To test whether the inhibitory actions of lidocaine on nAChRs are additive to those mediated by quaternary-ammonium molecules, lidocaine was co-applied with edrophonium, BW284c51, or QX-314, in the presence of 10 μM ACh. Given that lidocaine showed several inhibitory mechanisms of nAChRs, which, at pH 7, can be discriminated by dose (see above), we used for these experiments 20 μM lidocaine, which inhibits I_{ACh} mostly by open-channel

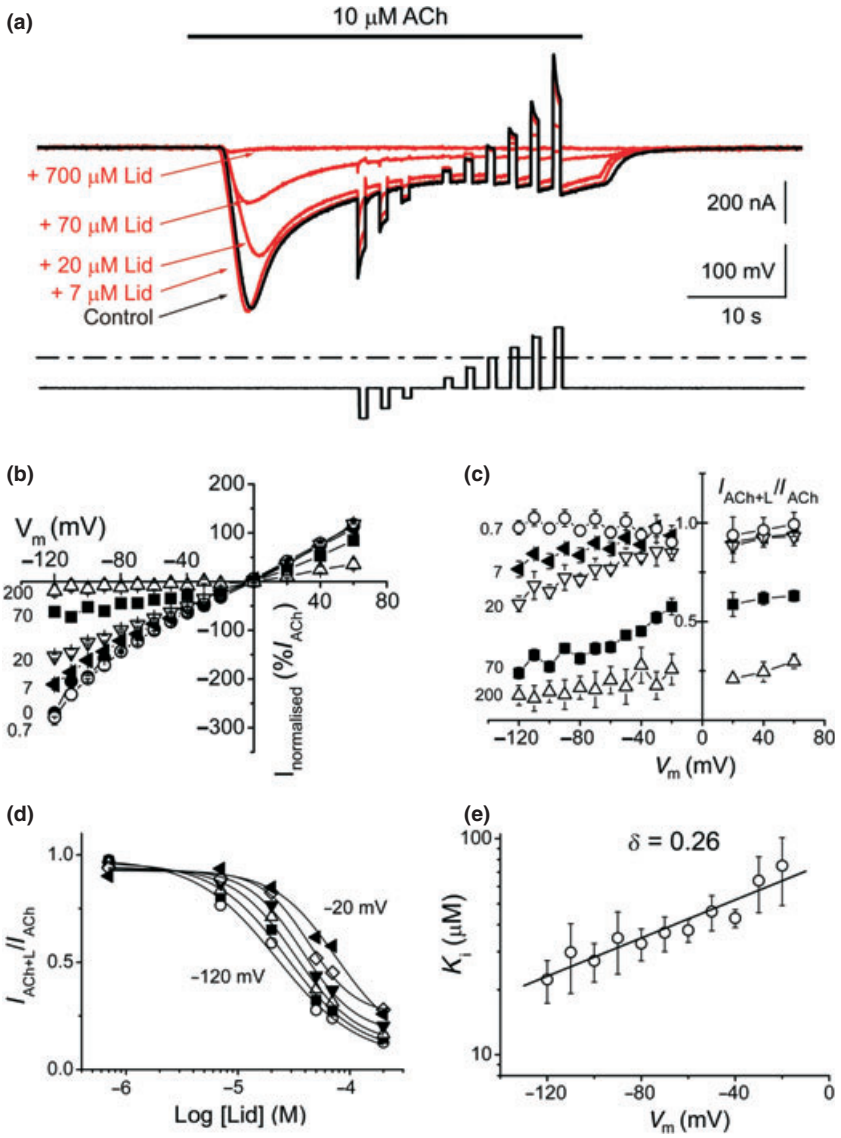

Fig. 6 Dose and voltage dependence of the I_{ACh} blockage by lidocaine. (a) Representative currents (upper traces) evoked, in the same oocyte, by voltage pulses from −120 to + 60 mV, in 20 mV steps (lower trace; the dotted line indicates the 0 voltage level) during the I_{ACh} plateau elicited by 10 μM ACh either alone (black) or plus lidocaine (red) at the indicated concentrations. The shown records are net I_{ACh}s. (b) Representative family of steady-state *i/v* plots of net I_{ACh}s elicited by 10 μM ACh either alone (filled circles) or plus lidocaine at different dosages (numbers on the left, in micromolar) while applying the voltage protocol shown in panel a. Net I_{ACh} values were normalised, for each oocyte, as the percentage of the control I_{ACh} at −60 mV. (c) Plot of the fraction of the I_{ACh} left by lidocaine (I_{ACh+L}) at different dosages, normalised to the control I_{ACh}, against the membrane potential, showing the voltage-dependent *nAChR* blockade by lidocaine. (d) Dose-dependent I_{ACh} blockade is illustrated by plotting I_{ACh+L}/I_{ACh} ratio against the logarithm of the lidocaine dosage; the obtained data were fitted to sigmoid curves to determine the apparent K_i (see Equation 5 of Data S1) for each membrane potential (values obtained at odd membrane potentials are not shown for clarity). (e) The K_i values were plotted versus membrane potential and the electrical distance (δ) of lidocaine binding was estimated from the slope of the fitted line. Each point in the panels (b–e) is the average of 4–12 oocytes (N = 2–9).

blockade. As this lidocaine dose caused a small I_{ACh} inhibition at −60 mV, these experiments were carried out at −100 mV. Figure 7 shows that co-application of 10 μM ACh with 20 μM lidocaine reduced I_{ACh} to 64.1 ± 1.8% of its control value (n = 14, N = 10); similar remaining I_{ACh}*s* were obtained when ACh (10 μM) was co-applied with 3 μM edrophonium (53.5 ± 3.9%, n = 4, N = 3), 0.5 μM BW284c51 (48.7 ± 2.4% n = 6, N = 3), or 10 μM QX-314 (54.2 ± 1.0% n = 6; N = 5). When ACh plus lidocaine were co-applied with edrophonium (Fig. 7bi), BW284c51 (Fig. 7bii), or QX-314 (Fig. 7biii), the I_{ACh} was significantly smaller (36.3 ± 1.3%, n = 4, N = 3; 32.2 ± 1.2%, n = 6,

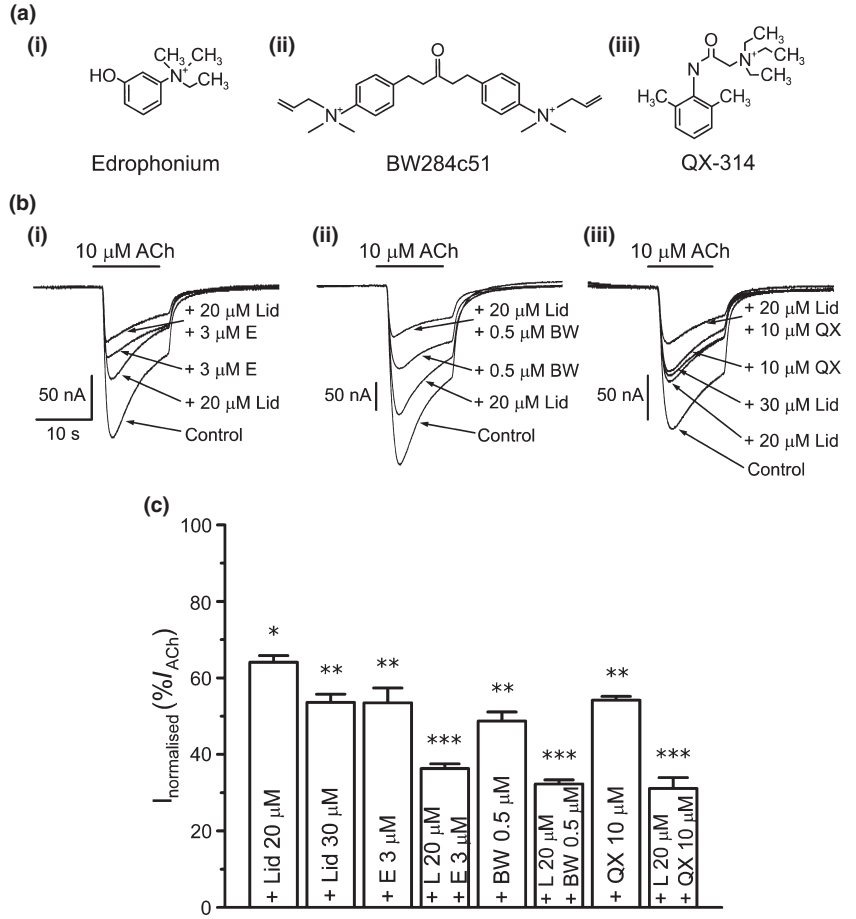

Fig. 7 Additive inhibitory effects of lidocaine and edrophonium, BW284c51 or QX-314 on I_{ACh}. (ai–aiii) Chemical structures of edrophonium, BW284c51 and QX-314. (bi–biii) Representative I_{ACh} records elicited by 10 μM ACh either alone (bi, bii, biii; Control), or plus 20 μM lidocaine (bi, bii, biii; + 20 μM Lid), 3 μM edrophonium (bi; + 3 μM E), 0.5 μM BW284c51 (bii; + 0.5 μM BW), 30 μM lidocaine (biii; + 30 μM Lid) or 10 μM QX-314 (biii; + 10 μM QX); and by 10 μM ACh co-applied with 20 μM lidocaine and 3 μM edrophonium (bi; + 20 μM Lid + 3 μM E), 20 μM lidocaine and 0.5 μM BW284c51 (bii; + 20 μM Lid + 0.5 μM BW), or 20 μM lidocaine and 10 μM QX-314 (biii; + 20 μM Lid + 10 μM QX). In these experiments, the holding potential was −100 mV. (c) Column graph showing an additive inhibitory action on I_{ACh} when lidocaine was co-applied with edrophonium, BW284c51 or QX-314. The height of the bar represents the normalised I_{ACh} amplitudes elicited by 10 μM ACh co-applied with the different combinations showed in panel b and indicated inside each column. Data are the mean ± SEM of 4–14 oocytes (N = 3–10). Different number of asterisks above the bars indicates significant differences between groups ($p < 0.05$, one-way ANOVA).

$N = 3$; and 31.1 ± 2.9%, $n = 6$, $N = 5$; respectively; $p < 0.05$, one-way ANOVA) than that obtained by applying separately each drug at the same concentration (Fig. 7c). Noteworthy, when ACh was co-applied with 30 μM lidocaine, the I_{ACh} decreased to 53.6 ± 2.2% ($n = 5$, $N = 4$), being this blockade significantly smaller than that mediated by 20 μM lidocaine plus 10 μM QX-314, in spite that the concentration of quaternary-ammonium molecules was similar in both cases.

Discussion

Despite of the interest in studying the mechanisms by which different drugs modulate nAChR function, to date, there are very few functional studies carried out to explore the effects of lidocaine on nAChRs. Most studies have been focused on its quaternary-ammonium derivatives QX-222 or QX-314, which share structural similarities, but also show important differences in nAChR modulation. So, for muscle-type nAChRs, the IC_{50} values of lidocaine (11–73 μM; Gentry and Lukas 2001; Wang *et al.* 2010; present data) are similar to those of QX-314 (19–78 μM; Pascual and Karlin 1998; Gentry and Lukas 2001) but markedly different to those of QX-222 (2.7–3.4 mM; Pascual and Karlin 1998; Gentry and Lukas 2001). It should be noted that, according to the Henderson–Hasselbalch equation, when lidocaine is added to a Ringer solution with pH 7.0, as in our experiments, roughly 86% of the molecules are in a charged form (with a

quaternary-ammonium group), and the remaining 14% are tertiary amines. Although only a small fraction of lidocaine molecules are in the neutral form, they can also play a relevant role in modulating nAChRs, especially at high lidocaine doses.

Lidocaine effects on I_{ACh} decay

Although it is well known that QX-314 enhances desensitisation of muscle-type nAChRs (Beam 1976; Sine and Taylor 1982), it has been reported that lidocaine exerts complex changes in the time course of the end plate potential (Steinbach 1968), increases desensitisation (Anwyl and Narahashi 1980) or has no effect on I_{ACh} decay (Wang *et al.* 2010). Our data show that lidocaine significantly accelerates I_{ACh} decay, although only at doses close to the IC_{50}, or higher. Similarly, other LAs of the same structural group, as procaine or tetracaine enhance muscle-type nAChR desensitisation (Koblin and Lester 1979), as well as some LAs with different molecular structure, such as adiphenine, although in this case it is only observed after drug pre-application to resting nAChRs (Spitzmaul *et al.* 2009). Interestingly, lidocaine effect on I_{ACh} decay was fully and quickly reversible, in contrast to the slow recovery of the I_{ACh} amplitude, even after strong depolarizing pulses (A. Morales, unpublished observations). This indicates that the increase in the rate of I_{ACh} decay is mediated by a mechanism different to those responsible for voltage-dependent blockade, present at lower ACh doses, and voltage-independent blockade, which shows slow recovery.

Closed-channel blockade by lidocaine

In contrast to QX-314, which mainly blocks nAChRs in the open state (Neher and Steinbach 1978), other LAs of the same structural group, such as procaine (Adams 1977) or tetracaine (Papke and Oswald 1989), block nAChRs in both resting and open states. The present experiments show, for the first time, that lidocaine caused both non-competitive and apparently competitive inhibition of nAChRs. The apparent competitive blockade was similar to that we previously reported for edrophonium and decamethonium (Olivera-Bravo *et al.* 2007), and can be explained by their binding to nAChR closed states. Strengthening this hypothesis, lidocaine pre-application before its co-application with 10 μM ACh, increased moderately the percentage of I_{ACh} inhibition respect to that obtained by direct ACh-lidocaine co-application; however, when co-applied with 1 mM ACh, a pronounced I_{ACh} blockade occurred (see Fig. 5b, filled symbols). The marked I_{ACh} blockade observed when pre-applying lidocaine before its co-application with 1 mM ACh can be explained assuming that at this high ACh concentration almost every unblocked nAChR present in the membrane opens, allowing open-channel blockade by lidocaine (acting within the channel). When lidocaine pre-application is followed by its co-application with a low ACh concentration, the same percentage of resting nAChRs blockage should be expected, but only a small fraction of the unblocked nAChRs (about 10% when using 10 μM ACh; see Fig. 4b) becomes activated and so, only these nAChRs can be blocked by open-channel blockade, resulting in a smaller percentage of I_{ACh} inhibition. However, the decrease in the percentage of I_{ACh} blockade observed when lidocaine (70 μM) is directly co-applied with a high ACh concentration (1 mM; see Fig. 5b, open symbols) could be due to the fast opening of most nAChRs by this high ACh concentration, which precludes the action of lidocaine on closed channels (in resting state), causing the apparent competitive inhibition.

The finding that resting nAChR inhibition required higher lidocaine doses than open-channel blockade apparently differs with the reported similar affinities of procaine for both open- and closed-channel states (Adams 1977). However, closed-channel blockade could be caused only by the uncharged fraction of lidocaine, which, at the pH used, has a concentration about six times lower than the protonated one. If so, the affinities of charged lidocaine for binding within the channel and neutral lidocaine for binding outside the pore would be similar. There are several reasons suggesting that the blockade of resting nAChRs was mediated by the neutral fraction of lidocaine: (i) this kind of blockade is observed with other tertiary-amine LAs (Adams 1977; Papke and Oswald 1989), but scarcely with the permanent charged molecules QX-222 and QX-314 (Neher and Steinbach 1978); (ii) tertiary-amine LAs are able to compete with phospholipids for sites at the lipid-protein interphase, and thus affecting nAChR function (Mantipragada *et al.* 2003); (iii) reversibility of resting nAChR blockade was very slow, and could not be surmounted by giving strong voltage pulses to positive potentials, which would expel charged lidocaine molecules from the channel pore.

Open-channel blockade by lidocaine

Quaternary-ammonium LAs, as QX-222 and QX-314 (Neher and Steinbach 1978; Horn *et al.* 1980; Pascual and Karlin 1998) and some tertiary-amine LAs, as procaine (Adams 1977) and tetracaine (Koblin and Lester 1979), induce non-competitive blockade of nAChRs by their binding into the channel pore. We have now shown that lidocaine blocked I_{ACh} in a voltage-dependent way and, at doses up to 20 μM, this open-channel blockade was the only significant effect on nAChRs. As most lidocaine molecules are protonated at pH 7.0, it seems likely that the voltage-dependent inhibition was mediated by the fraction of charged lidocaine, which is structurally very similar to its permanently charged derivate QX-314. Nevertheless, benzocaine, a derivative of procaine lacking the tertiary-amine moiety and so, permanently uncharged, is also able to block muscle-type nAChRs in the open state, although at higher

concentrations than procaine (Ogden *et al.* 1981) and does not elicit voltage-dependent blockade (Koblin and Lester 1979). Thus, we cannot fully rule out that, at high doses, some uncharged molecules of lidocaine contributed to the voltage-independent blockade of nAChRs acting into the channel pore.

Several molecules with quaternary-ammonium groups, including QX-314 and QX-222 (Neher and Steinbach 1978; Horn *et al.* 1980) and edrophonium (Olivera-Bravo *et al.* 2007), bind into the channel pore of nAChRs at a site located about 70% of the way through the membrane electrical field. However, the procaine binding site is half way through the channel (Adams 1977) and BW284c51 binds at a very shallow site (Olivera-Bravo *et al.* 2007). By contrast, our present results indicate that lidocaine binds at the external 30% of the channel span. These data indicate that there are several targets within nAChR channel where quaternary-ammonium molecules can bind, depending on their molecular structure. The multiplicity of binding sites in the nAChR for these molecules would justify their additive actions when co-applied together, since they do not compete for the same binding site, in spite of their similarities in molecular structure.

In conclusion, our results indicate that lidocaine exerts multiple inhibitory actions on nAChRs, which, at pH 7.0, can be discriminated by dose. The mechanisms involved in nAChR blockade by lidocaine are similar to those reported for different types of LAs, or even for other molecules with quaternary-ammonium groups. However the potency and the precise site of action on the nAChR are quite specific, allowing additive effects when co-applying lidocaine with related LAs, or other similar molecules. These results provide new insights into the diverse and complex modulation of nAChRs by different LAs, even structurally related. Besides, they highlight the importance of deepening in the knowledge of nAChR modulation to understand the possible side-effects of clinically used molecules, such as LAs and quaternary-ammonium cholinesterase inhibitors, and to contribute to the rational design of new therapeutic tools.

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The authors state no conflict of interest.

Supporting information

Additional supporting information may be found in the online version of this article:

Appendix S1. Data Analysis.

Figure S1. Effect of focal intracellular or extracellular application of lidocaine on outward-going I_{Ach}.

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