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RESEARCH PAPER

Diverse inhibitory actions of quaternary ammonium cholinesterase inhibitors on *Torpedo* nicotinic ACh receptors transplanted to *Xenopus* oocytes

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Background and purpose: This work was aimed at comparing and analysing the effects and mechanisms of action of the quaternary ammonium cholinesterase inhibitors (QChEIs) BW284c51, decamethonium and edrophonium, on nicotinic ACh receptor (nAChR) function.
Experimental approach: nAChRs purified from *Torpedo* electroplax were transplanted to oocytes and currents elicited by ACh (I_{ACh}) either alone or in presence of these QChEIs were recorded.
Key results: None of the QChEIs, by itself, elicited changes in membrane conductance; however, when co-applied with ACh, all of them decreased I_{ACh} in a concentration-dependent way. The mechanisms of nAChR inhibition were different for these QChEIs. BW284c51 blockade was non-competitive and voltage-dependent, although it also affected the n_H of the dose-response curve. By contrast, decamethonium and edrophonium inhibition, at –60 mV, was apparently competitive and did not modify either desensitisation or n_H. Decamethonium effects were voltage-independent and washed out slowly after its removal; by contrast, edrophonium blockade had strong voltage dependence and its effects disappeared quickly after its withdrawal. Analysis of the voltage-dependent blockade indicated that BW284c51 bound to a shallow site into the channel pore, whereas edrophonium bound to a deeper locus. Accordingly, additive inhibitory effects on I_{ACh} were found among any pairs of these QChEIs.
Conclusions and implications: The tested QChEIs bound to the nAChR at several and different loci, which might account for their complex inhibitory behaviour, acting both as allosteric effectors and, in the case of BW284c51 and edrophonium, as open channel blockers.
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Keywords: BW284c51; edrophonium; decamethonium; nicotinic acetylcholine receptors; quaternary ammonium molecules; allosteric modulation

Abbreviations: ACh, acetylcholine; ANR, normal Ringer with atropine; BW284c51, 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide; ChEI, cholinesterase inhibitor; I_{ACh}, acetylcholine current; M2, second membrane-spanning segment; nAChR, nicotinic acetylcholine receptor; QChEI, quaternary ammonium cholinesterase inhibitor; TEA, tetraethylammonium

Introduction

Nicotinic acetylcholine (ACh) receptors (nAChRs) are heteropentameric members of the 'Cys-loop' subfamily of ligand-gated ion channels, which play important functional roles at both peripheral and central nervous systems (Gotti and Clementi, 2004; Dani and Bertrand, 2006; Sine and Engel, 2006). So, alterations in nAChR number and/or function have been related to different pathologies, including certain types of epilepsy, congenital myasthenic syndrome, myasthenia gravis, schizophrenia and Parkinson's and Alzheimer's diseases (Gotti and Clementi, 2004; Sacco *et al*., 2004; Dani and Bertrand, 2006). This fact explains the current interest in studying the effects and mechanisms by which different drugs modulate nAChR function.

Quaternary ammonium compounds, including cholinesterase inhibitors (QChEIs), local anaesthetics and other small molecules, as tetraethylammonium (TEA), have shown significant effects on nACh currents (I_{ACh}), although some of their mechanisms of action remain yet uncertain. For example, the effects of TEA on nAChRs have been known for a long time (Koketsu, 1958), although the complexity of its inhibition, with at least three independent underlying

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mechanisms, has been only recently elucidated (Akk and Steinbach, 2003). Local anaesthetics containing ammonium groups, such as QX-314, QX-222 or procaine, act on nAChRs mainly as noncompetitive inhibitors, binding within the open channel and occluding it (Neher and Steinbach, 1978; Pascual and Karlin, 1998). However, these noncompetitive inhibitors can also interact with closed states of the nAChR and promote its desensitisation (Neher, 1983; Gage and Wachtel, 1984). Ethyl(3-hydroxyphenyl)dimethylammonium chloride, edrophonium, is a QChEI that, at clinical concentrations, reduces single-channel I_{ACh} in BC_3H1 cells expressing muscle-type nAChRs (Wachtel, 1990) and increases the I_{ACh} desensitisation rate of mouse muscle receptors expressed in *Xenopus* oocytes (Yost and Maestrone, 1994). Decamethylene{bis[trimethylammonium}dibromide], decamethonium, is a well-known QChEI (Harel *et al*., 1993), which exhibits heterogeneous actions on nAChRs, depending, at least in part, on the receptor subunit composition. So, it acts as a partial agonist of muscle nAChRs (del Castillo and Katz, 1957; Adams and Sakmann, 1978; Liu and Dilger, 1993), it blocks open endplate channels (Adams and Sakmann, 1978) or antagonises α7-nAChRs (Bertrand *et al*., 1992), without affecting other neuronal nAChRs (Lummis *et al*., 1992). In contrast, hexamethonium and dodecamethonium block both neuronal and muscle nicotinic responses, although tetramethonium fails to block ACh-induced responses, suggesting a relationship between the ability to block I_{ACh} and the size of the carbon chain (Lummis *et al*., 1992).

Recently, we have shown that 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide, BW284c51, a bisquaternary ammonium ChEI, inhibits *Torpedo* nAChRs with potency similar to that shown by d-tubocurarine (Olivera-Bravo *et al*., 2005). In contrast to curare, BW284c51 blockade of I_{ACh} is mainly due to an open-channel blockade, although it also modifies the desensitisation and the slope of the nAChR dose–response curve. This effect seems to be specific for the muscle subtype of nAChRs because BW284c51 showed no inhibitory actions either on homomeric or heteromeric nAChRs of CA1 hippocampal interneurones (Fayuk and Yakel, 2004).

Despite the similar effects of quaternary ammonium compounds, such as BW284c51, edrophonium and decamethonium (see chemical structure in Figure 1c), as inhibitors of cholinesterase, there are several pieces of evidence, as mentioned above, suggesting significant differences in their actions on nAChRs. Therefore, the aim of this work was to compare and elucidate the mechanisms underlying the BW284c51, edrophonium and decamethonium effects on purified *Torpedo marmorata* nAChRs transplanted to *Xenopus* oocytes (Morales *et al*., 1995). This experimental approach allowed us to test the effects of these compounds on the function of fully processed and correctly assembled nAChRs. We have found that QChEIs differ in their inhibitory actions on transplanted nAChRs, most likely due to their binding to different loci in the nAChR. Preliminary results have been published elsewhere (Olivera-Bravo *et al*., 2006).

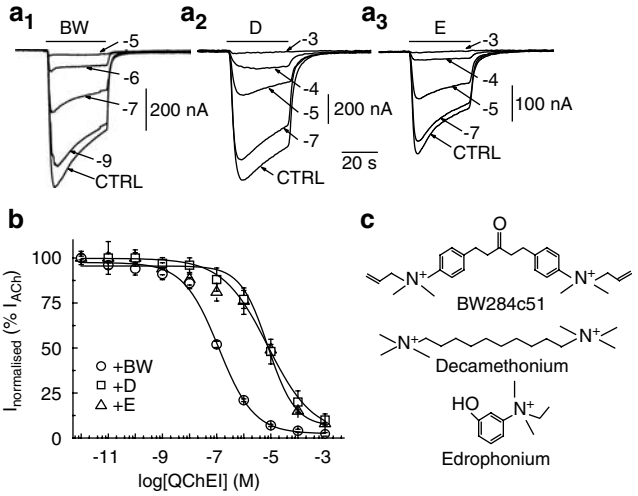

Figure 1 Effects of QChEIs on ACh-induced currents (I_{ACh}). Representative superimposed currents elicited by superfusing a single nAChR-injected oocyte with 10 μM ACh either alone (control; CTRL) or co-applied with different concentrations of either BW284c51 (**a**$_1$; BW), decamethonium (**a**$_2$; D) or edrophonium (**a**$_3$; E). The numbers on the right of the recordings indicate the logarithm of the drug concentration applied. Note that neither 10 μM decamethonium nor 10 μM edrophonium modified the time from the onset of the response to the I_{ACh} peak. (**b**) Relationship between BW284c51 (+BW), decamethonium (+D) or edrophonium (+E) concentration and the I_{ACh} peak amplitude elicited by 10 μM ACh co-applied with increasing concentrations of each drug at 5–10 min intervals. I_{ACh} values obtained for each drug were normalised as the percentage of the corresponding control I_{ACh}. Data are the average of 3 ($N=1$; hereafter 'N' indicate the number of donors), 7 ($N=3$) and 8 ($N=3$) oocytes for BW284c51, decamethonium and edrophonium, respectively. Each solid line in the graph represents a sigmoid curve with n_H close to 1. (**c**) Chemical structure of the QChEIs tested. In this and following figures, the holding potential was −60 mV, unless otherwise stated; downward deflections denote inward currents and horizontal bars indicate the time of drug application.

Materials and methods

Oocyte preparation and microinjection of purified nAChRs

Adult female *Xenopus laevis* (purchased from Blades Biological, Edenbridge, Kent, UK) were immersed in cold 0.17% MS-222 for 15 min and a piece of ovary was drawn out aseptically. Animal handling was carried out in accordance with the guidelines for care and use of laboratory animals adopted by the E.U. Stage V and VI oocytes were isolated and their surrounding layers removed manually, as described previously (Ivorra and Morales, 1997). 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 $MgSO_4$, 10.00 mM HEPES (pH 7.4), 100 U ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin) until used. Procedures employed for receptor purification and oocyte microinjection have been described previously (Morales *et al*., 1995; Ivorra *et al*., 2002). Briefly, enriched nAChR homogenates from *Torpedo marmorata* electroplax were purified by affinity chromatography and reconstituted in asolectin liposomes at a final concentration of 0.3–1.2 mg of protein per ml. Frozen aliquots of this sample were slowly thawed on ice and 100 nl were microinjected into oocytes.

Electrophysiological recordings

I_{ACh} recording methodology has been described previously (Morales *et al*., 1995; Ivorra *et al*., 2002; Olivera-Bravo *et al*., 2005). Briefly, membrane current recordings were performed at 21–25°C, 16–36 h after oocyte injection of purified nAChRs, using a high-compliance two-microelectrode voltage-clamp system (TurboTEC-10CD, npi). Oocytes were placed in a 150 μl recording chamber that was continuously superfused (3–15 ml min^{-1}) with normal frog Ringer's solution (NR; 115 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$ and 5 mM HEPES, pH 7.0) containing 0.5 μM atropine sulphate (ANR) to block muscarinic responses (Kusano *et al*., 1982). The membrane potential was held at −60 mV, unless otherwise stated. Membrane currents were low-pass filtered at 30–2000 Hz and after sampling at fivefold the filter frequency (Digidata 1200 Series; Axon Instruments, Foster City, CA, USA), recorded on both a chart recorder (Kipp and Zonen BD-112, Delft, Holland) and a PC-computer, using the WCP v. 3.2.8 package developed by J Dempster (Strathclyde Electrophysiology Software, University of Strathclyde, Scotland, UK).

Experimental design

To study the voltage dependence of the I_{ACh} blockade by QChEIs, series of 800 ms voltage pulses (in 10 mV steps from −120 to −20 mV, followed by 20 mV jumps from −20 to +60 mV) were given to the oocyte before ligand superfusion and during the I_{ACh} plateau elicited by 10 μM ACh either alone or co-applied with BW284c51, decamethonium or edrophonium. In a few cells, the −120 mV pulse duration was extended up to 1500 ms to allow a more complete current relaxation. Net *i*/*v* curves for I_{ACh} were obtained by subtracting, for each voltage, the steady-state currents attained in presence of ACh (measured during the last 100 ms of the pulse) from those obtained in its absence. I_{Ach} values were normalised for each oocyte to the ACh response at −60 mV. For *i*/*v* experiments, the rate of ACh superfusion was decreased to 3–5 ml min^{-1}, to reduce the apparent I_{ACh} desensitisation. The ACh concentration–I_{ACh} amplitude curves were obtained by exposing injected oocytes to increasing ACh concentrations, either alone or together with each tested drug at its respective IC_{50}. To reduce nAChR desensitisation, the interval between consecutive ACh applications was at least 5 min.

Data analysis and statistical procedures

For receptor activation, dose–response data were fitted to the following form of the Hill equation:

$$I/I_{max} = [1 + (EC_{50}/[ACh])^{n_H}]^{-1} \quad (1)$$

where I is the I_{ACh} peak elicited at a given ACh concentration, I_{max} is the maximum current recorded at the highest ACh concentration; EC_{50} the agonist concentration required to obtain one-half of the maximum current, and n_H is the Hill coefficient. Inhibition curves were determined by measuring I_{ACh} in presence of different BW284c51, decamethonium or edrophonium concentrations. Data were fitted to a single-site inhibition curve using the Origin 6.1 software (OriginLab Corp, Northampton, MA, USA); for this fitting, I_{min} was set to 0, to prevent the curve reaching negative values. The rate of desensitisation was determined by measuring the I_{ACh} amplitude elicited by 10 or 100 μM ACh, either alone or co-applied with each QChEI at its IC_{50}, at different times after the current peak. When measuring I_{ACh} desensitisation, the superfusion rate was set to 13–15 ml min^{-1}. The values of desensitisation were obtained using the equation:

$$D_{ti} = (1 - (I_{ti}/I_{peak})) \times 100 \quad (2)$$

where D_{ti} is the desensitisation value at the specified time, I_{peak} is the peak current amplitude, and I_{ti} is the current amplitudes remaining 2, 10 and 20 s after the peak. The percentage of I_{ACh} inhibition at different membrane potentials (V_m) was computed using the following equation:

$$In_{V_m} = (1 - (I_{(ACh+QChEI)\,at\,V_m}/I_{(ACh)\,at\,V_m})) \times 100 \quad (3)$$

where In_{Vm} is the percentage of I_{ACh} inhibition at the corresponding V_m, $I_{(ACh\,+\,QChEI)\ at\ Vm}$ is the I_{ACh} amplitude in presence of ACh and the QChEI at V_m, and $I_{(ACh)\ at\ Vm}$ is the I_{ACh} elicited by ACh alone at V_m. Values from the *i*/*v* relationship obtained at different blocker concentrations were fitted to equations 4 and 5 to estimate the fraction of voltage field (δ) sensed by each QChEI at its binding site. The apparent K_i, which is the concentration of each inhibitor that reduces the current amplitude to the half, was estimated from the following equation:

$$I_{ACh+I}/I_{ACh} = I_{min} + ((I_{max} - I_{min})/(1 + [I]/K_i)^n) \quad (4)$$

where $I_{ACh\,+I}$ is the current evoked by co-application of 10 μM ACh with different concentrations of either BW284c51 or edrophonium, I_{ACh} is the current elicited by 10 μM ACh alone (control conditions), I_{min} and I_{max} are the minimum and maximum fractional-current amplitudes evoked, [*I*] is the concentration of each inhibitor and n is the slope factor; for the curve fitting, the free variables were 'K_i', 'n', 'I_{max}' and 'I_{min}'. To estimate the fraction of the voltage field experienced by the blocking particle, we used the following form of the Woodhull equation (Woodhull, 1973):

$$\log K_i(V) = \log K_i\,(0\,mV) + (z\delta FV/2.303\,RT) \quad (5)$$

where K_i (V) is estimated from equation 4 at each voltage; K_i (0 mV) is the K_i value at 0 mV, z is the electric charge of the channel blocker and R, T and F have the usual thermodynamic meanings. For this curve fitting, the free variables were 'K_i (0 mV)' and 'δ'. The I_{ACh} obtained during voltage pulses in the presence of different edrophonium concentrations was fitted to a single exponential to estimate the channel closure time constant (τ).

Unless otherwise specified, values given are the mean ± s.e.m; n indicates the number of oocytes and N is the number of donors from which the values were obtained. When comparing two-group means of normally distributed data, the Student's *t*-test was used. Otherwise, the 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, BW284c51, decamethonium, edrophonium, MS-222, 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. Solutions containing BW284c51 were protected from light at all times.

Results

This work was aimed at characterising the effects of three prototypic QChEIs, BW284c51, decamethonium and edrophonium, on nAChRs transplanted to *Xenopus* oocytes. For this purpose, we have studied the actions of these QChEIs on the nAChR pharmacological profile, the concentration and voltage dependence of their effects, to estimate their binding site on the nAChR and, finally, whether or not they have synergic inhibitory actions.

Inhibition of I_{ACh} *by QChEI*

None of the QChEI tested showed by itself any effect on the cell membrane conductance of either native cells or oocytes injected with nAChRs, even at concentrations as high as 10 mM. However, in oocytes incorporating nAChRs, co-application of ACh (10 μM) with each QChEI tested, at concentrations ranging from 5 pM to 1 mM, caused I_{ACh} blockade in a concentration-dependent way, as shown in Figure 1. In concordance with our previous data, I_{ACh} inhibition by BW284c51 fitted to a simple sigmoid with an estimated IC_{50} of 0.5 μM and a n_H close to 1 (Figures 1a$_1$ and b; Olivera-Bravo *et al*., 2005). Likewise, decamethonium (Figures 1a$_2$ and b) and edrophonium (Figures 1a$_3$ and b) also caused a significant nAChR blockade, but with much lower potency than BW284c51, as indicated by their respective IC_{50} values, very close to 10 μM for both drugs (Figure 1b). The similarity in the slope of the dose–inhibition curves for the three QChEIs tested indicated that all of them caused inhibition by their binding to the nAChR in a molecular ratio of 1:1. Drug kinetics did not substantially affect the concentration–inhibition curves, because the inhibition time constant, measured in a few cells at low QChEI concentrations, was comparable to that of I_{ACh} activation at low ACh concentrations (10 μM; not shown).

Several differences were found among the effects of these QChEI on nAChRs. Neither decamethonium nor edrophonium, at their respective IC_{50} values, affected the I_{ACh} desensitisation pattern, measured 2, 10 and 20 s after the peak (see Materials and methods), even when they were co-applied with high doses of ACh (100 μM, Table 1). Moreover, neither decamethonium nor edrophonium modified the time lapsed from the onset of the response to the I_{ACh} peak (Table 1). The lack of effect of edrophonium and decamethonium on desensitisation and time to peak is in clear contrast with the effects of BW284c51, which reduced both parameters significantly (Olivera-Bravo *et al*., 2005). Concerning reversibility of QChEI blockade, both BW284c51 and edrophonium actions on I_{ACh} were immediately and completely reversed after the drug withdrawal (full I_{ACh} recovery took place in 12 ± 2 and 14 ± 2 s, for BW284c51 and edrophonium, respectively; four cells), as shown in Figures 2a and c. On the contrary, decamethonium blockade was not completely abolished after its removal, as was previously reported for homomeric α7-nAChRs (Bertrand *et al*., 1992). As shown in Figure 2b, the amplitude of the I_{ACh} plateau did not recover to the control values after decamethonium was washed out, suggesting a small, but significant, residual effect lasting for several minutes.

QChEI effects on nAChR pharmacological profile

ACh concentration–I_{ACh} amplitude curves were obtained by superfusing the cells with ACh either alone or together with each QChEI, individually, at its respective IC_{50}. As shown in

Table 1 Effects of edrophonium and decamethonium on I_{ACh} time to peak and desensitisation

Test	*Time to peak (s)*	*Desensitisation (%)*		
		2 s	*10 s*	*20 s*
10 μM ACh ($n=86$, $N=20$)	5.4 ± 0.2	5 ± 1	27 ± 2	41 ± 2
10 μM ACh + 10 μM D ($n=41$, $N=18$)	5.3 ± 0.3	4 ± 1	25 ± 2	37 ± 2
10 μM ACh + 10 μM E ($n=45$, $N=20$)	5.6 ± 0.3	5 ± 1	29 ± 2	44 ± 2
100 μM ACh ($n=114$, $N=14$)	2.6 ± 0.1	19 ± 1	67 ± 1	84 ± 1
100 μM ACh + 10 μM D ($n=58$, $N=11$)	2.3 ± 0.1	21 ± 1	70 ± 2	85 ± 1
100 μM ACh + 10 μM E ($n=56$, $N=11$)	2.5 ± 0.1	20 ± 2	70 ± 2	86 ± 1

Abbreviations: ACh, acetylcholine; I_{ACh}, acetylcholine current.

Data show I_{ACh} time to peak and desensitisation values elicited by 10 and 100 μM ACh either alone or co-applied with either 10 μM decamethonium (D) or 10 μM edrophonium (E). The time to the I_{ACh} peak was measured as the time between the onset of the current and its maximal value. Desensitisation values were obtained using equation 2 (see Materials and methods). Note that desensitisation increased with ACh concentrations and that neither decamethonium nor edrophonium modified the desensitisation rate. The number of cells tested and donors used for each group is given in parentheses as *n* and *N*, respectively. Significance level was determined as $P<0.05$.

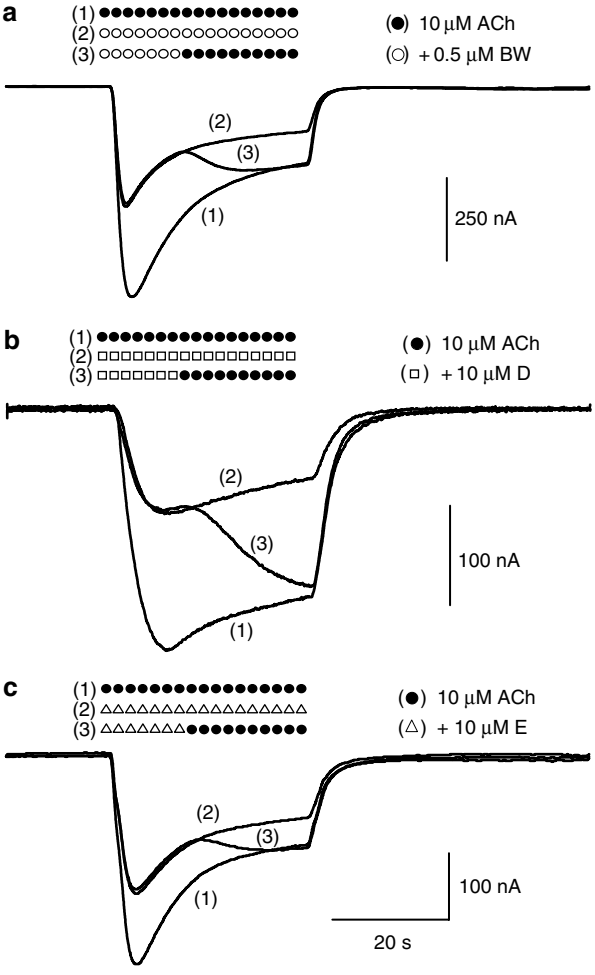

Figure 2 Reversibility of I_{ACh} blockade. I_{ACh} records elicited by 10 μM ACh either alone (1) or co-applied with 0.5 μM BW284c51 (**a**; 2); 10 μM decamethonium (**b**; 2) or 10 μM edrophonium (**c**; 2) in the same oocyte. For (**a**–**c**), mixed symbols (3) indicate the change from the ARN containing 10 μM ACh plus the corresponding QChEI to another one containing ACh alone. Note that I_{ACh} quickly recovered its control values after either BW284c51 or edrophonium washout, whereas after decamethonium withdrawal I_{ACh} did not recover to the control amplitude.

Figures 3a$_1$ and b, 0.5–1000 μM ACh applications to oocytes microinjected with nAChRs elicited I_{ACh} that fitted well to a two-site Hill equation (n_H of 1.8 ± 0.2; $n=13$, $N=7$), as reported previously (Morales *et al*., 1995; Olivera-Bravo *et al*., 2005). When the same ACh concentrations were co-applied with 0.5 μM BW284c51 (Figure 3a$_2$ and b), the I_{ACh} amplitude was about half of the control values, independent of the agonist concentration used, and the concentration–response relationship obtained fitted to a single sigmoid curve with a n_H close to 1 (1.2 ± 0.1; $n=5$, $N=3$), as reported previously (Olivera-Bravo *et al*., 2005). In contrast, when those ACh concentrations were applied in presence of 10 μM decamethonium (Figure 3a$_3$ and b) or edrophonium (Figure 3a$_4$ and b), the percentage of I_{ACh} blockade decreased as the ACh concentration increased, indicating that both of them show competitive antagonism on nAChRs. Noticeably, neither decamethonium nor edrophonium, at their IC_{50}, affected the pharmacological profile of the nAChRs response, because they did not modify the fitting to a two-site concentration–response curve (for decamethonium 1.7 ± 0.2, $n=4$, $N=3$; for edrophonium 1.9 ± 0.2, $n=6$, $N=3$; see slopes in Figure 3b). The different mechanisms of I_{ACh} blockade exhibited by BW284c51 and either edrophonium or decamethonium, at their IC_{50}, are clearly shown in Figure 3c, where the percentage of I_{ACh} inhibition is plotted against ACh concentration. Notice that at low ACh concentrations (10 μM) all these QChEI reduced I_{ACh} roughly to half. However, at higher ACh concentrations (100 or 1000 μM), there was a significant difference ($P<0.05$, Dunn's test) between the inhibition caused by BW284c51, which was maintained at roughly 50% (54 ± 3, $n=8$ and 49 ± 2, $n=6$ for 100 and 1000 μM ACh, respectively), and either edrophonium (23 ± 2, $n=6$ and 21 ± 2, $n=6$) or decamethonium (30 ± 2, $n=7$ and 20 ± 4, $n=4$) for the corresponding ACh concentrations. Thus, at -60 mV, I_{ACh} inhibition by both decamethonium and edrophonium has an apparent competitive component, whereas BW284c51 blockade is noncompetitive and, in addition, modifies the slope of the concentration–response curve.

Voltage dependence of the QChEI effects on nAChRs

To determine whether the I_{ACh} blockade caused by the QChEIs tested is voltage-dependent, *i/v* curves were obtained by applying voltage jumps from -120 to $+60$ mV (in 10 or 20 mV steps; see Materials and methods) in absence and presence of 10 μM ACh either alone or together with each QChEI. The steady-state *i/v* curve for the I_{ACh} elicited by ACh alone (Figures 4, 5 and 6, black curves), showed an inward rectification and a reversal potential that were in good agreement with our previous data (Morales *et al*., 1995; Olivera-Bravo *et al*., 2005). In presence of BW284c51 (Figure 4), the I_{ACh} amplitude was smaller than the control one at negative potentials, but almost no reduction was found at positive values, as reported previously (Olivera-Bravo *et al*., 2005). When this protocol was applied to oocytes superfused with 10 μM ACh together with 10 μM decamethonium (Figure 5), the I_{ACh} blockade was of about 50% in the whole range of voltage tested. Conversely, 10 μM edrophonium caused a strong I_{ACh} reduction at hyperpolarising potentials, but did not evoke any significant blockade at positive values (Figure 6; Table 2). The strong voltage dependence of edrophonium blockade caused the typical inward rectification of I_{ACh} to be changed to outward rectification at negative potentials (Figure 6b). The ion selectivity of the ACh-gated channel was not affected by any of the QChEI tested, because the reversal potential remained unchanged, as compared to control values.

The different voltage dependence of the I_{ACh} blockade elicited by BW284c51, edrophonium and decamethonium can be demonstrated by comparing the percentage of I_{ACh} blockade promoted by the QChEI at two membrane potentials, $+60$ and -60 mV (Table 2). These two potentials were chosen because they produced a similar current driving force, because I_{ACh} reversal potential is close to 0 mV (see Figures 4b, 5b and 6b). As Table 2 shows, BW284c51 and

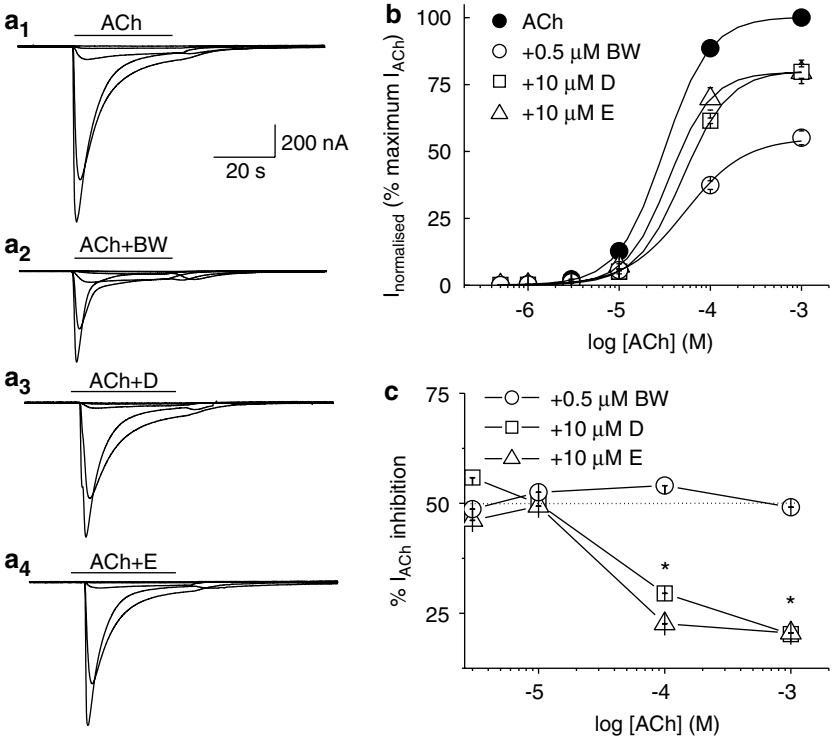

Figure 3 QChEI effects on the ACh concentration–I_{ACh} amplitude relationship. (**a$_1$**–**a$_4$**) Superimposed recordings obtained by applying sequentially to the same cell, 0.5, 1, 10, 100 and 1000 μM ACh either alone (**a$_1$**) or together with either 0.5 μM BW284c51 (**a$_2$**), 10 μM decamethonium (**a$_3$**), or 10 μM edrophonium (**a$_4$**). BW284c51 left roughly half the control I_{ACh} in each ACh concentration tested, whereas decamethonium and edrophonium exhibited an apparently competitive blockage, which was more evident at the highest agonist concentrations. (**b**) Averaged plots of concentration–response relationships evoked by ACh either alone ($n=13$; hereafter 'n' indicates the number of oocytes; $N=7$) or co-applied with either 0.5 μM BW284c51 ($n=5$; $N=3$); 10 μM decamethonium ($n=4$; $N=3$) or 10 μM edrophonium ($n=6$; $N=3$). To ensure complete recovery between trials, ACh pulses were given every 5–30 min, depending on ACh concentration. $I_{normalised}$ means the ratio between the I_{ACh} obtained for each ACh concentration (applied either alone or together with BW284c51, decamethonium or edrophonium) and the maximum I_{ACh} for each cell. (**c**) The different mechanisms of I_{ACh} blockade by QChEI are illustrated by plotting the percentage of I_{ACh} inhibition versus ACh concentration. Each point is the average and s.e.m. of 4–9 cells. Asterisks indicate significant differences ($P<0.05$; Dunn's test) between BW284c51 and either decamethonium or edrophonium groups.

edrophonium I_{ACh} blockade showed significant voltage dependence, in contrast to decamethonium, which exhibited similar I_{ACh} inhibition at both potentials. Given that membrane potential did not affect decamethonium I_{ACh} blockade, further studies on the voltage dependence of I_{ACh} inhibition by QChEIs were restricted to BW284c51 and edrophonium.

Concentration dependence of BW284c51 and edrophonium blockade of nAChRs at different membrane potentials

As BW284c51 and edrophonium effects were voltage-dependent at their respective IC_{50}, we extended the study of the blockade voltage dependence to other QChEI concentrations, because such knowledge provides a good estimation of the binding sites of the inhibitor in the channel (Sánchez *et al*., 1986; Qu and Hartzell, 2001). Figures 7a$_1$ and b$_1$, shows that the relative current amplitude evoked by ACh co-applied with BW284c51 (I_{ACh+BW}/I_{ACh}) decreased at negative potentials, confirming the voltage dependence of nAChR blockade by BW284c51, although, at 10 μM BW284c51 the I_{ACh} was almost completely abolished at any potential, suggesting another yet unknown blocking mechanism that seems to operate in the micromolar range. The curves obtained by plotting the relative amplitude of the I_{ACh} left by BW284c51 at each membrane potential versus BW284c51 concentrations fitted well to equation 4 (see Materials and methods), allowing an estimate of the apparent inhibition constant (K_i) for the different voltages tested (Figure 7c$_1$). The obtained K_i values were then used to estimate the fraction of the voltage field experienced by the inhibitor (δ), using the Woodhull equation (equation 5, Materials and Methods). When the K_i values for each voltage were plotted against the membrane potential in a semilogarithmic scale, a linear relationship was obtained (Figure 7d$_1$). The fitted line has a correlation coefficient of 0.776, giving a P-value (the probability for the t-test of the slope = 0) of 0.005, which corroborates the voltage dependence of the I_{ACh} blockade at negative potentials. The fraction of the voltage field (δ) experienced by the inhibitor can be calculated from the slope of the fitted line and depends on the number of charged groups that enter the voltage field. Given that BW284c51 has two quaternary ammonium groups (Figure 1c), it can be assumed that $z=2$ and then $\delta=0.06$; if only a single group enters the voltage field, then δ would be 0.12. Whatever the case, these δ values indicate

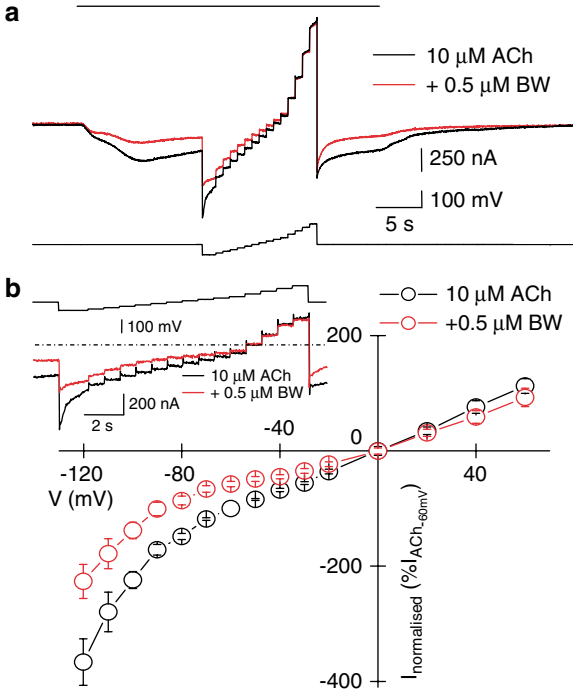

Figure 4 Voltage dependence of I_{ACh} blockade by BW284c51. (**a**) Representative membrane currents (upper traces) obtained by applying to the oocyte, voltage jumps (–120 to +60 mV, in 10 or 20 mV steps; lower panel) during the current plateau elicited by 10 μM ACh either alone (black) or together with 0.5 μM BW284c51 (red). (**b**) Inset displays, at an expanded scale, the same voltage protocol and recordings of net I_{ACh} and I_{ACh+BW} obtained by subtracting the whole currents elicited at each potential in presence of ACh (shown in **a**) from the corresponding ones in its absence (not shown). The dotted line indicates the 0 current level. Plot shows steady-state *i*/*v* relationship for the I_{ACh} elicited by 10 μM ACh either alone or together with BW284c51 (+0.5 μM BW) obtained by applying the voltage protocol shown in a. Net I_{ACh} values were plotted and values were normalised, for each oocyte, as the percentage of the control I_{ACh} at −60 mV. Note the lack of BW284c51 effects at positive potentials. Every point is the average and s.e.m. of nine oocytes ($N=4$).

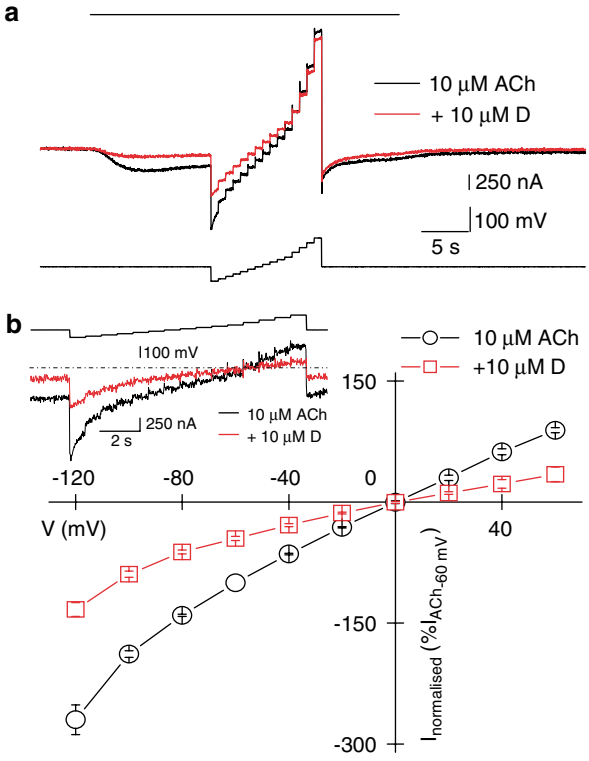

Figure 5 Lack of voltage dependence of I_{ACh} blockade by decamethonium. (**a**) Whole membrane currents (upper traces) obtained in an oocyte after applying the voltage protocol shown on bottom, during the current plateau elicited by 10 μM ACh, either alone (black) or with 10 μM decamethonium (red). (**b**) Net *i*/*v* relationship for I_{ACh} evoked by 10 μM ACh, either alone or co-applied with decamethonium (+10 μM D), obtained by applying the voltage protocol shown in a. Values represent the percentage of current referred to their control I_{ACh} at −60 mV and each point is the average of five cells ($N=3$). Note the similar I_{ACh} blockade by decamethonium in the whole range of voltage tested. Inset shows the voltage protocol and the net I_{ACh} values obtained from the recordings shown in (**a**). The dotted line indicates the 0 current level.

that there is a very shallow binding site for BW284c51 into the nAChR channel.

The same protocol was used to study the edrophonium voltage and concentration dependence of I_{ACh} blockade. These experiments showed that the strong inhibition caused at negative potentials by edrophonium at the IC_{50} was maintained in the whole range of concentrations tested (Figure 7a$_2$ and b$_2$) and that the apparent K_i values (Figure 7c$_2$) varied significantly with the membrane potential. When plotting the K_i versus membrane potential in a semilogarithmic scale, a linear relationship was also obtained, but with a steeper slope than the BW284c51 one ($\delta=0.74$; Figure 7d$_1$ and d$_2$). This indicated that edrophonium bound to the nAChR channel in a deeper site than BW284c51 and, consequently, that the nAChR has different sites for QChEI binding.

As current inhibition by voltage-dependent channel blockers is often time-dependent, because of the accumulation or loss of the blocker from the blocking site when changing the membrane potential (Qu and Hartzell, 2001), we studied the time dependence of edrophonium blockade, because it was the QChEI with the strongest voltage-dependent effects. In presence of edrophonium, the I_{ACh} displayed an inactivation time course that was not only affected by voltage, but also by drug concentration (Figure 8, inset). Thus, when applying 800 ms voltage pulses, from –60 to −120 mV, during the current plateau elicited by either ACh alone or co-applied with different edrophonium concentrations, we found that the I_{ACh} decayed faster as the drug concentration increased, following a sigmoid curve in a semilogarithmic plot (Figure 8). Time constant (τ) values decreased from 480 ± 50 ms, in control conditions (10 μM ACh), to 64 ± 2 ms in presence of 10 μM edrophonium ($n=6$, $N=4$). Besides, as it would be expected from the above results, I_{ACh} amplitude diminished as the QChEI concentration increased (Figure 8). These results are consistent with a model in which edrophonium would enter the pore of the channel and exert its blockade in a voltage-dependent manner (Qu and Hartzell, 2001).

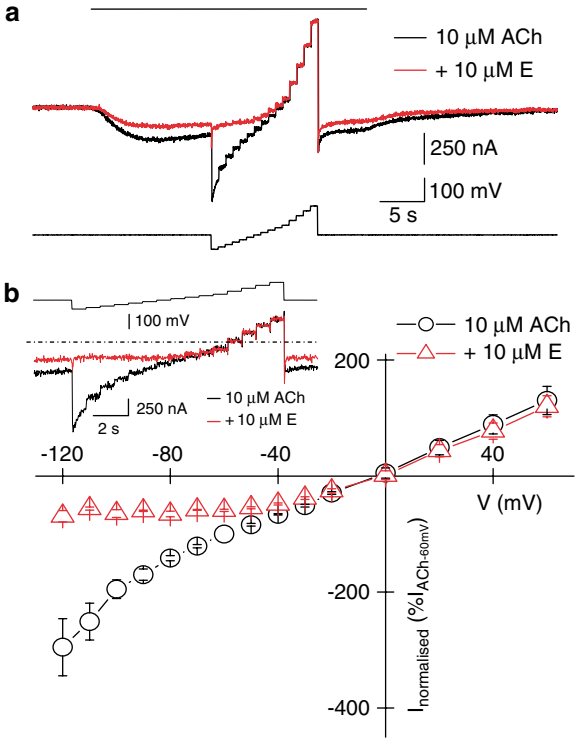

Figure 6 Strong voltage dependence of the I_{ACh} blockade by edrophonium. (**a**) Sequence of voltage steps (lower record) and the membrane currents evoked by this protocol when applied in the current plateau elicited by 10 μM ACh either alone or together with 10 μM edrophonium (upper panel). (**b**) Net *i*/*v* relationship for the I_{ACh} elicited by 10 μM ACh either alone or co-applied with 10 μM edrophonium (+10 μM E). Values were normalised to the control I_{ACh} at −60 mV. Note the powerful blocking effect of edrophonium at the most negative potentials. Inset shows the voltage protocol and the net I_{ACh} values obtained from the recordings shown in a. The dotted line indicates the 0 current level. Data are the mean and s.e.m. of seven oocytes ($N=4$).

Table 2 Voltage dependence of I_{Ach} blockade by BW284c51, decamethonium and edrophonium

Test	*Inhibition (%)*	
	−60 mV	*60 mV*
10 μM ACh + 0.5 μM BW ($n=9$; $N=4$)	43 ± 3	11 ± 3*
10 μM ACh + 10 μM D ($n=5$; $N=3$)	55 ± 3	51 ± 2
10 μM ACh + 10 μM E ($n=7$; $N=4$)	41 ± 2	6 ± 5*

Abbreviations: ACh, acetylcholine; I_{ACh}, acetylcholine current; QChEI, quaternary ammonium cholinesterase inhibitor.
I_{ACh} inhibition caused by different QChEIs was measured at −60 and +60 mV (potentials at which there is a similar current driving force). The percentage of inhibition was calculated using equation 3 (see Materials and methods). Note that there were significant differences of I_{ACh} inhibition at −60 and 60 mV for BW284c51 (BW) and edrophonium (E), whereas decamethonium (D) blockade was voltage-independent. The number of cells tested and donors used for each group is given in parentheses as *n* and *N*, respectively. Asterisks indicate significant differences ($P<0.05$; Student's *t*-test) between different potentials, for the same group.

Additive inhibitory effects between QChEI

We have previously shown that BW284c51 and tacrine, a ChEI lacking of permanent-charged quaternary ammonium groups, but with a tertiary amine usually charged at pH 7.4, have additive inhibitory effects on I_{ACh} (Olivera-Bravo *et al.*, 2005). Given that BW284c51, decamethonium and edrophonium share the same functional group in their structure, we have now tested the effects on I_{ACh} of ACh co-application with different pairs of these QChEIs, at their respective IC_{50}. To minimize errors due to the slow but progressive current decay, sometimes found after successive applications, we quantitated the I_{ACh} inhibition exerted by each mixture of QChEIs as the percentage of the remaining currents evoked in their presence relative to the average of the I_{ACh} elicited by ACh alone in the previous and the subsequent trials. Similar results were obtained when applying the selected combination of drugs in different oocytes or sequentially in the same cell. Nevertheless, except when decamethonium residual effects were noticeable (recovery smaller than 85% of the control value), the latter approach was preferred to reduce deviations due to differences between oocytes, even from the same donor. As shown in Figure 9a, when BW284c51 and ACh were co-applied together with either decamethonium or edrophonium, there was a further reduction of I_{ACh} amplitude. So, 0.5 μM BW284c51 alone decreased I_{ACh} amplitude to 44% ± 1 ($n=9$, $N=4$) of the control current, which decreased to 25% ± 1 ($n=9$, $N=3$), when it was co-applied with 10 μM decamethonium and to 28% ± 1 ($n=7$, $N=4$) when combined with 10 μM edrophonium, respectively (Figure 9a). Likewise, when decamethonium and edrophonium were co-applied at their respective IC_{50}, the remaining current amplitude, normalised to the control, was significantly smaller (21% ± 2, $n=20$; $N=8$) than that obtained by applying each drug separately at the same concentration (40% ± 1, $n=21$, $N=7$ for decamethonium and 52% ± 1, $n=21$, $N=7$ for edrophonium, respectively; Figure 9b). Interestingly, the degree of inhibition obtained when decamethonium and edrophonium were co-applied simultaneously (21% ± 2) was significantly greater than those obtained when each one was applied individually at twice their concentration (26% ± 1 and 37% ± 2 for decamethonium and edrophonium, respectively), which is equivalent to the sum of the concentrations of both QChEIs when they were applied simultaneously. Therefore, these results clearly indicate that BW284c51, decamethonium and edrophonium exhibited additive blocking effects on I_{ACh}, likely to be due to their different binding sites in the nAChR.

Discussion and conclusions

Our present results confirm previous studies indicating that different quaternary ammonium compounds have inhibitory actions on nAChRS and extend the knowledge of the mechanisms by which some QChEIs, such as BW284c51, decamethonium and edrophonium, block muscle-type nAChRs. In fact, one of the most striking results obtained is that in spite of the structural and functional similarities of these molecules, at least as ChEI, their mechanisms of action on nAChRs are diverse, even on the same subtype of nAChRs, resulting in a wide repertoire of actions. The complex inhibitory effects mediated by ChEIs on nAChR

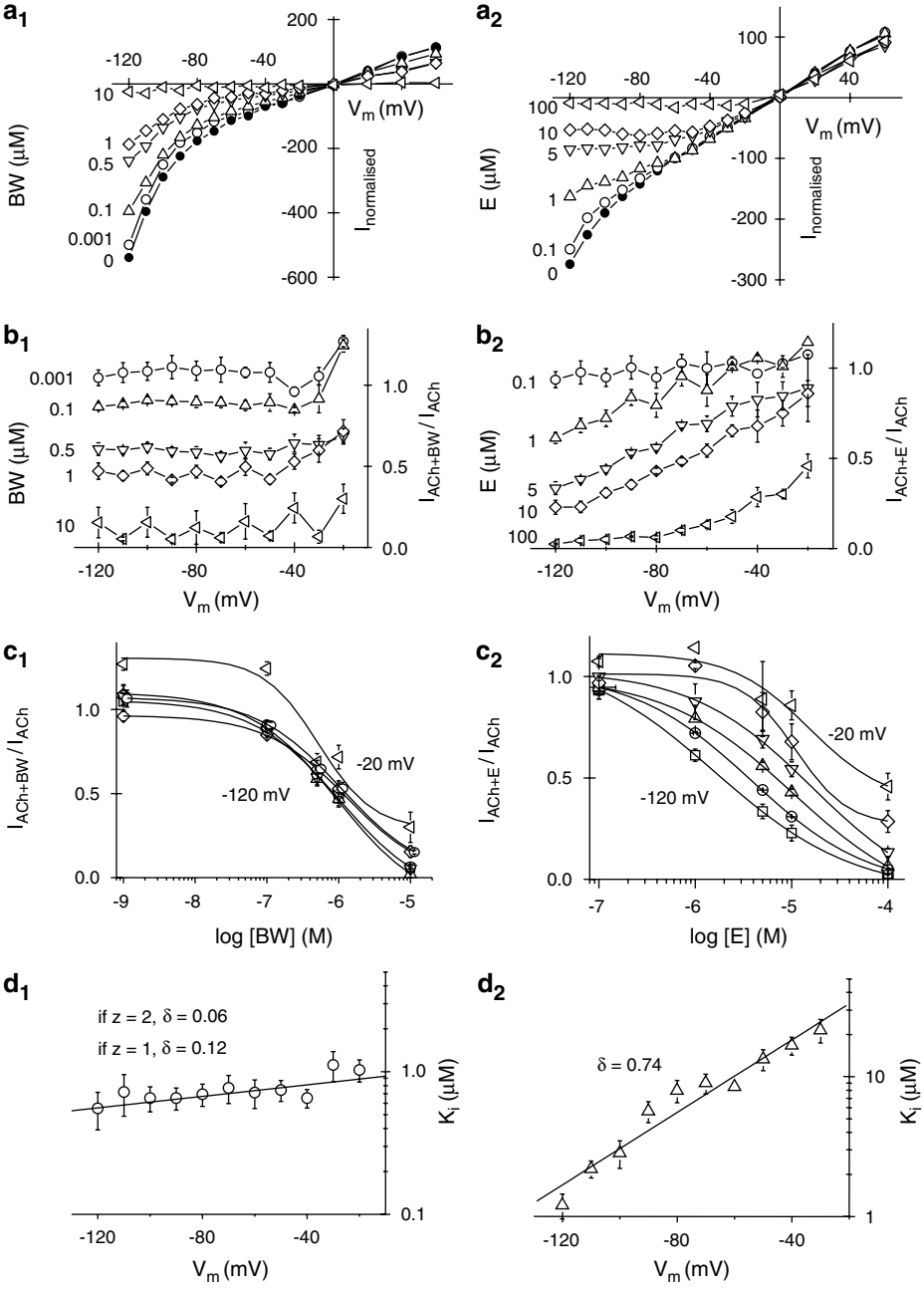

Figure 7 Concentration and voltage dependence of the I_{ACh} blockage by BW284c51 and edrophonium. (**a**) Representative family of *i–v* plots for I_{ACh}s obtained by applying the voltage protocol shown in Figures 4–6, whereas superfusing the oocyte with 10 μM ACh either alone (closed circles) or co-applied with increasing concentrations of either BW284c51 (**a$_1$**) or edrophonium (**a$_2$**). Open symbols correspond to I_{ACh} values obtained in presence of different QChEI concentrations, which are indicated on the left of each graph. Values plotted were normalised to their respective control I_{ACh} at −60 mV. (**b**) The voltage dependence of QChEI blockade is displayed by plotting the fraction of the I_{ACh} left by either BW284c51 (**b$_1$**, I_{ACh+BW}/I_{ACh}) or edrophonium (**b$_2$**, I_{ACh+E}/I_{ACh}) at different concentration, normalised to the control I_{ACh}, versus the membrane potential. (**c**) Concentration dependence of I_{ACh} blockade is illustrated by plotting both I_{ACh+BW}/I_{ACh} (**c$_1$**) and I_{ACh+E}/I_{ACh} (**c$_2$**) ratios against the logarithm of the concentrations of BW284c51 and edrophonium, respectively. Data obtained were fitted to a sigmoid curve to determine the apparent K_i (see equation 4 in Materials and methods) for each membrane potential. (**d**) The resulting apparent K_i values were plotted versus membrane potential and then fitted to the Woodhull equation (see equation 5 in Materials and methods). The electrical distance (δ) was estimated from the slope of the curve; for BW284c51 two possible δ values are given, depending on the number of charged groups that enter the voltage field (see text). Each point in the panels (**b–d**) is the average of five oocytes ($N = 5$).

can be explained assuming that they act as allosteric effectors, which can bind the receptor complex at many possible locations (Hogg *et al.*, 2005), besides blocking the open channels.

We have previously shown that BW284c51 is a powerful inhibitor of nAChRs that behaves as a typical open-channel blocker, because it causes a voltage-dependent and non-competitive inhibition of I_{ACh} as well as a decrease in the

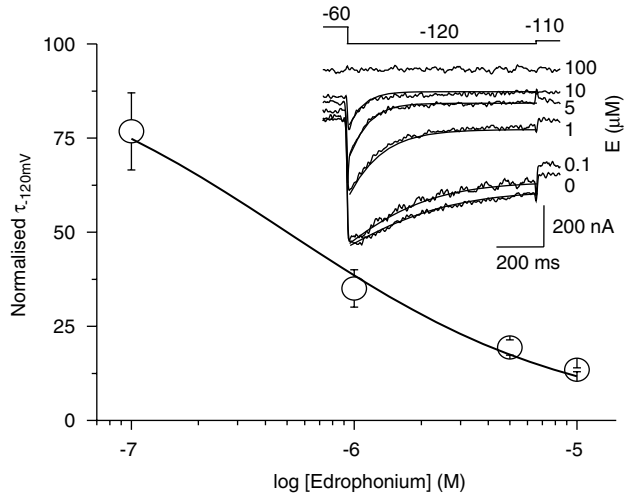

Figure 8 Time dependence of I_{ACh} blockade by edrophonium. The normalised I_{ACh} decay time constants (τ), obtained when stepping the membrane potential from -60 to -120 mV ($\tau_{-120\,mV}$) in presence of different edrophonium concentrations, were plotted against edrophonium concentration in a semilogarithmic scale. $\tau_{-120\,mV}$ values for each edrophonium concentration were normalised as the percentage of those obtained when applying ACh alone. Points are the mean$\pm$s.e.m. of six oocytes ($N=4$). Note that increasing the edrophonium concentration led to a more rapid current inactivation. Data were fitted to a sigmoid curve (line; the fitting parameters for this curve were: Y_{Max}: 100; Y_{Min}: 0; X at Y_{50}: 0.5 μM; power: 0.68). Inset shows I_{ACh} values sequentially evoked in a single cell when giving the voltage pulse shown on the top record, during the current plateau elicited by 10 μM ACh either alone or together with different edrophonium concentrations (values shown on the right). Note that I_{ACh} was completely blocked in this cell at 100 μM edrophonium. τ values were calculated by fitting the current inactivation to simple exponentials (shown in bold).

time to reach the I_{ACh} peak (Olivera-Bravo *et al*., 2005; present data). Nevertheless, it also enhances nAChR desensitisation and modifies the n_H for ACh, which are effects that cannot be easily explained by a single mechanism of action based on block of open channels. In general, the effects of BW284c51 are shared by many other noncompetitive inhibitors of the nAChR, including several molecules with quaternary ammonium groups. So, in the frog neuromuscular junction, TEA, a simple quaternary ammonium molecule, reduces the I_{ACh} peak amplitude and accelerates the decay course of spontaneous endplate currents, although both effects show quite different concentration ranges (Adler *et al*., 1979). Something similar has been reported for local anaesthetics with quaternary ammonium groups, such as QX-314 and QX-222, that bind to and inhibit nAChR via noncompetitive mechanisms involving open-channel blockade (Neher, 1983; Pascual and Karlin, 1998), although they can also promote desensitisation (Neher, 1983). Nevertheless, there are also important differences between all these quaternary ammonium compounds, for instance in their IC_{50}, which is submicromolar for BW284c51, micromolar for QX-314 (19–78 μM; Pascual and Karlin, 1998; Gentry and Lukas, 2001) and in the milimolar range for both TEA (2.7 mM; Akk and Steinbach, 2003) and QX-222 (2.7–3.4 mM; Pascual and Karlin, 1998; Gentry and Lukas, 2001).

However, decamethonium and edrophonium effects on nAChRs seem quite different to those mentioned above and cannot be solely explained by an open-channel blockade mechanism, even though the molecules all have quaternary ammonium groups. There are at least five sound arguments indicating that decamethonium and/or edrophonium effects on nAChRs differ from those caused by BW284c51: (i) at -60 mV, both decamethonium and edrophonium mainly block nAChRs in an apparently competitive way. This might arise from a direct interaction between these ChEIs and the nAChR-binding site but, alternatively, it can also result from the binding of these molecules to the closed states of nAChRs. In that case, their preferential binding to closed receptors could appear as competitive interaction, because increasing agonist concentrations would take the channels away from the state for which the antagonist would have the highest affinity (Papke *et al*., 2001). In any case, the fact that the inhibition caused by either decamethonium or edrophonium, at their IC_{50}, could not be fully surmounted by increasing ACh concentrations would argue against inhibition arising solely from competitive interactions at the ACh-binding site. Furthermore, decamethonium and edrophonium have additive inhibitory effects on nAChRs, because their co-application at their IC_{50} resulted in greater inhibition than that caused by each of them, applied individually, at twice their IC_{50}. This result implies that there are different binding sites for each of these QChEIs on nAChRs and hence rules out that both of them solely compete at the agonist-binding site. (ii) Neither decamethonium nor edrophonium affected both nAChR desensitisation and the time to I_{ACh} peak, at least, at the concentrations tested. Conversely, it has been reported that edrophonium increased desensitisation of muscle-type nAChRs expressed in *Xenopus* oocytes (Yost and Maestrone, 1994), but these authors used dimethylphenyl piperazinium iodide (DMPP) as agonist, which induces a very small desensitisation (about 30–34% current decrease after 30 s application of 50 μM DMPP, which is close to the EC_{80}) as compared with ACh (Table 1). (iii) The inhibition potency of decamethonium and edrophonium was roughly equal, but it was much smaller than that of BW284c51 (Olivera-Bravo *et al*., 2005; present data). (iv) Whereas the blocking effects of BW284c51 and edrophonium were quickly and completely reversed after drug withdrawal, decamethonium effects vanished slowly, usually requiring several minutes to disappear fully. This result support the hypothesis that decamethonium-binding site on nAChRs differs to those of either BW284c51 or edrophonium, although differences in drug dissociation rates do not necessarily imply different binding sites. (v) Decamethonium blocking effects were voltage-independent, as also has been reported for α7-nAChRs (Bertrand *et al*., 1992), whereas decamethonium inhibition of both synaptic currents of frog autonomic ganglia (Ascher *et al*., 1979; Lipscombe and Rang, 1988) and avian α4/nα-nAChRs (Bertrand *et al*., 1990) displayed clear voltage dependence. The lack of voltage dependence would again argue against open-channel blockade as the main mechanism of decamethonium I_{ACh} inhibition. By contrast, edrophonium blockade of nAChRs showed marked voltage dependence, more evident at potentials more negative than -60 mV, which was much stronger than that

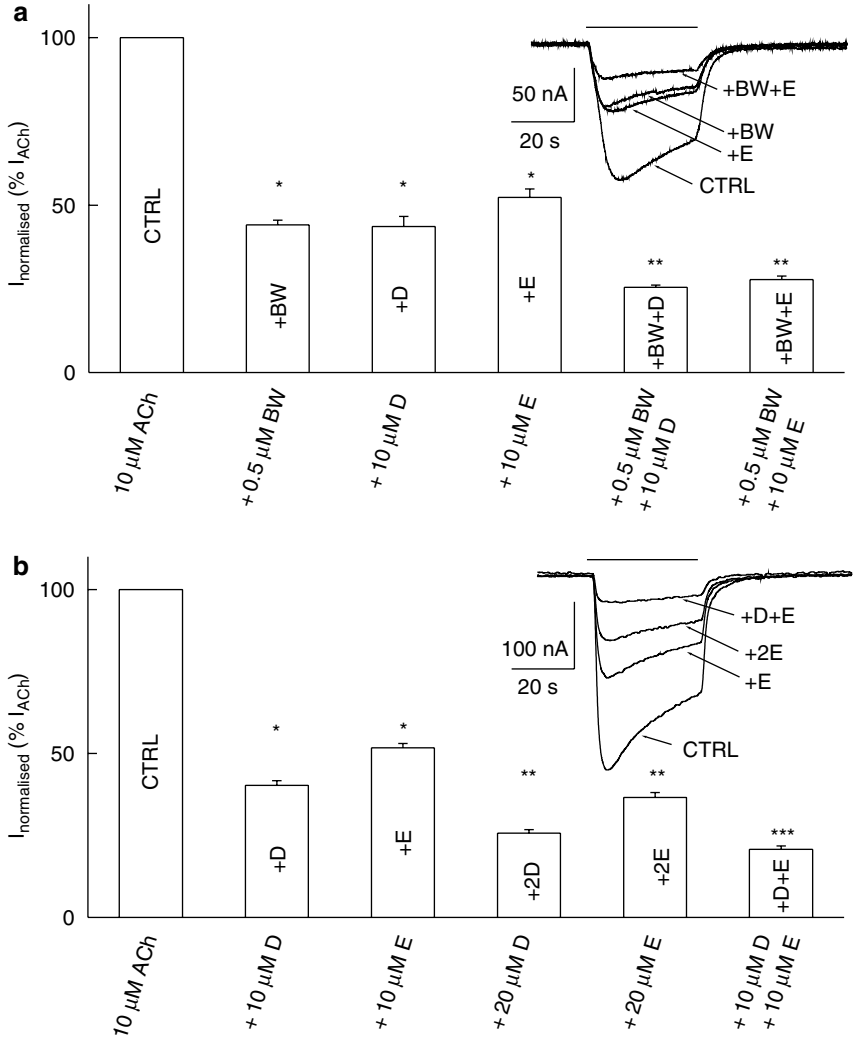

Figure 9 Additive effects of QChEIs on I_{ACh}. (**a**) Column graph showing the additive inhibitory effects on I_{ACh} of BW284c51 when co-applied with either decamethonium or edrophonium. The height of the bars represents the I_{ACh} amplitude elicited by 10 μM ACh either alone (CTRL) or co-applied with the QChEIs, either individually or combined pairwise. Data are the mean$\pm$s.e.m. of 7–9 oocytes ($N=4$). The inset shows representative currents evoked in an oocyte superfused with 10 μM ACh either alone (CTRL) or together with 0.5 μM BW284c51 (+BW), 10 μM edrophonium (+E) or both of them simultaneously (+BW+E). As shown, the I_{ACh} blockade caused by each of these drugs was comparable, but their co-application resulted in an increased inhibition. (**b**) Potentiating effects of edrophonium and decamethonium. The inset shows representative I_{ACh} values evoked in an oocyte superfused with 10 μM ACh either alone (CTRL) or together with 10 μM edrophonium (+E), 20 μM edrophonium (+2E) or decamethonium (10 μM) plus edrophonium (10 μM) (+D+E). Note the enhanced inhibition when both drugs were applied simultaneously. The column-graph displays the quantitation of I_{ACh} blockade elicited by these QChEIs when applied either alone or combined pairwise. Each bar gives the mean$\pm$s.e.m. of 20 or 21 cells ($N=8$). Different number of asterisks above the bars indicates significant differences between groups ($P<0.05$).

displayed by BW284c51. This suggested that, at very negative potentials, the main inhibitory action of edrophonium on nAChRs becomes open-channel blockade and hence a noncompetitive mechanism.

As BW284c51 and edrophonium blockade of the nAChR was voltage-dependent, it allowed us to determine the putative binding sites of these molecules within the ion-conducting pore, by determining the fraction of the voltage sensed by the blocker. As it could be expected from their i/v relationship, the δ values for BW284c51 and edrophonium were markedly different, indicating that whereas BW284c51 bound to a very shallow site, close to the channel mouth ($\delta=0.06$–0.12), edrophonium bound deeper in the channel ($\delta=0.74$), most likely at the same site as other quaternary ammonium molecules such as QX-222 (δ values ranging from 0.65 to 0.80; Neher and Steinbach, 1978; Charnet *et al*., 1990; Pascual and Karlin, 1998) and TEA ($\delta=0.7$; Akk and Steinbach, 2003). Interestingly, philanthotoxin-343 and philanthotoxin-(12), which are two similar molecules, only differing in two internal ammonium groups substituted with methylenes, have quite different inhibiting effects on muscle-type nAChRs (Brier *et al*., 2003) and they bind differentially to the receptor at a shallow and a deep site within the ion channel (Brier *et al*., 2003; Tikhonov *et al*., 2004). The edrophonium-binding site in the nAChR channel seems to correspond to the common binding site for

noncompetitive inhibitors, which is located in the narrow portion of the pore, lined by polar side chains in the second membrane-spanning segment (M2), specifically by αS248 and the aligned residues in the other M2 subunits (Pascual and Karlin, 1998; Tikhonov *et al*., 2004). The presence of different binding sites for BW284c51 (shallow) and edrophonium (deep) could explain why these two QChEIs show synergic inhibitory effects on nAChRs. On the other hand, because decamethonium effects were voltage-independent, it follows that its binding site is not located within the pore domain and so it might explain its synergic inhibitory effects with both BW284c51 and edrophonium on nAChRs.

It is noteworthy that none of the QChEI tested were partial agonists at the transplanted nAChRs, in contrast to previous observations in which decamethonium behaved as a partial agonist of either endplate nAChRs (Adams and Sakmann, 1978) or muscle-type nAChRs expressed in either oocytes (Aoshima, 1990) or BC3H-1 cells (Liu and Dilger 1993). Notwithstanding, it is also known that decamethonium blocks endplate nAChRs (Adams and Sakmann, 1978) and does not activate either α7- or α4/nα-nAChRs, even when applied at high concentrations (Bertrand *et al*., 1990, 1992). Likewise, TEA binds to the nAChR agonist-binding site with low affinity (1.5 mM), but gates the channel with very low efficacy and blocks the channel with an IC_{50} of about 3 mM (Akk and Steinbach, 2003). Therefore, both the low putative efficacy of decamethonium in activating nAChR and its concomitant channel blocking effect are likely to be the reasons why we failed in evoking decamethonium-activated currents.

To conclude, BW284c51, decamethonium and edrophonium blocked muscle-type nAChRs through different mechanisms, probably reflecting differences in their binding sites in the nAChR, which would explain the synergic inhibitory actions found among them. Moreover, we postulate that BW284c51 and edrophonium have at least two mechanisms of action on nAChRs, as allosteric effectors and open-channel blockers, binding to different nAChR loci. Even more, the open-channel blockade induced by BW284c51 and edrophonium takes place at different sites within the ion-conducting pore. It remains to be explained why these ChEIs, which seem to act on different nAChR sites, caused their inhibitory effects by the binding of a single molecule to the nAChR, as deduced from the dose–inhibition curve, either for BW284c51 (Olivera-Bravo *et al*., 2005; present data) or edrophonium (Yost and Maestrone, 1994; present data). Anyway, these results strongly suggest that nAChRs have several loci to which these, and probably other, molecules with quaternary ammonium groups might bind with different affinity. This would explain the large heterogeneity of inhibitory effects of QChEIs and related molecules on nAChRs.

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Conflict of interest

The authors state no conflict of interest.

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