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Effects of benzothiazepines on human neuronal nicotinic receptors expressed in *Xenopus* oocytes

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1 We have investigated the effect of diltiazem and its newly synthesized derivative (+,−)-trans-3-acetoxy-8-chloro-2,3-dihydro-5[2-diisopropylamine)ethyl]-2-(4-methoxyphenyl)-1,5-benzothiazepin-4-(5*H*)-ona hydrochloride (JAC-65) on several recombinant human neuronal nicotinic acetylcholine receptors (nAChRs) expressed in *Xenopus* oocytes.

2 At 3 μM, both drugs have little effect on the maximal currents evoked by brief pulses of acetylcholine (ACh) in five subtypes of nAChRs ($\alpha7$, $\alpha3\beta2$, $\alpha4\beta2$, $\alpha3\beta4$, and $\alpha4\beta4$), showing little selectivity among subtypes.

3 However, both drugs accelerate the decay of the ionic currents evoked upon continuous stimulation of ACh, being this effect larger with JAC-65, and in $\beta4$*-nAChRs. Such an effect was dependent on the concentrations of both the drug and of the agonist used, and showed the characteristics of a non-competitive antagonism.

4 We have further investigated the effect of both drugs when combined with submicromolar concentrations of nicotine, such as those present in plasma of cigarette smokers, and found that JAC-65, but not diltiazem, is able to greatly enhance the desensitizing effect of these low concentrations of nicotine, specially in $\beta4$*-nAChRs.

5 Experiments in $\alpha4\beta4$-nAChRs failed to show voltage dependence of the action of JAC-65. Moreover, recovery from desensitization followed the same time course regardless of the presence of the drug, suggesting that the main mechanism of action of JAC-65 does not involve open channel block.

6 In summary, both drugs, diltiazem and JAC-65, seem to act through a non-competitive mechanism, accelerating the decay of the ionic currents, being JAC-65 more effective than diltiazem at the concentrations used in $\beta4$*-nAChRs. Thus, the differences between both benzothiazepines when measuring various parameters suggest that their mechanisms of action could be slightly different. This would require further investigation.

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Abbreviations: ACh, acetylcholine; CNS, central nervous system; HEPES, N′- 2-Hydroxyethyl[piperazine-N′-2-ethanesulphonic acid]; JAC-65, (+,−)-trans-3-acetoxy-8-chloro-2,3-dihydro-5-[2-(diisopropylamine)ethyl]-2-(4-methoxyphenyl)-1,5-benzothiazepin-4-(5*H*)-ona hydrochloride; nAChR(s), nicotinic acetylcholine receptor(s)

Introduction

Nicotinic acetylcholine receptors (nAChRs) are members of the superfamily of ligand-gated ion channels formed by the pentameric arrangement of one or more structurally homologous subunits, which have been cloned from different species including the human. All of these subunits are found in the nervous system of mammals, but the exact stoichiometry of the native receptors is not completely determined. The $\alpha3\beta4$ subtype (probably combined with the $\alpha5$ subunit) is the predominant subtype in the peripheral nervous system, including adrenal chromaffin cells and peripheral ganglia, and its physiological role is relatively well-understood (Sargent, 1993; Colquhoun & Patrick, 1997). A different landscape is seen in the central nervous system (CNS) where, although the most abundant subunits are $\alpha4$, $\beta2$, and $\alpha7$, the establishment of the possible functions of neuronal nAChRs is less clear. Neuronal nAChRs have been involved in physiopathological processes as varied as memory, neurodegeneration, analgesia, tobacco smoking addiction (Jones *et al*., 1999; Paterson & Nordberg, 2000), and even ocular growth and myopia (Stone *et al*., 2001). However, only recently it has been possible to demonstrate the modulatory role of some neuronal nAChRs in synaptic transmission (McGehee *et al*., 1995; Pidoplichko *et al*., 1997). In many of these processes it seems that, in addition to their activation, desensitization of nAChRs could be involved (Pidoplichko *et al*., 1997). For instance, desensitization of neuronal nAChRs could play a predominant role in shaping synaptic currents in a variety of neural structures, in particular those involved in the reinforcing effects of nicotine (Dani & Heinemann, 1996; Pidoplichko *et al*., 1997). Chronic exposure to nicotine or to other agonists causes neuronal nAChRs desensitization, which depends on the duration of agonist exposure and the receptor subtype (Fenster *et al*.,

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1997; Reitstetter *et al*., 1999), and also induces upregulation of $\alpha4\beta2$-nAChRs expressed in oocytes or cell lines. Both effects seem to be connected through processes of phosphorylation and dephosphorylation of the receptors, which are controlled by intracellular calcium, protein kinase A and protein kinase C (Gopalakrishnan *et al*., 1997; Hsu *et al*., 1997; Fenster *et al*., 1999a; see Paterson & Nordberg (2000) for a review). However, few studies on desensitization have been made using low concentrations of nicotine, such as those related to tobacco consumption, and none in human nAChRs. Within the concentration range achieved by smokers (100–1000 nM; Benowitz *et al*., 1989), it has been reported the influence of subunit composition on the kinetics and extent of desensitization in rat neuronal nAChRs (Hsu *et al*., 1996; Fenster *et al*., 1997). To most of these studies, the molecular cloning of the neuronal nAChR subunits, and its expression in heterologous expression systems have been helpful. In spite of some discrepancies between native and expressed receptors, which depend on the expression system (Chavez-Noriega *et al*., 1997; Colquhoun & Patrick, 1997; Shafaee *et al*., 1999; Sivilotti *et al*., 1997; Stauderman *et al*., 1998), the combination of these tools has been useful in widening the knowledge of the intrinsic properties of neuronal nAChRs, and in discovering new roles in physiology as well as in drug therapy.

Diltiazem and other benzothiazepines are well known L-type calcium channel antagonists and widely used in the therapy of cardiovascular diseases. On the other hand, they have been used as specific pharmacological tools for L-type calcium channels. However, diltiazem can affect ligand-gated ion channels as well. In particular, diltiazem has been reported to block 5-HT_3 receptors (Hargreaves *et al*., 1996), and neuronal nAChRs present in bovine chromaffin cells (Gandia *et al*., 1996), or heterologously expressed in *Xenopus* oocytes (Herrero *et al*., 1999; Houlihan *et al*., 2000). Its action seems to be restricted to cationic channels (Houlihan *et al*., 2000).

JAC-65 is a novel derivative of diltiazem which, in bovine chromaffin cells, blocks the calcium uptake evoked by nicotinic agonists more potently than that evoked by high potassium, suggesting a certain selectivity over nicotinic receptors (Villarroya *et al*., 1998). The present work studies the effects of two benzothiazepines, diltiazem and JAC-65, on several human neuronal nAChRs expressed in the oocyte system with well-defined stoichiometry, focusing in the process of the decay of the ionic currents upon continuous application of agonists, which will be called desensitization thereafter, and in the interactions of both benzothiazepines with low, tobacco-related concentrations of nicotine. We present evidence of benzothiazepines blocking different human neuronal nAChRs, affecting their function by means of a non-competitive mechanism. But our results also suggest that there are differences between the mechanisms of action of diltiazem and its derivative JAC-65.

Methods

Oocyte expression

DNAs of human neuronal nicotinic acetylcholine receptor subunits receptors ($\alpha7$, $\alpha3\beta2$, $\alpha4\beta2$, $\alpha3\beta4$, and $\alpha4\beta4$) were inserted into the pSP64T vector (Krieg & Melton, 1984). Capped mRNA was synthesized *in vitro* using SP6 RNA polymerase. Defolliculated *Xenopus* oocytes were injected with 5 ng of total RNA in 50 nl of sterile water. The two subunits of heteromeric receptors were injected in an equimolar ratio. All measurements were made within 3–6 days after injection.

Electrophysiological recordings

Two electrode voltage clamp recordings were obtained as described (Stühmer, 1998). Oocytes were located in a chamber (0.9 ml of volume) and perfused by gravity with a modified frog Ringer containing (in mM): NaCl 82.5, KCl 2.5, $BaCl_2$ 2.5, $MgCl_2$ 1 and HEPES 5 (pH 7.4). Perfusion rate was 12–15 ml min^{-1}. With barium as the permeant divalent cation no calcium-activated chloride currents were detected, as no significant difference was found when BAPTA was injected into the oocytes. Agonists and drugs were applied through a gravity-driven pipette with an internal diameter of 1.2 mm and located close to the animal hemisphere of the oocyte, where larger currents were consistently found with respect to the vegetal hemisphere. The velocity of application was 18–22 ml min^{-1}. The solution exchange rate was measured as the junction potential change detected by the voltage electrode after switching to a solution with 8 mM NaCl. The electrode was located at 16 evenly spaced points on the oocyte surface. The junction potential change detected on the hemispheric surface facing the application tube followed an exponential time course with $\tau_f = 90 \pm 5$ ms. The change on the opposite surface was best fitted with an exponential with $\tau_s = 992 \pm 58$ ms, raised to the third power to account for the delay in the arrival of the applied solution to this less exposed side. With such a slow solution exchange rate, it is clear that rapid processes such as fast desensitization or channel activation cannot be either detected or studied properly. Therefore, peak responses should not be taken as absolute measurements of channel activation, but rather as relative to their control. However, we have performed computer simulations of several kinetic models of nAChR including at least three different kinetic states (closed, open and desensitized) with the unfavourable cases of an even distribution of nAChRs on the oocyte surface, and with a slow solution exchange time course up to $\tau_f = 1000$ ms and $\tau_s = 4000$ ms (not shown). Even with such a slow exchange, the simulations showed that desensitization decay kinetics, as studied here, is practically unaffected.

Holding potential was usually −80 mV. Currents were filtered at 50 Hz with a low pass 8-pole Bessel filter, sampled at 100–500 Hz and stored on hard disk for later analysis. Data acquisition and agonist application were controlled by a DigiData 1200 interface driven by PClamp 6.0 software (Axon Instruments, Foster City, CA, U.S.A.).

Drugs

Diltiazem was purchased from Research Biochemical International (Natick, MA, U.S.A.). (+,−)-trans-3-acetoxy-8-chloro-2,3-dihydro-5-[2-(diisopropylamine)ethyl]-2-(4-methoxyphenyl)-1,5-benzothiazepin-4-(5*H*)-ona hydrochloride (JAC-65) was a novel derivative of diltiazem synthesized by Professor Julio Alvarez Builla (Villarroya *et al*., 1998). Unless

otherwise specified, all other drugs were purchased from Sigma.

Analysis

Results are presented as means and standard errors. Data analysis was performed with the software package Prism 3.0 (GraphPad, San Diego, CA, U.S.A.). EC_{50} and IC_{50} values were calculated by non-linear regression analysis using the equation for a sigmoid concentration-response curve. Statistical significance was calculated by one-way ANOVA test and, when a significant F value was obtained, by Bonferroni's multiple comparison test. The null hypothesis was rejected when $P<0.05$.

Results

Effects of diltiazem and JAC-65 on recombinant human nicotinic receptors

Five human nicotinic acetylcholine receptor subunits were expressed in *Xenopus* oocytes forming five different receptors subtypes ($\alpha7$, $\alpha3\beta2$, $\alpha4\beta2$, $\alpha3\beta4$, and $\alpha4\beta4$). Robust inward currents were produced when any of these receptors was stimulated with 1 mM acetylcholine (ACh) at a holding potential of -80 mV (Figure 1). Stimulation lasted 1 s for $\alpha7$-nAChRs, and 10 s for all other receptors. This protocol allowed the analysis of two parameters: the peak current and the extent of decay upon continuous application of an agonist. Although the magnitude of the control currents was usually stable, sometimes they rundown slowly along the experiment and then percentages of the control currents were calculated over the interpolated control current.

Figure 1 shows that the co-application of 3 μM JAC-65 with 1 mM ACh, produces both a small reduction of the peak currents and some changes on the desensitization, depending on the receptor subtype. Similar results were obtained with 3 μM diltiazem. Figure 2A shows that such a blocking action of benzothiazepines was significant, depending on both the drug and the receptor studied. For instance, no significant blockade was achieved by 3 μM of either drug in $\alpha3\beta2$-nAChRs. The extents of blockade obtained by 3 μM benzothiazepines in all other receptors were small but significant, except for JAC-65 in $\alpha7$-nAChRs. When comparing the blocking action of both benzothiazepines, significant differences appeared in $\alpha7$-nAChRs (blockades of $21\pm3\%$ ($n=4$), and $3\pm5\%$ ($n=5$), for diltiazem and JAC-65, respectively), and $\alpha4\beta4$-nAChRs (blockades of $32\pm2\%$ ($n=10$), and $14\pm3\%$ ($n=9$), for diltiazem and JAC-65, respectively). The extent of blockade was similar when the drug was present in the bathing solution for 3 min before the challenge with ACh, regardless of its presence during the agonist application (not shown). This suggests that both benzothiazepines are able to bind to the resting state of all receptors, and that their dissociation rates are relatively slow with respect to the agonist binding. However, as explained below, estimates of the peak currents might be distorted by the presence of a faster desensitization.

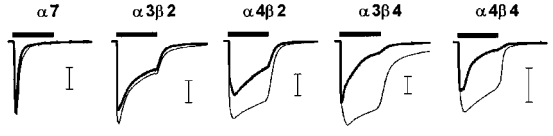

Figure 1 Representative inward currents evoked by continuous application of 1 mM ACh in five different human nAChRs expressed in *Xenopus* oocytes, in the absence (thin traces), or in the presence of 3 μM JAC-65 (thick traces). In all the experiments shown, holding potential was -80 mV. Vertical scale bars are 5 μA for all receptors. Horizontal application bars are 1 second for $\alpha7$-nAChRs, and 10 s for all other nAChRs.

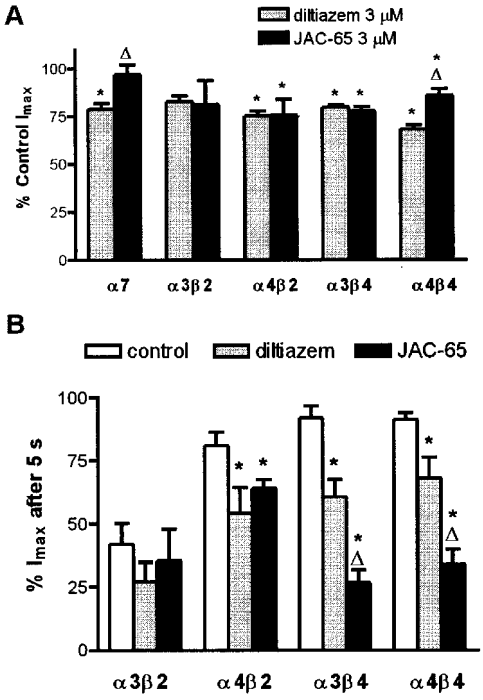

Figure 2 (A) Blockade of peak inward currents caused by 3 μM of either diltiazem or JAC-65 in five nAChR subtypes stimulated by 1 mM ACh. (B) Desensitization of neuronal nAChRs. Oocytes expressing heteromeric nAChRs were stimulated by a 9 s-application of 1 mM ACh, in the absence (control) or in the presence of 3 μM of benzothiazepine drugs. Values are the percentage of the peak inward current remaining at 5 s after the peak. Data are means and standard errors of 4–14 oocytes of, at least, two donors. * Represents statistically significant differences between control and drug. Δ represents statistically significant differences between JAC-65 and diltiazem.

Ionic currents evoked in homomeric $\alpha7$-nAChRs quickly decayed upon continuous stimulation, and the kinetics were not altered by the presence of the drugs. In contrast, decay kinetics in heteromeric receptors were slower and more complex, depending on the receptor subtype. Often, it hardly could be fitted by a single- or double-exponential function. Therefore, we have used the relative decay of the current observed 5 s after the peak as a comparison of the relative extents of desensitization (Bohler *et al*., 2001). At this time, peak currents in control conditions were reduced to $42\pm9\%$ ($n=6$), $81\pm6\%$ ($n=7$), $92\pm5\%$ ($n=12$) and $92\pm3\%$ ($n=14$), in $\alpha3\beta2$-, $\alpha4\beta2$-, $\alpha3\beta4$- and $\alpha4\beta4$-nAChRs, respectively. However, in the presence of 3 μM of either diltiazem or JAC-65, the decay kinetics were altered to different extents (Figures 1 and 2B). For instance, both benzothiazepines showed little, if any, effect on $\alpha3\beta2$-nAChRs, which were the

faster desensitizing subtype. In contrast, diltiazem reduced significantly the percentage of current remaining 5 s after the peak to 54–66% in the other receptor subtypes. The effect of JAC-65 was equivalent to that of diltiazem in $\alpha4\beta2$-nAChRs, but was significantly more marked in $\beta4$*-nAChRs, as it reduced the remaining currents to $26\pm5\%$ ($n=6$) and $34\pm6\%$ ($n=7$) of the peak responses, in $\alpha3\beta4$- and $\alpha4\beta4$-nAChRs, respectively. A qualitatively similar effect has been described for 3 μM diltiazem in rat $\alpha3\beta4$-nAChRs, but not in $\alpha3\beta2$-nAChRs (Herrero *et al*., 1999).

Drug concentration dependence of the effects of benzothiazepines

When compared to rat neuronal nAChRs (Herrero *et al*., 1999), peak currents of human neuronal nAChRs seem to be less sensitive to benzothiazepines, as the extent of blockade produced by 3 μM of the drugs was only in the range of 20–30% (Figure 2A). Moreover, the reduction of the peak currents might be partially due to the effect of the drugs on decay kinetics combined with slow solution exchange. Therefore, in order to study the concentration dependence of blockade within the range of clinically relevant concentrations of benzothiazepines (Yeung *et al*., 1996) a more suitable parameter is the decay of the currents upon continuous application of ACh. We have chosen $\beta4$*-nAChRs, where this effect is more conspicuous, and JAC-65, which produced the greatest effect. Figure 3 shows that there is a drug concentration dependence of the desensitization measured as the fraction of current remaining 5 s after the peak response. Whereas 1 mM ACh produces little desensitization after 5 s in both $\alpha3\beta4$- and $\alpha4\beta4$-nAChRs, the presence of JAC-65 accelerates the decay of the currents in a dose-dependent manner. When fitting these results with a one-site competition model, JAC-65 showed up equally potent in accelerating decay kinetics of both $\alpha3\beta4$- and $\alpha4\beta4$-nAChRs, as its IC_{50} values were 1.16 and 1.20 μM, respectively. These values are close to those reported for the diltiazem blockade of peak currents in rat $\alpha3\beta4$-nAChRs (Herrero *et al*., 1999), and in human $\alpha7$-nAChRs (Houlihan *et al*., 2000).

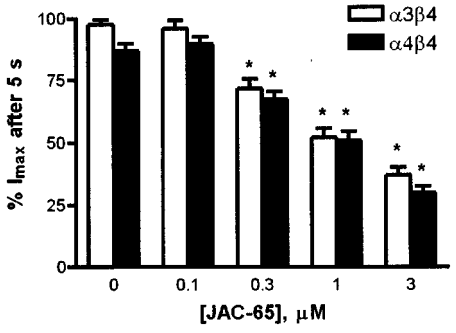

Figure 3 Dependence of drug concentration. Effect of increasing concentrations of JAC-65 on the extent of desensitization of $\alpha3\beta4$- and $\alpha4\beta4$-nAChRs upon continuous application of 1 mM ACh. Values are the percentage of the peak inward current remaining at 5 s after the peak. When measuring this parameter, the calculated IC_{50} values for JAC-65 were 1.16 and 1.20 μM in $\alpha3\beta4$- and $\alpha4\beta4$-nAChRs, respectively. For each point, data shown are means and standard errors of 8–48 oocytes of, at least, three donors. *Represents statistically significant differences between control and JAC-65.

Dependence of acetylcholine concentration

To explore the nature of JAC-65 interaction with the human neuronal nAChRs, experiments were designed to find out the characteristics of the blockade of the ACh-evoked currents. Oocytes expressing either $\alpha3\beta4$- or $\alpha4\beta4$-nAChRs were stimulated with different concentrations of ACh applied during 9 s, in the absence or in the presence of 3 μM JAC-65. From these experiments we measured the peak currents and their remaining fraction after 5 s in every condition. Peak responses are normalized to the peak current obtained with 1 mM ACh in the absence of the blocker. Figure 4A shows dose-response curves for peak currents evoked by ACh in $\alpha3\beta4$-nAChRs and the effect of 3 μM JAC-65. The calculated EC_{50} values for ACh in the absence or presence of JAC-65 were 72 and 73 μM, respectively. Figure 4B shows the same kind of data in $\alpha4\beta4$-nAChRs. In this case, the EC_{50} values for ACh in the absence or presence of JAC-65 were 102 and 105 μM, respectively. Since the estimates of the peak currents might be distorted by the slow solution exchange, the fact that the blockade is not overcome by higher concentrations of ACh can not be considered as a strong evidence against a competitive antagonism, but together with the lack of shift in the EC_{50} values, it suggests a non-competitive mechanism of action between JAC-65 and ACh in both receptors.

ACh-evoked currents in $\beta4$*-nAChRs desensitize slowly and, depending on the receptor subtype and ACh concentration, only a 10–30% of the peak current has inactivated after 5 s (Figure 4C and D, open symbols). When this parameter is measured in the presence of JAC-65 there are no significant differences with respect to controls when low concentrations of ACh are used (up to 30 μM). However, when the ACh concentration is high enough, the extent of decay is significantly enhanced by JAC-65 in both $\alpha3\beta4$- or $\alpha4\beta4$-nAChRs. This feature strongly argues against a competitive mechanism of blockade for JAC-65.

Voltage dependence of the effect of JAC-65

As the effects of benzothiazepines on ACh-evoked currents could be accounted for by an open channel block mechanism, we have performed experiments in order to explore the voltage dependence of these effects. Figure 5A shows currents obtained at -100 and -40 mV in the absence and in the presence of 3 μM JAC-65 in an oocyte expressing $\alpha4\beta4$-nAChRs. It could be observed that, in spite of the large difference in current magnitude obtained at the two holding potentials (note the different vertical scale), the effect of JAC-65 is roughly the same regardless of the membrane potential. Figure 5B summarizes the results obtained in several oocytes expressing $\alpha4\beta4$-nAChRs at different holding potentials. The extents of blockade observed, either at the peak response or after 5 s, suggest that there is no voltage dependence, as the linear regressions through the data did not show slopes significantly different from zero. In agreement with these results, stimulation with ACh at positive potentials did not produce relief of block (data not shown), as would be expected for typical nAChRs open channel blockers like mecamylamine or hexamethonium (Buisson & Bertrand, 1998; Giniatullin *et al*., 2000).

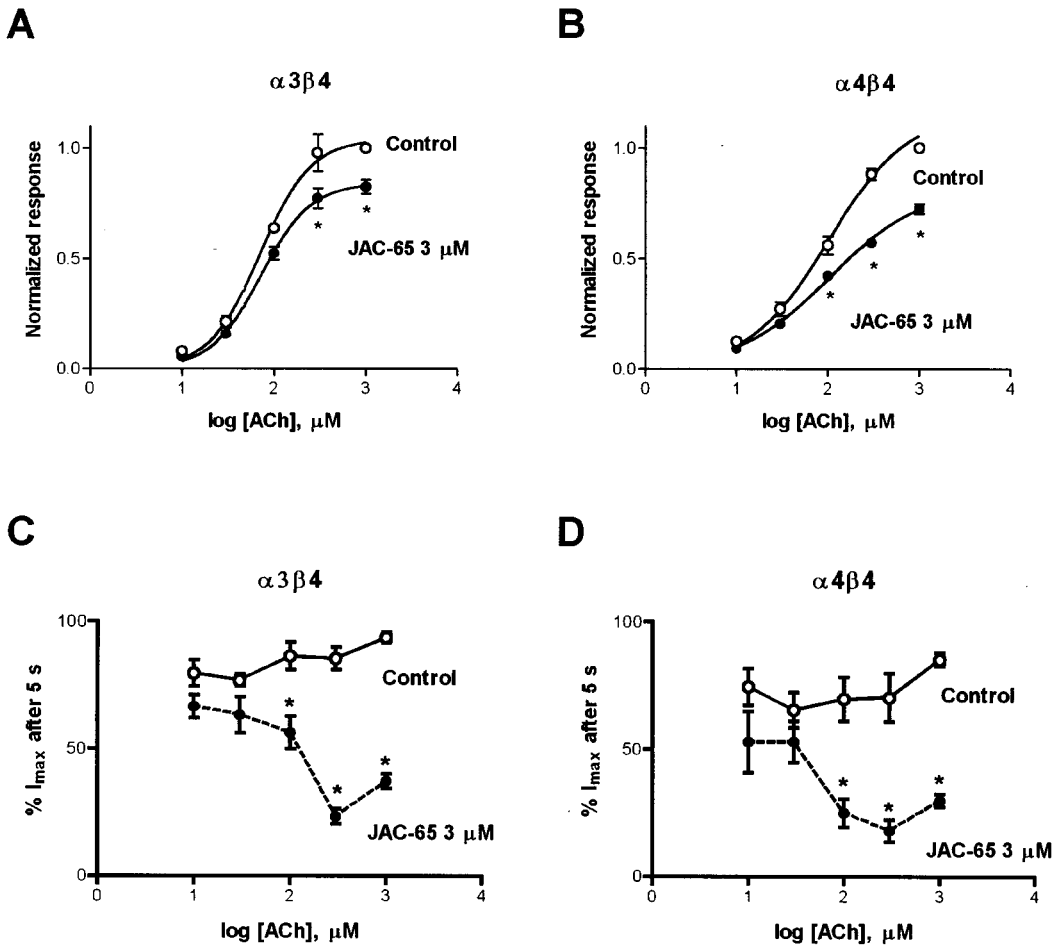

Figure 4 A-B: ACh concentration-response curves in the absence (open circles), or in the presence of 3 μM JAC-65 (closed circles), in $\alpha 3\beta 4$- (A) and $\alpha 4\beta 4$-AChRs (B). Continuous lines are fits with the Hill equation. EC_{50} values and Hill coefficients in $\alpha 3\beta 4$-nAChRs were 72 and 73 μM, and 1.53 and 1.56, in the absence or in the presence of JAC-65, respectively. EC_{50} values and Hill coefficients in $\alpha 4\beta 4$-nAChRs were 102 and 105 μM, and 1.00 and 0.89, in the absence or in the presence of JAC-65, respectively. (C–D) Dependence on ACh concentration of the effect of JAC-65 in $\alpha 3\beta 4$- (C) and $\alpha 4\beta 4$-nAChRs (D). Values are the percentage of the peak inward current remaining at 5 s after the peak, in the absence (control, open symbols) or in the presence of 3 μM JAC-65 (closed symbols). For each point, data are means and standard errors of 4–33 oocytes of, at least, two donors. Error bars are shown if larger than symbols. *Represents statistically significant differences between control and JAC-65 with the same ACh concentration.

Time course of recovery from desensitization

We have also investigated whether the conformational state induced by the coapplication of ACh and JAC-65 was different to that induced by the continuous application of ACh alone. Were that true it might be detected by changes in the time course of recovery from desensitization. Figure 6A shows a typical experiment in $\alpha 4\beta 4$-nAChRs in which application of very brief pulses of ACh (1 mM, 100 ms) every 15 s, evoked very reproducible control peak responses. During a longer pulse with 1 mM ACh coapplied with 3 μM JAC-65 (9 s, large arrow), ionic currents fully developed and showed the typical effect of JAC-65 on desensitization. Subsequent brief pulses allowed to study the time course of recovery from desensitization produced by the long pulse, and showed a full recovery of the control responses after 5 min. Figure 6B shows data obtained in experiments similar to that of A in which the long ACh pulses were applied in the absence or in the presence of 3 μM JAC-65. Independent fits with single exponential functions to both groups of data gave time constants of 48 ± 5 and 68 ± 6 s, for control and JAC-65, respectively. However, an *F*-test did not reveal significant differences between both time courses. Thus, all data were best fitted with a shared single exponential with time constant 61 ± 5 s, as shown in the figure. We have also studied the time course of recovery from block after 3 min pre-exposure of 300 nM nicotine and 3 μM JAC-65 in $\alpha 4\beta 4$-nAChRs (see protocols below). In this case, the recovery followed a single exponential time course with a time constant of 51 ± 4 s (4 oocytes, two donors; data not shown), which is not significantly different of the time course reported above.

Interactions of benzothiazepines with low concentrations of nicotine

The results presented so far showed that diltiazem and its derivative, JAC-65, are able to accelerate current decay of several subtypes of human nAChRs, possibly by facilitating

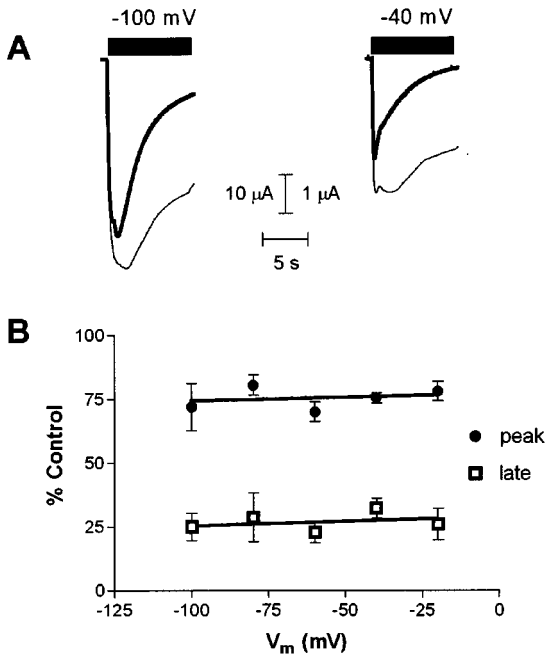

Figure 5 Voltage dependence of block. (A) Currents obtained upon 9 s stimulation with 1 mM ACh in an oocyte expressing $\alpha4\beta4$-nAChRs at two holding potentials, in the absence (thin traces) or in the presence of 3 μM JAC-65 (thick traces). (B) Extent of the blockade produced by 3 μM JAC-65 on the currents evoked by 1 mM ACh in $\alpha4\beta4$-nAChRs at different holding potentials, and measured at the peak response (filled circles) and 5 s after the start of stimulation (hollow squares). Data are means and standard errors of 8–10 oocytes of two donors and expressed as fractions of control currents. Both continuous lines are linear regressions whose slopes are not significantly different from zero.

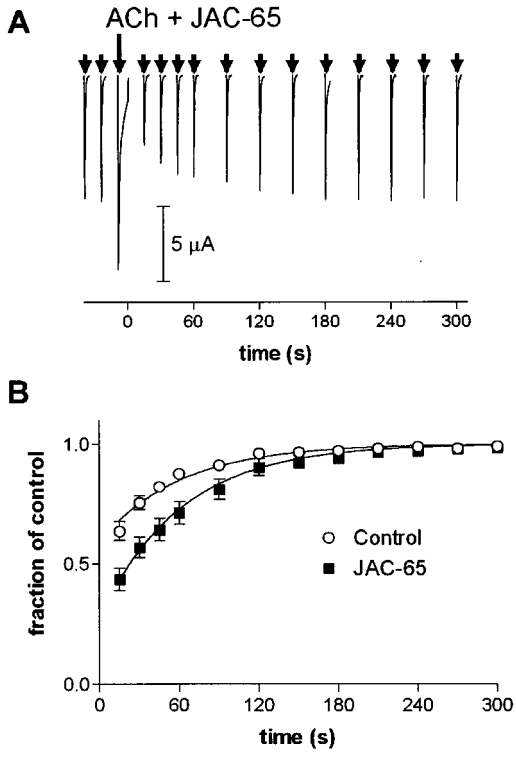

Figure 6 Recovery from desensitization upon continuous stimulation with ACh. (A) Currents evoked by brief ACh pulses (1 mM, 100 ms, short arrows) in an oocyte expressing $\alpha4\beta4$-nAChRs, before and after a 9 s coapplication of 1 mM ACh and 3 μM JAC-65 (large arrow), showing the time course of recovery of the inactivated current. (B) Summary of data obtained in $\alpha4\beta4$-nAChRs with protocols similar to that shown in (A). Long pulses of ACh were applied in the absence (hollow circles) or in the presence of 3 μM JAC-65 (filled squares). Data represent fractions of the current evoked by the brief pulses before the 9 s application, and are means and standard errors of 8 oocytes from two donors. Error bars are shown if larger than symbols. Both continuous lines are fits with a single-exponential function with time constant of 61 s.

agonist-induced desensitization. Therefore, we decided to focus on the possible interactions between these benzothiazepines and nicotine used at low, tobacco-related, concentrations. Figure 7A shows a typical experiment, performed in an $\alpha4\beta4$-expressing oocyte and exploring the interaction between nicotine and JAC-65. After two control pulses of 1 mM ACh (arrows), the oocyte is exposed to 300 nM nicotine for 3 min. Then, the application of an ACh pulse gives rise to an inward current that is slightly smaller than the control (3rd pulse). If the oocyte is exposed to 3 μM JAC-65 the current obtained is decreased by 20% (5th pulse). But when nicotine and JAC-65 are applied together the ACh-evoked current is diminished by 60% (7th pulse). The line, indicated as control baseline, represents the slow rundown of ACh-evoked control currents sometimes found in long-lasting experiments. Figure 7B shows a similar protocol in $\alpha4\beta4$-nAChRs with diltiazem. In this case the blockade caused by diltiazem alone is greater than that of JAC-65, but when diltiazem is coapplied with nicotine, no additional blocking effect can be observed. These results did not depend on the sequence of exposure to the drugs because similar results were obtained in experiments where the sequence was permuted.

We have further characterized the interaction of nicotine and JAC-65 in $\alpha4\beta4$-nAChRs. Figure 8 shows the blockade of peak control responses to 1 mM ACh produced by the pre-exposure to low concentrations of nicotine, in the absence and in the presence of 3 μM JAC-65. After pre-exposure of the oocyte to 10–1000 nM nicotine, control responses to ACh are maintained, suggesting that, in our experimental conditions, $\alpha4\beta4$-nAChRs are not significantly blocked (or desensitized) by nicotine up to 1 μM. In contrast, when concentrations of nicotine above 30 nM are applied together with JAC-65, the responses to ACh are significantly reduced below the level of blockade produced by JAC-65 alone, in a nicotine concentration dependent manner.

With identical experimental protocols, we have also characterized the interaction of both benzothiazepines with low concentrations of nicotine in receptors other than the $\alpha4\beta4$-nAChR subtype. Figure 9A and B summarize the results obtained in four receptor subtypes for diltiazem and JAC-65, respectively. The concentration of nicotine used (never above 1 μM), and the time of pre-exposure for each receptor subtype were chosen to induce a mild desensitization which might be modulated by benzothiazepines (see figure legend for details), but no systematic study was performed (Fenster *et al.*, 1997; Reitstetter *et al.*, 1999). With such exposures to nicotine, the activation of nAChRs was negligible or very low (less than 5% of the maximal current, and that can be blocked by nicotinic antagonists; data not shown), but the different nAChR subtypes are blocked to different extents (Fenster *et al.*, 1997). In $\beta2$*-nAChRs, the

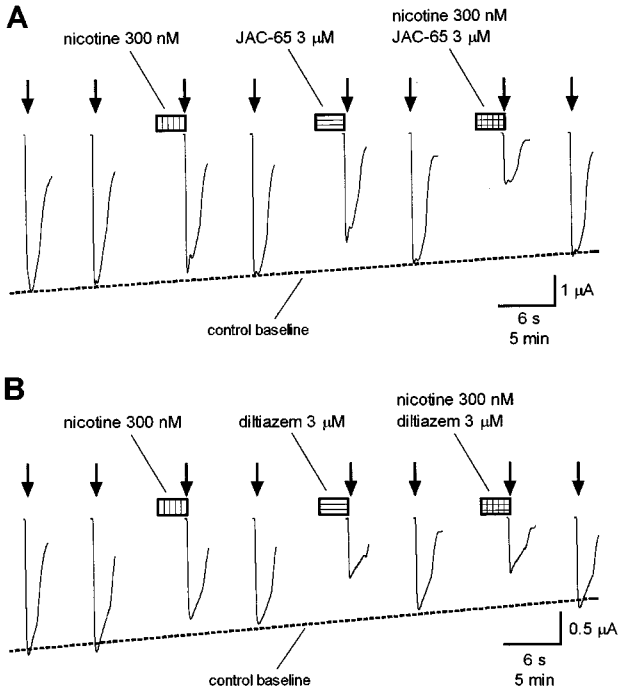

Figure 7 Inward currents evoked by 2-s applications of 1 mM ACh (arrows) in two different oocytes expressing human $\alpha4\beta4$-nAChRs. Holding potential was -80 mV in both experiments. At the times marked by the boxes, oocytes were exposed to 3-min applications of either low concentrations of nicotine (300 nM), 3 μM benzothiazepines (JAC-65 in (A), diltiazem in (B), or both, nicotine and benzothiazepines. Dotted lines in both panels represent the slow decline of the maximal currents evoked in control conditions, whose interpolated values are used to calculate the extent of blockade caused by the drugs, individually or combined. Notice that the time scale is 6 s for the ACh applications (current traces) and 5 min for the intervals between ACh pulses.

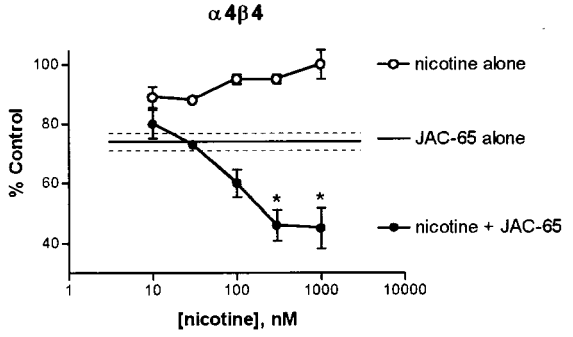

Figure 8 Synergic effects of pre-exposure to nicotine and JAC-65. Data are extracted from experiments similar to that shown in Figure 5A, exposing the $\alpha4\beta4$-nAChRs to different nicotine concentrations, either alone (open circles) or in combination with 3 μM JAC-65. Data are percentages of control peak response (no pre-exposure of drugs) and are means and standard errors of 4–11 oocytes of, at least, two donors. Error bars are shown if larger than symbols. Horizontal lines through the middle of the graphic represent the mean and standard error of the blockade caused by the pre-exposure of 3 μM JAC-65 alone in the same group of experiments (29 oocytes, four donors). *Represents statistically significant differences between the blockade achieved by JAC-65 alone, and those by JAC-65 plus nicotine.

pre-exposure to nicotine and benzothiazepines together does not shown synergic block. This is not surprising in $\alpha3\beta2$-nAChRs, because neither diltiazem nor JAC-65 modified the desensitization time course of these receptors. However, although in $\alpha4\beta2$-nAChRs both diltiazem and JAC-65 promoted a faster desensitization (Figure 2B), no significant enhancement of their blockade is observed when co-applied with nicotine. This same behaviour is observed with diltiazem in $\beta4$*-nAChRs. In contrast, as seen before, the blockade of JAC-65 in $\alpha4\beta4$-nAChRs (blockade of $26\pm3\%$, $n=29$), is significantly enhanced by 300 nM nicotine (blockade of $55\pm7\%$, $n=5$), which only slightly blocks the receptors when applied in the absence of JAC-65 (blockade of $9\pm3\%$, $n=8$). Curiously, the blockade produced by diltiazem alone in these receptors ($45\pm6\%$, $n=6$, Figure 2A) is significantly higher than that produced by JAC-65. The differences in the blocking effect of both benzothiazepines when pre-applied alone are similar to those shown in Figure 2, and can not be attributed to the solution exchange system, because this artifact would overestimate the most the blocking effect of JAC-65.

Discussion

We have studied the effects of two benzothiazepines, diltiazem and JAC-65, on human neuronal nAChRs expressed in the oocyte system with well-defined stoichiometry. In general, both benzothiazepines have mild blocking effects on the peak currents evoked by ACh in five subtypes of neuronal nAChRs, but the observed block can be simply attributed to a combination of a faster decay kinetics and slow solution exchange. Nevertheless, when the action of both drugs are compared in the same receptor subtype, a higher extent of blockade is found with diltiazem in $\alpha7$ and $\alpha4\beta4$ receptors, which can not be accounted for by methodological problems. In any case, the blocking effect attained by the drugs at 3 μM in human receptors is smaller than those previously reported for bovine or rat receptors (Gandia *et al*., 1996; Herrero *et al*., 1999). These quantitative differences might be due to species peculiarities (Peng *et al*., 1994), to the different nicotinic agonists used, or to slower solution exchange system.

However, benzothiazepines show striking effects when desensitization is studied. Both drugs accelerate desensitization during long agonist applications in $\alpha4\beta2$-, $\alpha3\beta4$-, and $\alpha4\beta4$-nAChRs. Herrero *et al*. (1999) have reported that desensitization of rat $\alpha3\beta4$-, but not $\alpha3\beta2$-nAChRs, is facilitated by diltiazem. Here, we have shown that human receptors are more sensitive to diltiazem than rat receptors, and that JAC-65 is significantly more efficient than diltiazem in $\beta4$*-nAChRs.

We have characterized the effect of JAC-65 on desensitization of $\beta4$*-nAChRs in more detail. It not only depends on the drug concentration, but on the ACh concentration as well, because the effect is larger when receptors are challenged with higher ACh concentrations. In contrast, the peak dose-response curves in the presence and in the absence of JAC-65 are roughly scaled, with no significant changes in the EC_{50} values. Taken together, these results argue against any competitive mechanism involved in the blocking effect, but are compatible with a coupling of channel opening with the blocking action, complying with one of the criteria defining the drug class of open-channel blockers (Buisson &

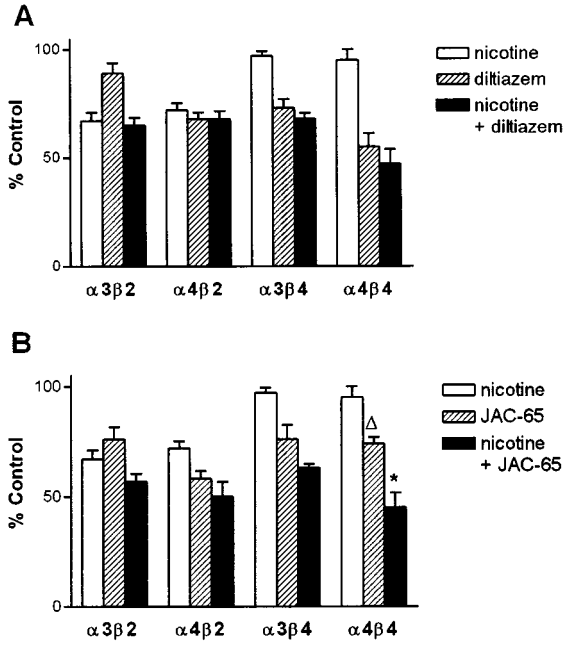

Figure 9 Summary of blocking effects produced in human neuronal nAChRs by the pre-exposure to either low concentrations of nicotine (same data on both A and B, for comparative purposes), diltiazem (A), JAC-65 (B), or a combination of nicotine and diltiazem (A), or nicotine and JAC-65 (B). Experimental procedures were as shown in Figure 5. Nicotine concentrations were 1 μM or 300 nM for α3*- or α4*-nAChRs, respectively. Pre-exposure times were 30 s in $\alpha3\beta2$-nAChRs, 1 min in $\alpha3\beta4$- and $\alpha4\beta2$-nAChRs, and 3 min in $\alpha4\beta4$-nAChRs. Data are percentages of control peak response (no pre-exposure of drugs) and, for each bar, are means and standard errors of 4–29 individual oocytes from at least two donors. *Represents statistically significant differences between the blockade achieved by JAC-65 alone, and that by JAC-65 plus nicotine. Δ Represents statistically significant differences between the blockades achieved by JAC-65 and diltiazem (different panels).

Bertrand, 1998). Nevertheless, other criteria are not complied because the effects produced by JAC-65 are not voltage-dependent, in agreement with other reports studying diltiazem (Herrero *et al*., 1999; Houlihan *et al*., 2000). Although incomplete recovery from desensitization induced by benzothiazepines upon continuous application of nicotinic agonists might indicate trapping block as shown for mecamylamine (Giniatullin *et al*., 2000), in the experiments presented here the recovery was usually complete (except the small fraction of rundown which appeared sometimes, and which did not depend on the presence of benzothiazepines). Moreover, the recovery time course is not affected by the presence of JAC-65 during the pulse, in contrast with results of substance P that suggested a concomitant open channel block (Boyd & Leeman, 1987). However, this possibility could not be totally ruled out without single-channel data. Furthermore, our experiments on recovery from desensitization do not rule out the possibility that the conformational state induced by JAC-65 was different from control, but the results suggest that the process of recovery from this state would not be a rate limiting one. Another interesting issue would be the study of the process of recovery from desensitization as a potential target for benzothiazepine action, but unfortunately our experimental limitations did not allow us to address the question unequivocally.

The effects of diltiazem and JAC-65 are qualitatively similar to those caused by some putative endogenous modulators, such as substance P and somatostatin, in nicotinic receptors of chick sympathetic neurons (Valenta *et al*., 1993), progesterone in α4-containing nicotinic receptors expressed in oocytes (Valera *et al*., 1992), and 5-hydroxytryptamine in muscle nicotinic receptors (Cross *et al*., 1995). Therefore, the action of benzothiazepines could be explained in terms of both drugs favoring the transitions to desensitized states, either from the resting or from the open states, by means of a non-competitive mechanism. However, since the effects of diltiazem and JAC-65 are not qualitatively the same (faster desensitization but less effect, if any, on peak responses with JAC-65), the mechanism of both drugs should differ in spite of being structurally very similar. Such a difference could also account for the quantitative differences of both drugs when coapplied with low concentrations of nicotine.

Because competitive antagonists, such as dihydro-β-erytroidine or methyllycaconitine, but not open-channel blockers, are also capable of inducing upregulation of nicotinic receptors, it seems that is the mere occupancy of the ACh site which triggers upregulation (El-Bizri & Clarke, 1994; Molinari *et al*., 1998; Fenster *et al*., 1999b; Buisson & Bertrand, 2001). This raises the interesting question of whether some non-competitive drugs, such as the benzothiazepines studied here, would have synergic effects with concentrations of nicotine in the smoker's level range (Benowitz *et al*., 1989) in regulating the number of nicotinic receptors. Moreover, benzothiazepines might modify both the addictive properties and the acute tolerance to nicotine's effects. Although the expression of the human nAChRs has been made in frog oocytes and the correspondence with their properties in the native receptors is an open issue, the results presented here might have also more direct clinical implications. As the plasma concentrations of diltiazem could reach the micromolar range during the treatment of cardiovascular diseases with therapeutic doses (Yeung *et al*., 1996), the effects of diltiazem described here, and eventually of JAC-65, might take place in patients. Particularly, diltiazem and JAC-65 can block nAChRs present in human sympathetic ganglia and adrenal medulla (containing α3 and β4 subunits), thus reducing the adrenergic drive present during stress situations. Other effects on the CNS, not easily predicted, could be produced during the treatment with benzothiazepines as these drugs can affect the function of many nicotinic receptor subtypes. For instance, the $\alpha4\beta4$ subtype, which is the most sensitive to the action of benzothiazepines, has been found to be present in chick retina in considerable amount, but the relevance of these findings awaits further investigation (Barabino *et al*., 2001).

Our results also justify previous reports calling for caution when interpreting experiments, which use benzothiazepines as selective pharmacological tools for calcium channels. This could be especially important in systems where the presence of neuronal nAChRs has been demonstrated or is suspected.

In conclusion, our data show that benzothiazepines have evident effects on the decay kinetics of human neuronal nAChRs, being specific of some receptor subtypes. However, the limitations of our experimental setup do not allow to exclude other, additional effects on other receptor conformations, including fast desensitization.

Diltiazem and its novel derivative JAC-65 seem to act differently from each other, also depending on the receptor subtype studied. Performing single-channel experiments could be helpful for understanding in more depth the mechanism of action of benzothiazepines on nAChRs.

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