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The acetylcholinesterase inhibitor BW284c51 is a potent blocker of *Torpedo* nicotinic AchRs incorporated into the *Xenopus* oocyte membrane

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1 This work was aimed to determine if 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW284c51), the most selective acetylcholinesterase inhibitor (AchEI), affects the nicotinic acetylcholine (Ach) receptor (AchR) function.

2 Purified *Torpedo* nicotinic AchRs were injected into *Xenopus laevis* oocytes and BW284c51 effects on Ach- and carbamylcholine (Cch)-elicited currents were assessed using the voltage-clamp technique.

3 BW284c51 (up to 1 mM) did not evoke any change in the oocyte membrane conductance. When BW284c51 (10 pM–100 μM) and Ach were coapplied, Ach-evoked currents (I_{Ach}) were reversibly inhibited in a concentration-dependent manner (Hill coefficient, 1; IC_{50}, 0.2–0.5 μM for 0.1–1000 μM Ach). Cch-elicited currents showed a similar inhibition by BW284c51.

4 I_{Ach} blockade by BW284c51 showed a strong voltage dependence, being only apparent at hyperpolarising potentials. BW284c51 also enhanced I_{Ach} desensitisation.

5 BW284c51 changed the Ach concentration-dependence curve of *Torpedo* AchR response from two-site to single-site kinetics, without noticeably affecting the EC_{50} value.

6 The BW284c51 blocking effect was highly selective for nicotinic over muscarinic receptors. BW284c51 inhibition potency was stronger than that of tacrine, and similar to that of *d*-tubocurarine (*d*-TC). Coapplication of BW284c51 with either tacrine or *d*-TC revealed synergistic inhibitory effects.

7 Our results indicate that BW284c51 antagonises nicotinic AchRs in a noncompetitive way by blocking the receptor channel, and possibly by other, yet unknown, mechanisms.

8 Therefore, besides acting as a selective AchEI, BW284c51 constitutes a powerful and reversible blocker of nicotinic AchRs that might be used as a valuable tool for understanding their function.
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Keywords: BW284c51; nicotinic acetylcholine receptors; *Xenopus* oocytes; cholinesterase inhibitors; acetylcholine; carbamylcholine; *Torpedo marmorata*

Abbreviations: Ach, acetylcholine; AchE, acetylcholinesterase; AchEI, acetylcholinesterase inhibitor; AchR, acetylcholine receptor; ANR, normal Ringer with atropine; BW284c51, 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide; Cch, carbamylcholine; ChEI, cholinesterase inhibitor; *d*-TC, *d*-tubocurarine; I_{Ach}, acetylcholine current

Introduction

Nicotinic acetylcholine receptors (AchRs) are widespread ligand-gated ion channels that mediate fast cholinergic transmission at both the peripheral and central nervous systems. Thus, in addition to their involvement in neuromuscular and autonomic ganglia synaptic transmission, they play an important role in cognitive and addictive processes (Wonnacott, 1997; Lena & Changeux, 1998; Clementi *et al*., 2000). Furthermore, their dysfunction has been linked to a number of human diseases, including congenital myasthenia, schizophrenia, familial epilepsy and neurodegenerative disorders such as Alzheimer's and Parkinson's diseases (Lena & Changeux, 1998; Hogg *et al*., 2003). A special attention is currently given to numerous compounds that are able to modulate AchR function through their interaction with allosteric sites in the receptor molecule (Clapham & Neher, 1984; García-Colunga & Miledi, 1996; Pereira *et al*., 2002). At present, acetylcholinesterase (AchE) inhibitors (AchEIs) constitute, in fact, the preferred strategy for Alzheimer's treatment (Small, 2004), not only because of their reinforcing action on cholinergic transmission through AchE inhibition but also due to their modulatory effects on nicotinic AchRs, including allosteric potentiation (Pereira *et al*., 2002). Actually, distinct AchEIs can evoke different effects on nicotinic AchRs depending on their chemical nature and on receptor subunit composition (Zwart *et al*., 2000). Tacrine (Zwart *et al*., 2000), physostigmine (Van den Beukel *et al*., 1998; Zwart *et al*., 2000) neogstigmine (Nagata *et al*., 1997; Smulders *et al*., 2003) and pyridostigmine (Bradley *et al*., 1986) can alternatively block or potentiate the activity of nicotinic AchRs, depending on

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the concentrations used and the biological sample tested. The actions of quaternary ammonium AchEIs on nicotinic Ach currents (I_{Ach}) have also been extensively studied. Clinical concentrations of edrophonium reduced single channel I_{Ach} amplitude in BC_3H1 cells (Wachtel, 1990) and in mouse muscle receptors expressed in *Xenopus* oocytes (Yost & Maestrone, 1994). Decamethonium, a bisquaternary compound, acts as a partial agonist of muscle nicotinic AchRs (del Castillo & Katz, 1957; Adams & Sakmann, 1978; Aoshima, 1990; Bertrand *et al*., 1992; Liu & Dilger, 1993), and it can also antagonise α7 responses (Bertrand *et al*., 1992) or block open endplate channels (Adams & Sakmann, 1978). Other bisquaternary AchEIs such as hexamethonium and dodecamethonium block both neuronal and muscle nicotinic responses (Bertrand *et al*., 1992; Lummis *et al*., 1992).

Over the years, 1,5-bis(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide, BW284c51, a bisquaternary ammonium AchEI, whose chemical structure is shown in Figure 1a, has been used to ascertain the tissue localisation of the AchE, despite the significant structural and functional homology found between the different vertebrate cholinesterases (Koelle, 1963; Mikalsen *et al*., 1986, Radic *et al*., 1993; Dupree & Bigbee, 1994). Additionally, its high specificity allows it to discriminate between AchEs from different species, since BW284c51 binding depends on the presence of specific amino-acid residues in the catalytic and peripheral sites of the enzyme (Radic *et al*., 1993; Eichler *et al*., 1994). Even though no actions have been described for BW284c5s besides those related to AchE inhibition, there are significant structural and functional similarities between this compound and other cholinesterase inhibitors (ChEIs) that behave as nicotinic AchR modulators, especially those with quaternary ammonium groups. Therefore, this work was addressed to determine whether BW284c51 has any action on nicotinic AchR function and, should this be the case, to unravel the mechanisms underlying such interaction. With this purpose, we transplanted *Torpedo marmorata* nicotinic AchRs, reconstituted in asolectin lipid vesicles, to *Xenopus* oocytes (Morales *et al*., 1995). This procedure allows us to study the behaviour of native nicotinic AchRs incorporated into the oocyte membrane, overcoming any post-translational modification that could occur when heterologous nicotinic AchRs are expressed from exogenous mRNA in oocytes (Buller & White, 1990; Sivilotti *et al*., 1997). The effect of BW284c51 on the function of nicotinic AchR was assessed using the voltage-clamp technique, which provides a detailed functional assay of receptor activity and allows quantitative pharmacological studies.

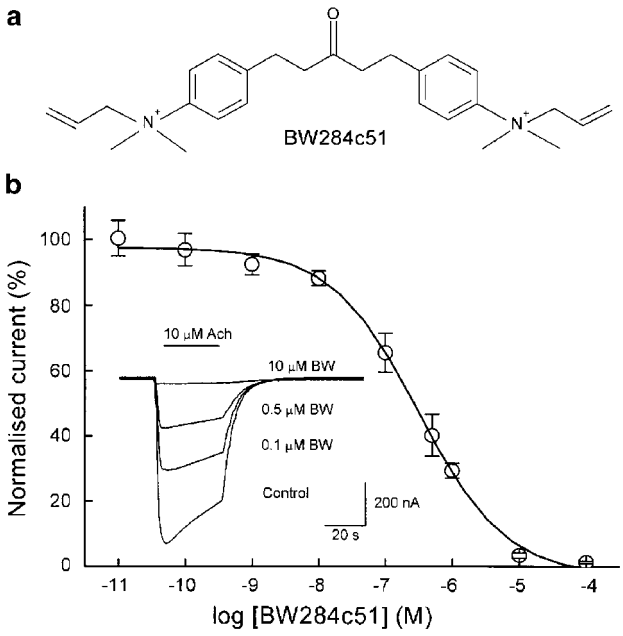

Figure 1 Effects of BW284c51 on Ach-induced currents (I_{Ach}). (a) Chemical structure of BW284c51. (b) Relationship between BW284c51 dose and I_{Ach} peak amplitude in *Xenopus* oocytes injected with purified *T. marmorata* nicotinic AchRs. Repeated I_{Ach} were evoked by 32 s pulses of 10 μM Ach, either alone (control) or coapplied with increasing concentrations of BW284c51 (BW). The interval between applications was of 10 min. Values obtained for each BW284c51 concentration were normalised as the percentage of the maximum I_{Ach} response, and data represent the mean $\pm$ s.e.m. corresponding to five oocytes from three donors. The solid line represents a simple sigmoidal curve that fits to the data with a Hill coefficient close to 1. The inset shows representative, superimposed I_{Ach} elicited at a holding potential of -60 mV by Ach alone (control) or coapplied with the indicated BW284c51 concentrations to the same oocyte. In this and following figures, downward deflections denote inward currents and the horizontal bars indicate the time of drug application.

Methods

Purification and reconstitution of nicotinic AchRs

The methodology employed has been described previously (Morales *et al*., 1995; Ivorra *et al*., 2002). Briefly, membranes from the electric organ of *T. marmorata* were solubilised in cholate and nicotinic AchRs isolated by bromoacetylcholine-affinity chromatography carried out in the presence of asolectin lipids. After elution with carbamylcholine (Cch), purified receptors were dialysed and reconstituted in asolectin vesicles at a final protein concentration of 0.3–1.2 mg ml^{-1}. Aliquoted samples were stored in liquid nitrogen until use.

Oocyte preparation and microinjection

Adult *Xenopus laevis* (purchased from Blades Biological, U.K.) were anaesthetised by immersion in 0.17% MS-222 for 15 min and a piece of ovary was aseptically removed, thereafter allowing the recovery of the toad. Fully grown immature oocytes were isolated from the ovary and their surrounding layers removed either manually or by collagenase treatment, as described previously (Ivorra & 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) supplemented with penicillin (100 U ml^{-1}) and streptomycin (0.1 mg ml^{-1}) until used for electrophysiological recordings. Oocytes were microinjected with 100 nl of an aliquot of reconstituted nicotinic AchRs, after being thawed on ice and rehomogenised before injection (Morales *et al*., 1995).

Electrophysiological recordings

Membrane current recordings were performed at 21–25°C, 16–72 h after injection, using a high-compliance two-microelectrode voltage-clamp system (TurboTEC-10CD, npi). The

recording methodology has been described previously (Morales *et al*., 1995). Briefly, intracellular electrodes (0.8–3 MΩ) were filled either with 3 M KCl or potassium acetate for voltage recording and current injection, respectively. Oocytes were placed in a 150 μl recording chamber that was continuously superfused with normal frog Ringer's solution (NR, 115 mM NaCl, 2 mM KCl, 1 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, Cch and other tested drugs were diluted in ANR solution and applied for 32 s while superfusing the oocyte at a flow rate of 7–15 ml min^{-1}. Membrane currents were low-pass filtered at 30–1000 Hz and recorded on both a chart recorder (Kipp & Zonen BD-112) and a PC computer, after sampling (Digidata 12 bits, 4096 points per record) at five times the filter frequency using the WCP v. 3.2.8 package developed by J. Dempster (Strathclyde Electrophysiology Software, University of Strathclyde, U.K.). Current–voltage relationships (*i–v* curves) were obtained by giving 800 ms voltage pulses from −120 to +60 mV in 20 mV steps) to the oocyte while superfusing it with Ach alone or coapplied with BW284c51. Concentration–response curves were obtained by exposing injected oocytes to increasing Ach concentrations, alone or together with the indicated drugs. In order to reduce nicotinic AchR desensitisation, the interval between two consecutive Ach applications was of, at least, 7 min.

Data analysis

For receptor activation, dose–response data were fitted to Hill's equation as described by the ratio $I/I_{max} = [1 + (EC_{50}/[Ach])^n]^{-1}$, where I is the peak I_{Ach} elicited at a given Ach concentration, I_{max} the maximum current recorded, EC_{50} is the concentration of agonist required to obtain one-half of the maximum current and n the Hill coefficient (n_H). Inhibition curves were determined by measuring I_{Ach} in the presence of different BW284c51 concentrations. Each value given corresponds to the average of, at least, two consecutive measurements obtained under the same conditions. Data were fitted to a single-site inhibition curve using the Origin 6.1 software (OriginLab Corp., Northampton, MA, U.S.A.). For graphical representations, the values of the dose–response curves were normalised as the percentage of the largest induced current for each oocyte. Unless otherwise specified, values given are the mean ± s.e.m. 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 groups differences were determined by the Kruskal–Wallis analysis of variance on ranks, and the comparison of groups by using the Dunn's test. A significance level of $P < 0.05$ was considered for all cases.

Drugs

Ach, atropine sulphate, BW284c51, Cch, MS-222, penicillin, streptomycin, tacrine sulphate and *d*-tubocurarine (*d*-TC) chloride were purchased from Sigma (St Louis, MO, U.S.A.). HEPES was obtained from Acros Organics (New Jersey, NJ, U.S.A.). Reagents of general use were purchased from Scharlau Chemie SA (Barcelona, Spain). Unless otherwise stated, all drugs used were dissolved from stock solutions in ANR just before each application. BW284c51-containing solutions were protected from light at all times.

Results

BW284c51 inhibition of nicotinic currents

In both oocytes bearing nicotinic AchRs and uninjected cells, with the membrane potential being held at −60 mV, BW284c51 (0.1 nM–1 mM) superfusion did not appreciably modify the cell membrane conductance. However, when it qwas coapplied with Ach, the amplitude of the elicited I_{Ach} was reduced and the current decay pattern became significantly altered. Figure 1b shows the blocking effect of different BW284c51 concentrations on the I_{Ach} elicited by 10 μM Ach. The normalised dose-inhibition curve obtained could be fitted to a sigmoidal function with an estimated IC_{50} of 0.5 μM (pIC_{50} 6.3 ± 0.1) and an n_H value of 0.7, suggesting that BW284c51 exerts its inhibitory action through its binding to the nicotinic AchR in a molecular ratio of 1 : 1.

The effect of BW284c51 on I_{Ach} desensitisation was determined by measuring, for each oocyte, the I_{Ach} amplitude elicited by 10 or 100 μM Ach either alone or coapplied with 0.5 μM BW284c51, at different times after the current peak. As previously reported (Morales *et al*., 1995), under control conditions (Ach alone), I_{Ach} displayed a slow desensitisation at low agonist concentrations (10 μM), whereas at higher Ach doses (100 μM) I_{Ach} showed a fast peak followed by a plateau. The presence of BW284c51 markedly increased I_{Ach} desensitisation, especially at the higher Ach concentrations (Table 1), and decreased the time between the beginning of the response and the current peak (Table 1). Interestingly, the fast desensitisation phase of the I_{Ach} was more pronouncedly affected by BW284c51 than the slow one (Table 1; compare the desensitisation values at 2 and 20 s for currents elicited by Ach alone or coapplied with BW284c51), although both components were significantly speeded up for 100 μM Ach.

Although the presence of 0.5 μM atropine in the bathing solution, to block any muscarinic response, has no effect on muscle type nicotinic AchRs (Miledi & Sumikawa, 1982), it might contribute to the observed effects of BW284c51 on I_{Ach}. To rule out this possibility, we recorded the I_{Ach} elicited by 100 μM Ach (in a Ringer without atropine added) either alone or coapplied with 0.5 μM BW284c51 in five oocytes of a donor lacking muscarinic receptors in their membrane. In these experiments, BW284c51 showed similar blocking effects on AchRs than those observed in the presence of atropine, indicating that BW284c51, by itself, was responsible for I_{Ach} blockade.

The BW284c51 inhibitory effect on I_{Ach} was immediately and completely reversed after BW284c51 removal as shown in Figure 2. Furthermore, the I_{Ach} elicited by Ach after its coapplication with BW284c51 (up to 10 μM) showed the same amplitude and time-course kinetics as the initial control response (Figure 2) for any of the Ach concentrations tested, thus indicating the lack of any residual effect of BW284c51 on nicotinic AchRs.

Table 1 BW284c51 effects on I_{Ach} time to peak and desensitisation

Test	*Time to peak* (s)	*Desensitisation* (%)		
		2 s	*10* s	*20*s
10 μM Ach ($n=34, 7$)	7.1±0.5	3±1	14±3	25±3
10 μM Ach + 0.5 μM BW284c51 ($n=34, 7$)	5.2±0.5**	5±1**	17±2*	23±2
100 μM Ach ($n=67, 13$)	3.4±0.2	14±2	49±2	67±2
100 μM Ach + 0.5 μM BW284c51 ($n=67, 13$)	2.0±0.1**	44±2**	75±1**	83±1**
100 μM Cch ($n=21, 6$)	9.3±1.1	2±1	12±2	30±4
100 μM Cch + 0.5 μM BW284c51 ($n=21, 6$)	9.4±1.2	4±1*	11±2	26±5
1000 μM Cch ($n=13, 2$)	3.4±0.2	16±2	55±4	72±3
1000 μM Cch + 0.5 μM BW284c51 ($n=13, 2$)	2.6±0.2**	33±5**	60±5	71±6

Data show desensitisation values for either Ach (10 and 100 μM) or Cch (100 and 1000 μM) currents, elicited by each agonist alone or coapplied with 0.5 μM BW284c51. The time to I_{Ach} peak data is the time between the current onset and its maximal value. Desensitisation values were obtained using the equation: $D_{ti}=100-((I_{ti}/I_{peak})*100)$, where D_{ti} is the desensitisation value at the specified time, I_{peak} the peak current amplitude, and I_{ti} the current amplitudes remaining at 2, 10 and 20 s after the peak. Note that desensitisation increased with concentration for both Ach- and Cch-elicited responses, and that BW284c51 mainly enhanced the fast desensitisation component (measured as the 2 s desensitisation), although the slow one (measured as the 20 s desensitisation) was also increased for currents elicited by 100 μM ACh. The numbers of cells tested and donors used for each group are given within parentheses. Values from control and the corresponding BW284c51 groups were compared (*$P<0.05$; **$P<0.005$).

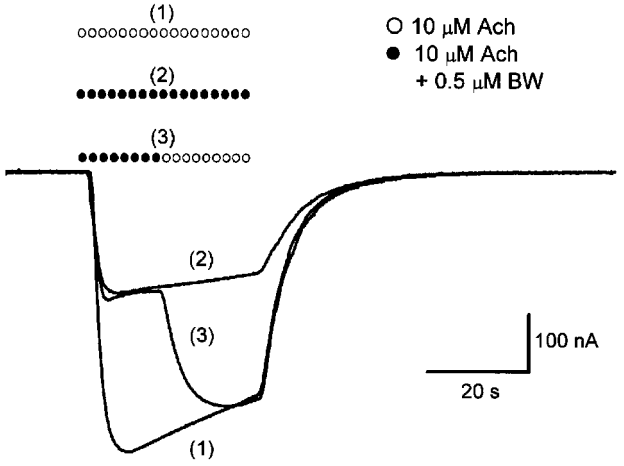

Figure 2 Fast and reversible I_{Ach} blockade by BW284c51. I_{Ach} records elicited by 10 μM Ach alone (1) or coapplied with 0.5 μM BW284c51 (2) in the same oocyte. Mixed symbols (3) indicate substitution of the Ringer's solution containing Ach plus BW284c51 by another with Ach alone. Note that once the washout had started, the I_{Ach} quickly reached the control amplitude.

Noncompetitive antagonism of BW284c51

To better characterise BW284c51 effects on I_{Ach}, dose–response curves were obtained in the absence and presence of 0.5 μM BW284c51. As shown in Figure 3, bath applications of 0.5–1000 μM Ach to oocytes injected with nicotinic AchRs elicited responses similar to those previously described for these receptors (Morales *et al*., 1995). The relationship obtained was well fitted to a two-site nonlinear Hill equation with a value of n_H of 2.0±0.3 and an EC_{50} of 41 μM ($n=7$, four donors).

When the same Ach concentrations were coapplied with BW284c51 at a 0.5 μM dose (approximately its IC_{50}), I_{Ach} was reduced by about 50%, as compared to values obtained in the absence of the inhibitor, independent of the agonist concentration used (48±1%, 34 cells from seven donors and 49±1, 67 cells of 13 donors, for 10 and 100 μM Ach, respectively; $P>0.457$, *t*-test; Figure 3a), thus indicating a noncompetitive action of BW283c51 in the tested range of Ach concentrations. The concentration–response curve in the presence of BW284c51 was fitted to a simple sigmoidal curve with a slope corresponding to an n_H value of 1.0±0.2 and an EC_{50} for Ach of 55 μM ($n=9$, six donors), this suggesting that the affinity of the nicotinic AchRs towards its agonist was barely affected by the presence of BW284c51.

Since *Xenopus* oocytes bear intrinsic AchE activity (Soreq *et al*., 1982; Gundersen & Miledi, 1983), we set to address whether the BW284c51 effects on nicotinic currents were somehow associated to its activity as AchEI. With this purpose, we used Cch instead of Ach as the agonist of nicotinic AchRs, since Cch is not hydrolysable by AchE (Sung *et al*., 1998). This approach also allowed us to test whether a possible interaction between BW284c51 and Ach occurred when both compounds were coapplied. As shown in Figure 4b, superfusion of an injected oocyte with Cch (100 μM) evoked nicotinic currents of smaller amplitude and slower desensitisation than those elicited, in the same cell, by an identical Ach concentration (Figure 4a; Table 1). However, BW284c51 effects on Cch currents were comparable in their potency to those evoked on I_{Ach}, since 0.5 μM BW284c51 when coapplied with 100 or 1000 μM Cch evoked currents that were reduced to 49±2% ($n=21$, six donors) and 46±1% ($n=13$, two donors), respectively, of the values obtained at the same concentrations of Cch alone (Figure 4b). These values were neither significantly different between them ($P>0.05$, Mann–Whitney rank sum test) nor from those obtained when Ach was used as the agonist ($P>0.05$, Kruskal–Wallis analysis of variance on ranks). As shown in Table 1, BW284c51 also increased the rate of desensitisation of Cch currents, but in contrast to Ach currents, only the fast desensitisation component (2 s desensitisation) was significantly affected.

Comparison of the blocking effects of BW284c51 with those of other antagonists

BW284c51 actions on nicotinic AchR receptors were compared to those mediated by other compounds with well-known inhibitory actions. Tested drugs were tacrine, a powerful ChEI of clinical use (Cantí *et al*., 1998), and *d*-TC (Jenkinson, 1960; O'Leary *et al*., 1994), both potent antagonists of muscle and

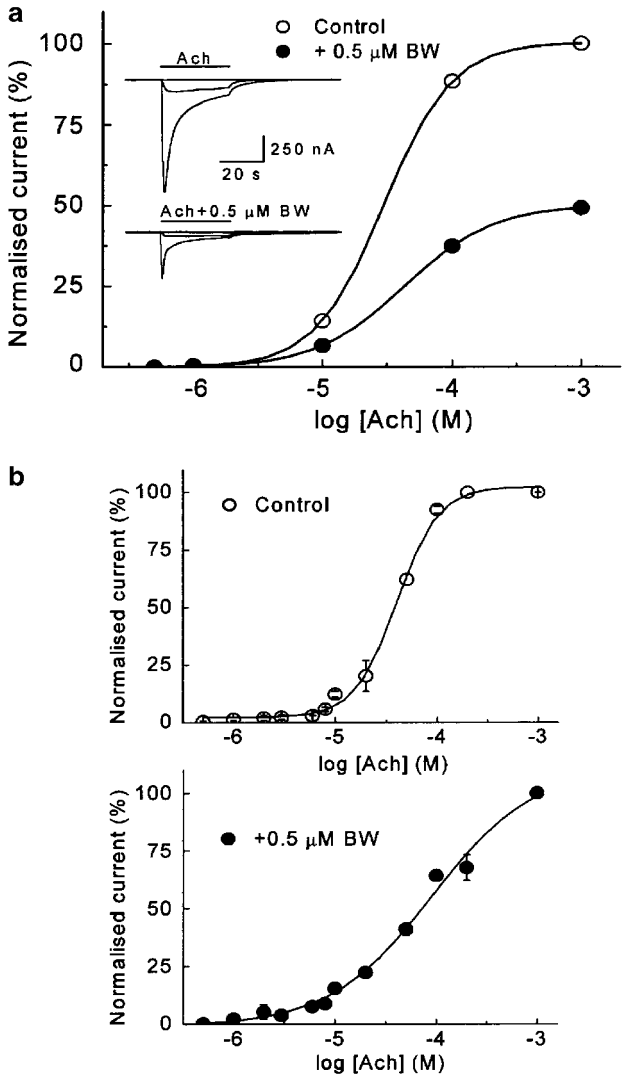

Figure 3 Ach dose–I_{Ach} relationship for currents evoked by Ach alone or together with BW284c51 in oocytes bearing nicotinic AchRs. (a) The plot shows Ach dose–I_{Ach} relationships in an oocyte challenged with increasing Ach concentrations, applied alone or together with 0.5 μM BW284c51. Ach pulses were given for 32 s every 5–30 min, depending on concentrations, to ensure complete recovery between trials. The inset shows superimposed recordings obtained by applying to the same cell, at −60 mV, 0.5, 10 or 1000 μM Ach, either alone (upper records) or together with 0.5 μM BW284c51 (lower records). Note that in the presence of BW284c51, the values are roughly half of those obtained with Ach alone. (b) Graphs were obtained by averaging the I_{Ach} from seven oocytes (four donors) covering the total range of Ach concentrations tested. I_{Ach} was elicited by either Ach alone (upper graph) or coapplied with BW284c51 (lower graph). The dose–response curve for currents elicited by Ach alone best fitted to a two-site Hill equation, whereas in the presence of BW284c51, the best fit corresponded to a single-site equation.

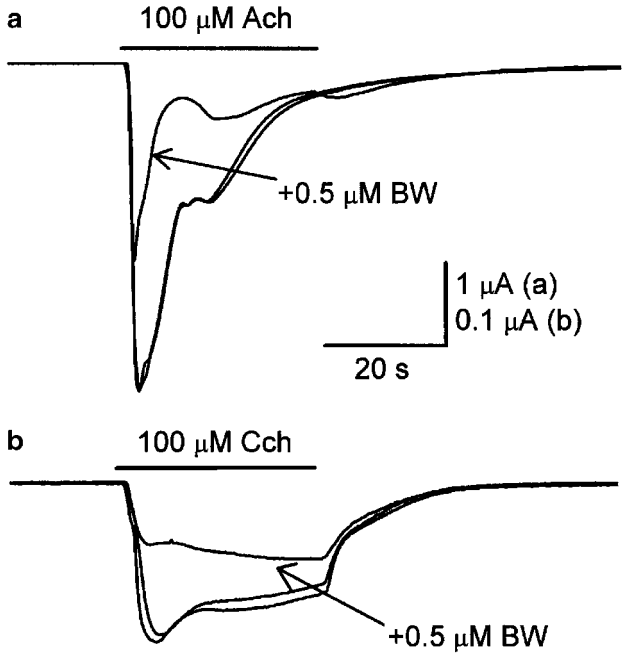

Figure 4 BW284c51 effects on Ach- and Cch-elicited currents. Recordings show nicotinic currents elicited in the same oocyte by 100 μM Ach either alone (pre- and post-control responses) or together with 0.5 μM BW284c51 (a), and by 100 μM Cch either alone (pre- and post-control responses) or coapplied with 0.5 μM BW284c51 (b). Note that as for I_{Ach}, BW284c51 blocked about half of the Cch-induced current. In both cases, the blockade was fully reversible upon removal of BW284c51.

Torpedo nicotinic I_{Ach}. For comparative purposes, all antagonists were used at the same concentration, together with 100 μM Ach. Similar results were obtained when applying the selected drugs in different oocytes and sequentially in the same cell, although the latter approach was preferred to decrease deviations due to differences between oocytes, even from the same donor. However, repeated applications of agonist for long periods usually caused a slow, but progressive, decrease in the current amplitude, likely due to receptor desensitisation. To minimise the possible errors derived from this unwanted effect in the quantitation of the inhibitory potency of the drugs on the I_{Ach}, the degree of the inhibition elicited by each tested compound was expressed as the percentage of the response obtained in presence of the antagonist with respect to that evoked by Ach alone in the previous and subsequent trials.

Tacrine at 0.5 μM had a weak blocking effect on I_{Ach}, whose amplitude fell to 82 ± 5% ($n = 7$, from three donors; $P < 0.005$) of the control values (see histogram of Figure 5b). The I_{Ach} inhibition promoted by 0.5 μM tacrine was much smaller than that obtained in the same cells with BW284c51 at the same concentration (Figure 5b). When both drugs were coapplied at 0.5 μM, the I_{Ach} inhibition was similar to that found with BW284c51 alone (Figure 5b). The IC_{50} for tacrine was close to 10 μM (see Figure 5a middle panel, and Figure 5b). When tacrine and BW284c51 were coapplied at concentrations close to their IC_{50} values (10 and 0.5 μM, respectively), the I_{Ach} was significantly smaller than that obtained with any of these blockers alone ($P < 0.005$; Figure 5a and b). Although these results indicate that tacrine and BW284c51 exerted an additive blocking effect on I_{Ach}, the resulting inhibition was smaller than the sum of their individual blocking effects.

The inhibitory effect of 0.5 μM BW284c51 on I_{Ach} was also compared to that of *d*-TC, used at the same concentration. As shown in Figure 6, the I_{Ach} blockade elicited by BW284c51 (to 49 ± 1% of the control I_{Ach} value; $n = 67$, 15 donors) was comparable to that evoked by *d*-TC (to 53 ± 5% of the control I_{Ach}, $n = 10$, four donors; $P = 0.083$), when both drugs were

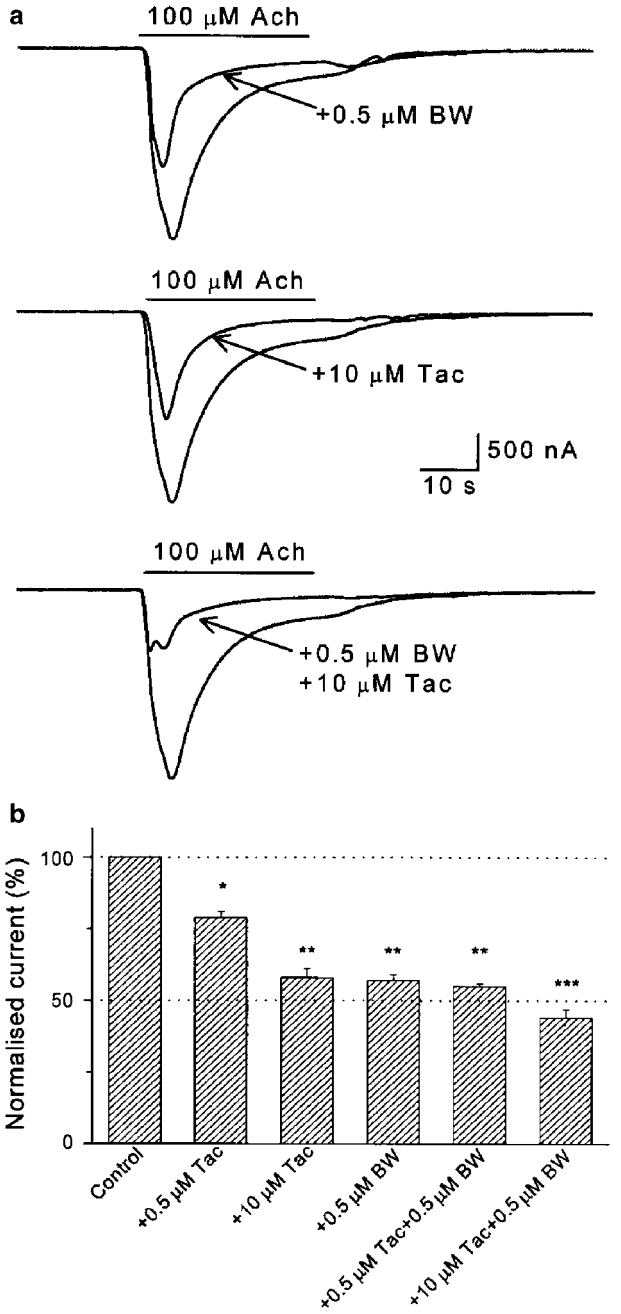

Figure 5 Additive blockade of I_{Ach} by BW284c51 and tacrine. (a) Representative currents evoked in an oocyte upon superfusion either with 100 μM Ach alone or together with 0.5 μM BW285c51 (upper records), 10 μM tacrine (Tac; middle records) or both inhibitors simultaneously (lower panel). As shown, the blockade caused by individual application of these drugs was comparable, but their coapplication resulted in an increased inhibition. (b) Column graph showing the inhibitory effects of BW284c51 and/or tacrine on I_{Ach}. The height of the bars indicates the fraction of the control I_{Ach} remaining after the application of Ach with the indicated drugs. Data are the mean $\pm$ s.e.m. (five oocytes from two donors). Different number of asterisks atop the bars indicate significant differences between two groups ($P<0.05$, as determined by the analysis of variance on ranks).

separately coapplied with 100 μM Ach. As above, experiments were carried out in oocytes from the same donor injected with a common sample of reconstituted receptors. Both drugs

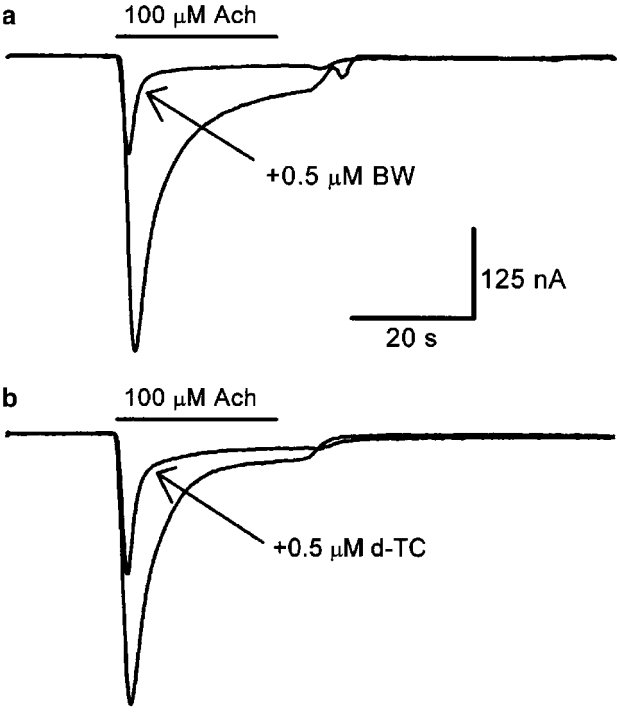

Figure 6 BW284c51 and *d*-TC exhibit similar antagonist potencies on I_{Ach}. Records obtained in the same oocyte by superfusing the cell with 100 μM Ach, either alone or together with 0.5 μM BW284c51 (a) or 0.5 μM *d*-TC (b). Note that both drugs had similar inhibiting effects on I_{Ach}.

potentiated each other's inhibitory effects on I_{Ach} and their synergistic effects were stronger than those obtained by BW284c51 and tacrine (not shown). Interestingly, although BW284c51 and *d*-TC exerted a fast antagonistic action on I_{Ach}, the BW284c51 blockade was quickly and completely removed after this drug was washed out (Figure 2), whereas the recovery from the *d*-TC block in the same cells ($n=10$, four donors) was much slower (not shown).

Selectivity of BW284c51 effects on nicotinic over muscarinic AchRs

Muscarinic currents can be elicited by Ach in some noninjected oocytes, since M1 and M3 receptors are functionally expressed in the membrane of about 40% of *X. laevis* oocytes (Lupu-Meiri *et al*., 1990; Davidson *et al*., 1991). There is experimental evidence that compounds representative of the main ChEI classes interact with muscarinic Ach receptors (Bakry *et al*., 1988; Zhang *et al*., 1997; Lockhart *et al*., 2001). Since it has been shown that physostigmine, tacrine, edrophonium and galantamine are able to displace the binding of the muscarinic AchR agonist [^{3}H]oxotremorine-M from its receptor in rat cortex and brain stem homogenates (Lockhart *et al*., 2001), we studied the effects of 0.5–10 μM BW284c51 on muscarinic responses. Figure 7a shows a muscarinic I_{Ach} elicited by 100 μM Ach, recorded in NR solution in an uninjected oocyte. This slow current shows some oscillations and is mainly carried by Cl^-, as a result of the activation of the inositol trisphosphate signalling cascade (Oron *et al*., 1985; Parker & Miledi, 1986), which raises intracellular Ca^{2+} concentration and, subse-

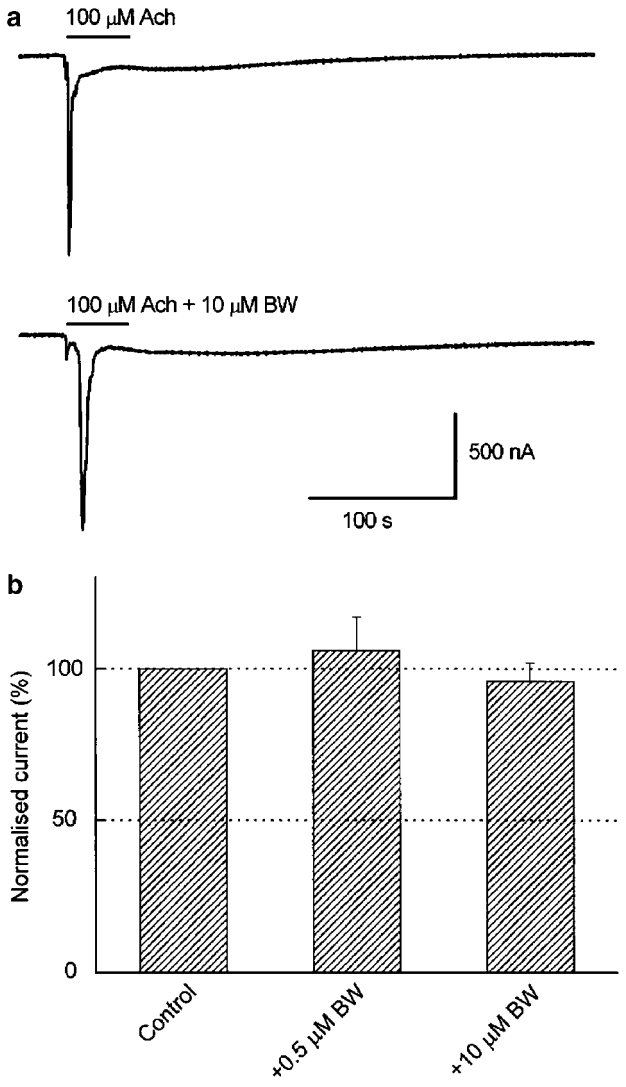

Figure 7 Lack of effect of BW284c51 on muscarinic AchRs. (a) Muscarinic currents elicited in a noninjected oocyte, with the membrane potential held at −60 mV, by superfusing it with an NR solution containing 100 μM Ach, either alone (upper record) or together with 10 μM BW284c51 (lower record). (b) Column graph showing the effect of Ach coapplication with either 0.5 or 10 μM BW284c51 on the muscarinic current amplitude. Data were normalised as the percentage of the control responses (obtained in the same cell in the absence of the drug) and expressed as the mean ± s.e.m. of five cells from three donors. There were not significant differences between any of these three groups.

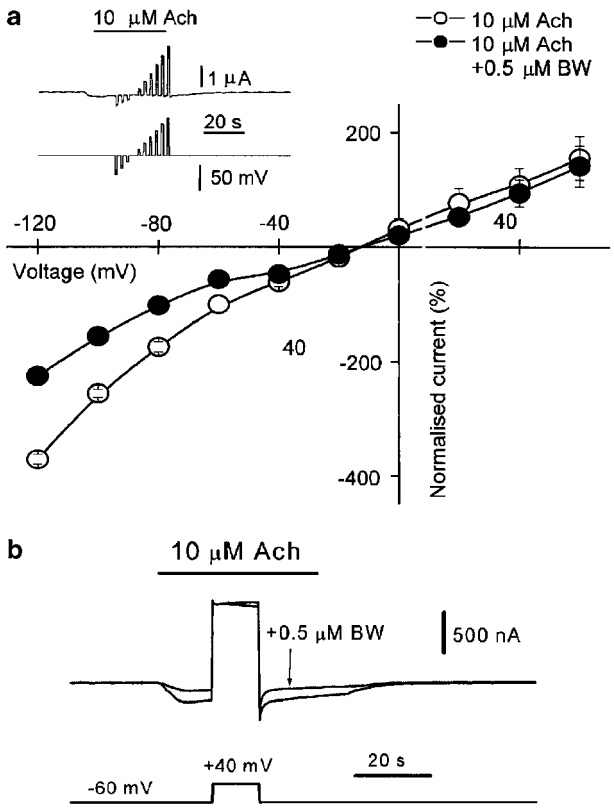

Figure 8 Voltage dependence of BW284c51 effects on I_{Ach}. (a) The *i–v* relationship for the I_{Ach}, elicited by 10 μM Ach either alone or coapplied with 0.5 μM BW284c51, was obtained by giving voltage pulses from −120 up to +60 mV, in 20 mV steps, during the current plateau induced by Ach. Values were normalised, for each oocyte, as the percentage of the I_{Ach} obtained at −60 mV while superfusing it with Ach alone. Every point is the mean ± s.e.m. of five oocytes from two donors. Note that the blocking effect of BW284c51 vanished at positive potentials. The inset shows the I_{Ach} plateau elicited by 10 μM Ach and the time at which voltage pulses were given. (b) Effect of a 10 s depolarising pulse (from the holding potential, −60 mV, up to +40 mV) on the I_{Ach} elicited by 10 μM Ach either alone or coapplied with 0.5 μM BW284c51. Note that I_{Ach} blockade by BW284c51 was completely abolished at +40 mV and that once the depolarising pulse had finished, the blocking action of BW284c51 was immediately, and completely, recovered.

quently, activates endogenous Ca^{2+}-dependent Cl^- channels present in the oocyte membrane. When the Ach pulse was repeated 15 min later in the presence of BW284c51 (0.5–10 μM), there were not significant differences neither in the current amplitude nor in the time course of the response (Figure 7a). Similar results were obtained in other cells isolated from different donors (Figure 7b; $P = 0.128$).

Voltage dependence of the BW284c51 blockade

To determine whether the I_{Ach} blocking effect of BW284c51 exhibited any voltage dependence, an *i–v* curve was obtained by jumping the membrane potential to different voltages during the plateau of the I_{Ach} elicited by Ach (10 μM) either alone or coapplied with BW284c51 (0.5 μM). The control I_{Ach}, *i–v* relationship showed an inward rectification at hyperpolarising potentials and a reversal potential of −13 ± 2 mV ($n = 5$, two donors), which was in good agreement with our previous data (Morales *et al.*, 1995). In the presence of BW284c51, I_{Ach} was markedly reduced at potentials below −40 mV, but no blocking effect was found at positive potentials (Figure 8a). The voltage dependence of the BW284c51 blockade of I_{Ach} could be plainly observed by giving a long-lasting pulse from −60 to +40 mV at the I_{Ach} plateau elicited by 10 μM ACh either alone or coapplied with BW284c51 (0.5 μM). As Figure 8b illustrates, the I_{Ach} blockade elicited by BW284c51, evident at −60 mV, was fully reversed by the depolarising pulse. After repolarisation, I_{Ach} was again quickly blocked, showing a similar degree of inhibition to that found before

membrane depolarisation. The ion selectivity of the Ach-gated channel was not affected by BW284c51, since the I_{Ach} reversal potential remained unchanged (-12 ± 1 mV, $n = 5$, two donors; $P = 0.547$).

Discussion

Our results show that BW284c51 blocks *Torpedo* nicotinic AchRs in a noncompetitive and voltage-dependent manner, and also increases I_{Ach} desensitisation. This blocking effect was selective for nicotinic over M1 and M3 muscarinic AchRs and was not related to its ability as ChEI, since a similar inhibition was obtained using Cch as the agonist, a compound which is not hydrolysed by AchE (Sung *et al*., 1998).

Noncompetitive inhibitors are thought to inhibit receptor function by different mechanisms, including blockade of the open channel and promotion of desensitisation (for a review see Popot & Changeux, 1984). The strong voltage dependence of the I_{Ach} inhibition by BW284c51, only present at negative potentials, and the faster I_{Ach} peak observed when Ach was coapplied with BW284c51, are consistent with an open-channel blockade by this compound exerted at the vestibule or within the channel itself. On the other hand, though BW284c51 induced a faster I_{Ach} decay, it cannot account for the observed blocking effect, since changes in desensitisation were more evident at high Ach doses, whereas the degree of inhibition was dose-independent.

It is well known that some AchEIs exert an inhibitory effect on I_{Ach}, including tacrine (Cantí *et al*., 1998; Prince *et al*., 2002), its derivatives CI-1002, CI-1017 (Ros *et al*., 2000) and bis(7)-tacrine (Ros *et al*., 2001a), and other inhibitors such as physostigmine (Cantí *et al*., 1998), huprines (Ros *et al*., 2001b), edrophonium, neostigmine and pyridostigmine (Yost & Maestrone, 1994). Interestingly, most of these compounds, which have quite different molecular structures, share the property of mediating a noncompetitive antagonism with remarkable voltage dependence, suggesting that they operate through an open-channel blocking mechanism. However, the inhibitory potency of different AchEIs on nicotinic I_{Ach} was highly variable. Thus, whereas (±)-huprine Y, bis(7)-tacrine and BW284c51 bear IC_{50} values in the submicromolar range (Ros *et al*., 2001a, b; our present data), the IC_{50} values for the rest of the above AchEIs are at least one order of magnitude higher. So, I_{Ach} inhibition by BW284c51 was stronger than that mediated by tacrine (Cantí *et al*., 1998; Prince *et al*., 2002), and was of a similar potency to that found for *d*-TC (O'Leary *et al*., 1994; our present data). Interestingly, the blocking effect of BW284c51 and tacrine or curare were additive, suggesting that these drugs differ, at least partially, in their mechanisms of I_{Ach} inhibition. However, since both BW284c51 and tacrine show a noncompetitive antagonism on nicotinic currents, their additive effect might be achieved through interactions with different sites on the receptor. In this sense, it is worth noting to mention that multiple binding sites have been proposed for the tacrine action on human muscle nicotinic AchRs (Prince *et al*., 2002), and that BW284c51 in addition to plugging the channel it is able to modify the n_H for Ach. Nevertheless, our data support a unique binding site for BW284c51, which might be located close to the pore of the channel. Moreover, the additive blocking effects of BW284c51 and *d*-TC, a competitive inhibitor, and the specificity on nicotinic over muscarinic receptors, agree well with an action of the BW284c51 exerted mainly on, or close to, the channel pore. The change observed in the n_H value for AchRs when Ach was coapplied with BW284c51 remains to be explained.

BW284c51 inhibitory effects on *Torpedo* nicotinic AchRs strongly resembled those elicited by edrophonium, an AchEI with only one quaternary ammonium group, on mouse muscle nicotinic AchRs expressed in *Xenopus* oocytes. Thus, edrophonium enhanced AchR desensitisation and caused a voltage-dependent blockade, which was more potent at hyperpolarising membrane potentials, this also suggesting a channel blockade within the ion-conducting pore (Yost & Maestrone, 1994). The main difference between these two compounds on their action on nicotinic AchRs is at their IC_{50} value, which is about two orders of magnitude higher for edrophonium than for BW284c51 (Yost & Maestrone, 1994; S. Olivera-Bravo, I. Ivorra & A. Morales, unpublished results); nevertheless, its effects were also completely reversible upon washout of the inhibitor.

Significant differences were found, however, between the BW284c51 effects on nicotinic AchRs and those exerted by other bisquaternary ammonium drugs, such as hexamethonium and decamethonium. Thus, whereas decamethonium behaves as a partial agonist of nicotinic AchRs (Adams & Sakmann, 1978; Aoshima, 1990; Bertrand *et al*., 1992; Liu & Dilger, 1993), BW284c51 did not elicit any agonistic effect on these receptors, even at concentrations as high as 1 mM. Unlike BW284c51, hexamethonium and decamethonium inhibitory effects on AchRs could not be immediately reverted upon their washing out; in fact, their effects only disappeared either by depolarising the membrane or by extensive, long-lasting washes (Bertrand *et al*., 1990). This fact suggests that these two bisquaternary compounds interact with the nicotinic AchR in a site located deeper within the channel than the site for BW284c51 binding. Since BW284c51 effects are more alike to those evoked by edrophonium, a molecule with a single quaternary ammonium, than those elicited by hexamethonium or decamethonium, which are bisquaternary ammonium compounds, it follows that BW284c51 blockade seemingly depends mainly on the presence of this functional group rather than on its number.

To conclude, this is the first report showing a set of BW284c51 actions on AchRs not directly related to its activity as an AchEI (Mikalsen *et al*., 1986, Radic *et al*., 1993; Dupree & Bigbee, 1994). It should be pointed out that the morphogenic effects of BW284c51, suppressing neurite outgrowth in several model systems, have been attributed to its interaction with the peripheral site of AchE (Eichler *et al*., 1994; Sharma & Bigbee, 1998; Brimijoin & Koenigsberger, 1999). Putatively, this interaction might distort the enzyme shape, and/or surface charge, which in turn could disturb critical protein–protein interactions, including those involved in neurites outgrowth (Bigbee *et al*., 1999). Although BW284c51 constitutes an excellent pharmacological tool for inhibiting AchE activity with high selectivity, our findings must be taken into account in view of its powerful effect on nicotinic receptors. Moreover, this compound should be useful as a tool for the study of nicotinic AchR function given its high blocking potency and immediate recovery of receptor activity after its removal, two properties in which BW284c51 has proven to be similar, or even better, than the classical inhibitor *d*-TC.

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