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# Tricyclic antidepressants inhibit homomeric Cys-loop receptors by acting at different conformational states

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**Abstract**

Tricyclic antidepressants not only inhibit monoamine reuptake but also modulate Cys-loop receptors. However, it is not understood how this modulation is involved in their therapeutic effects. We analyzed the mechanisms of inhibition of homomeric 5-$HT_{3A}$ and α7-5$HT_{3A}$ receptors by tricyclic antidepressants at the single-channel and macroscopic current levels. These drugs reduce agonist-evoked currents in a noncompetitive and concentration-dependent manner. When they act on the open state, the reduction is similar for both receptors and it is voltage-dependent, thus suggesting an open-channel block process in which the blocked channel can either close or remain stabilized. By acting on the resting state, tricyclic antidepressants reduce the peak current in a voltage-independent manner, with a potency 6-fold higher for 5-$HT_{3A}$ than for α7-5$HT_{3A}$ ($IC_{50}$: 6 μM and 1 μM for α7-5$HT_{3A}$ and 5-$HT_{3A}$, respectively). Thus, tricyclic antidepressants may act on closed channels at the unshared extracellular domain from where they inhibit channel opening. Single α7-5$HT_{3A}$ channels in the continued presence of tricyclic antidepressants show: i) reduced open durations, compatible with open-channel block; ii) reduced burst durations, compatible with closing of blocked channels; and iii) reduced frequency of opening events, compatible with both impaired opening and stabilization of a closed state. In summary, our study reveals that tricyclic antidepressants inhibit homomeric Cys-loop receptors by acting through different mechanisms at open and closed conformational states and probably at two different domains, namely, the pore in the open state and the extracellular domain in the closed state.


*Keywords:* Nicotinic receptor; Cys-loop receptors; Tricyclic antidepressant; Noncompetitive inhibitors

## 1. Introduction

Tricyclic antidepressants not only inhibit monoamine reuptake but may also modulate neuronal excitability through interference with Cys-loop receptors. This family of ligand-gated ion channels mediates communication among neurons by the conversion of neurotransmitter signals into a transmembrane flux of ions. Cys-loop receptors are complexes of homologous subunits which co-assemble to form homo- or hetero-pentameric proteins. In vertebrates, members of this family include the nicotinic acetylcholine receptor and the serotonin type 3 receptor (5-$HT_3$), which are permeable to cations, and GABA (A and C) and glycine receptors, which are permeable to anions (Lester et al., 2004). Both neuronal α7 nicotinic receptors and 5-$HT_{3A}$ receptors function as homomeric receptors.

Neuronal nicotinic receptors contribute to a wide range of neurophysiological processes. They are also involved in neurological diseases and they are targets for pharmacological agents (Gotti and Clementi, 2004). Previous studies suggest that nicotinic receptors are involved in major depression and in the effects of antidepressants. Cholinergic hyperactivity has been shown to play a role in the pathophysiology of depression (Janowsky et al., 1972; Shytle et al., 2002). It has been postulated that nicotine may have antidepressant properties and that smokers self medicate the underlying depression (Markou et al., 1998).

5-$HT_3$ receptors are found in the central and peripheral nervous system. They are located predominantly in regions that are involved in the integration of vomiting reflex, pain processing, the reward system and anxiety control (Faerber et al., 2007).

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Presynaptic 5-$HT_3$ receptors are involved in modulating the release of neurotransmitters, such as dopamine, GABA, glutamine, acetylcholine (Chameau and van Hooft, 2006; Faerber et al., 2007). Although the precise physiological role of 5-$HT_{3A}$ receptor is still elusive, it has been shown to modulate certain behavioral functions, including cognition, anxiety, and depression (Costall and Naylor, 2004; Iidaka et al., 2005; Faerber et al., 2007). 5-$HT_3$ receptors may also play a role in alcohol addiction and in the neural responses to drugs of abuse (Grant, 1995; Hodge et al., 2004; Johnson, 2004; McBride et al., 2004; Alex and Pehek, 2007; Faerber et al., 2007).

Several lines of experimental evidence have demonstrated that antidepressants modulate nicotinic and 5-$HT_{3A}$ receptors. Early studies have shown that tricyclic antidepressants act as noncompetitive inhibitors of nicotinic receptors (Schofield et al., 1981; Arita et al., 1987; Rana et al., 1993). We demonstrated that tricyclic antidepressants act at different conformational states of the muscle nicotinic receptor, and that their inhibitory actions are mediated by the enhancement of desensitization and/or by a slow channel blockade (Gumilar et al., 2003). Tricyclic antidepressants also inhibit 5-$HT_{3A}$ currents in a noncompetitive dose-dependent manner (Eisensamer et al., 2003). Fluoxetine, a selective serotonin reuptake inhibitor, has been shown to inhibit membrane currents elicited by the activation of muscle and neuronal nicotinic receptors (García-Colunga et al., 1997), as well as to block native 5-$HT_3$-mediated currents (Choi et al., 2003).

To further contribute to the understanding of the role of these receptors as targets for antidepressants, we investigated the mechanistic bases for their actions on neuronal α7 nicotinic receptor using α7-5$HT_{3A}$ chimeric receptor as a model of α7 extracellular domain (Rayes et al., 2005; Bartos et al., 2006), and on 5-$HT_{3A}$ receptors. We conclude that tricyclic antidepressants inhibit 5-$HT_{3A}$ and α7-5$HT_{3A}$ receptors by acting on open and closed states. Inhibition at each state occurs by the interaction of the drug with a different domain, i.e. the pore in the open state and the extracellular domain in the closed state.

## 2. Materials and methods

### 2.1. Materials

Imipramine hydrochloride, amitriptyline hydrochloride, doxepin hydrochloride, acetylcholine chloride, serotonin chloride were purchased from Sigma Chemical CO (St. Louis, MO).

### 2.2. Expression of α7-5$HT_{3A}$ and 5-$HT_{3A}$ receptors

Although chimeric α7-5$HT_{3A}$ receptors (Eisele et al., 1993) express well in mammalian cells single-channel currents are too small to be detected using the patch clamp. To overcome this limitation, we used the high conductance (HC) form of the α7-5$HT_{3A}$ receptor (α7-5$HT_{3A}$(HC)), which was constructed as described before (Rayes et al., 2005; Bartos et al., 2006). Briefly, three arginine residues responsible for the low conductance (LC) of the serotonin type 3A receptor (5-$HT_{3A}$) were mutated as described by Kelley et al. (2003). We have previously shown that the $EC_{50}$ for activation and the dissociation constant for acetylcholine binding (Kd) for the HC form of α7-5$HT_{3A}$ are similar to those of the LC form, thus revealing that the mutations do not affect significantly the activation properties (Rayes et al., 2005). The same mutations were introduced into the mouse 5-$HT_{3A}$ receptor to increase the peak currents elicited by 5-HT. We refer to the high conductance form of the receptors as 5-$HT_{3A}$(HC).

BOSC-23 (human cell line derived from HEK 293 cells) cells were transfected with the high conductance forms of α7-5$HT_{3A}$ or 5-$HT_{3A}$ cDNAs using calcium phosphate precipitation, as described previously (Bouzat et al., 1994; Bouzat et al., 2000). A plasmid encoding green fluorescent protein (pGreen lantern) was also included to allow identification of transfected cells under fluorescence optics. Cells were used for single-channel measurements 1 or 2 days after transfection.

### 2.3. Patch-clamp recordings

Single-channel currents were recorded in the cell-attached configuration (Hamill et al., 1981) at 20 °C. The bath and pipette solutions contained 140 mM KCl, 5.4 mM NaCl, 0.2 mM $CaCl_2$, and 10 mM HEPES (pH 7.4) (Rayes et al., 2005). Patch pipettes were pulled from 7052 capillary tubes (Garner Glass, CA) and coated with Sylgard (Dow Corning, Midland MI, USA). Single-channel currents were recorded using an Axopatch 200 B patch-clamp amplifier (Molecular Devices), digitized at 5 μs intervals with the PCI-6111E interface (National Instruments, Austin, TX), recorded in the computer hard disk using the Acquire program (Bruxton Corporation, Seattle, WA), and detected by the half-amplitude threshold criterion using the TAC 4.0.10 program (Bruxton Corporation, Seattle, WA) at final bandwidth of 10 kHz (Bouzat et al., 2000). Open- and closed-time histograms were plotted using a logarithmic abscissa and a square root ordinate and fitted to the sum of exponential functions by maximum likelihood using the program TACFit (Bruxton Corporation, Seattle, WA). Bursts of channel openings, each reflecting the activity of a single channel, were identified as a series of closely separated openings preceded and followed by closings longer than a critical duration, which was taken as the point of intersection of the second and third closed component in the closed-time histogram (Rayes et al., 2005).

Macroscopic currents were recorded in the whole-cell configuration for α7-5$HT_{3A}$(HC) and in the outside-out patch configuration for 5-$HT_{3A}$(HC) receptors. The currents of α7-5$HT_{3A}$(HC) recorded from outside-out patches are too small to accurately evaluate the effects of drugs (Rayes et al., 2005). For outside-out patch or whole-cell recordings the pipette solution contained 134 mM KCl, 5 mM EGTA, 1 mM $MgCl_2$, and 10 mM HEPES (pH 7.3). The extracellular solution (ECS) contained 150 mM NaCl, 5.6 mM KCl, 0.5 mM $CaCl_2$ and 10 mM HEPES pH 7.3. The agonist concentrations were 1 mM acetylcholine and 10 μM serotonin (5-HT) for α7-5$HT_{3A}$(HC) and 5-$HT_{3A}$(HC), respectively. These concentrations are ~4-fold higher than the $EC_{50}$ for activation (220 μM acetylcholine

for α7-5HT$_{3A}$(HC) (Rayes et al., 2005) and 2.7 μM 5-HT for 5-HT$_{3A}$(HC) (see below)). By using saturating concentrations of agonist we can ensure that most of the receptors are activated simultaneously and that the decay is mainly given by desensitization.

A series of applications of ECS containing agonist (acetylcholine or 5-HT) was applied to the patch (Liu and Dilger, 1991; Gumilar et al., 2003). The duration of the agonist pulse was 500 ms for α7-5HT$_{3A}$(HC) and 1500 ms for 5-HT$_{3A}$ (HC). We recorded the responses following different protocols: +/−protocol: the patch was exposed 2 min to bath solution containing different concentrations of tricyclic antidepressants before the application of the agonist-containing solution; −/+protocol: pulse of agonist solution containing tricyclic antidepressant without preincubation; +/+protocol: 2-min exposure to bath solution containing a specified concentration of tricyclic antidepressant followed by a pulse of agonist solution also containing tricyclic antidepressant. All currents were referred to those recorded in the same cell in the absence of tricyclic antidepressant (−/−protocol). In these experiments, control currents were also recorded after each protocol to assess recovery of the original peak current. For whole cell and outside-out patch recordings the solution exchange time was estimated by stepping the cell held at 0 mV, in the absence of agonist, during a switch from bath solution to a diluted (1:2 with water) bath solution. Typical times vary between 0.1 and 1 ms.

Macroscopic currents were filtered at 5 kHz. Data analysis was performed using the IgorPro software (WaveMetrics Inc., Lake Oswego, Oregon). The ensemble mean current was calculated for 5–10 individual current traces. Current records were aligned with each other at the point where the current reached 50% of maximum. Peak currents correspond to the value obtained by extrapolation of the decay current to this point. Current from α7-5HT$_{3A}$(HC) are fitted by a single exponential function: $I_{(t)}=I_0$ exp $(-t/\tau_d)+I_\infty$ where $I_0$ and $I_\infty$ are peak and the steady state current values, respectively, and $\tau_d$ is the decay time constant. Currents from 5-HT$_{3A}$(HC) receptors activated by 10 μM 5-HT show two exponential components, similarly to those described before for wild-type 5-HT$_{3A}$ receptors (Mott et al., 2001).

We determined the dose–response relationship for serotonin activation of 5-HT$_{3A}$(HC) receptors in the outside-out patch configuration. Expressing the peak response as a function of serotonin concentration produces a sigmoidal relationship that can be fitted by the Hill equation: $I/I_{max}=1/[1+(EC_{50}/L)^{nH}]$, where $EC_{50}$ is the agonist concentration that elicits the half-maximal response, nH is the Hill coefficient, and $L$ is the agonist concentration. The calculated $EC_{50}$ value for 5-HT$_{3A}$ (HC) is 2.7±0.5 μM (nH=2.5). This value is similar to that reported before for the low conductance form of 5-HT$_{3A}$ (Mott et al., 2001).

The ratio between the current in the presence ($I_{drug}$) and absence of the drug ($I_{cont}$) was plotted as a function of tricyclic antidepressant concentration and the curve was fitted to the Hill equation: $I_{drug}/I_{cont}=IC_{50}^{nH}/(IC_{50}^{nH}+[drug]^{nH})$, where $IC_{50}$ is the concentration of tricyclic antidepressant that produces 50% inhibition of the peak currents.

## 3. Results

### 3.1. Effects of tricyclic antidepressants on single-channel currents of chimeric α7-5HT$_{3A}$(HC) receptors

We first evaluated the effects of tricyclic antidepressants on α7-5HT$_{3A}$(HC) at the single-channel level. Our previous work has shown that the mutations that increase channel conductance do not affect significantly activation properties (Rayes et al., 2005). At the saturating concentration of 1 mM acetylcholine and in the absence of tricyclic antidepressants, channel activity occurs in bursts of 4–6 closely-spaced openings together with isolated events (Rayes et al., 2005) (Fig. 1). The open time distributions are fitted by 3 components whose mean durations and relative areas are: $\tau_1=114\pm8$ μs (0.410±0.05), $\tau_2=1.1\pm0.3$ ms (0.160±0.01), and $\tau_3=6.9\pm1.0$ ms (0.430±0.07) (Fig. 2). 10 μM

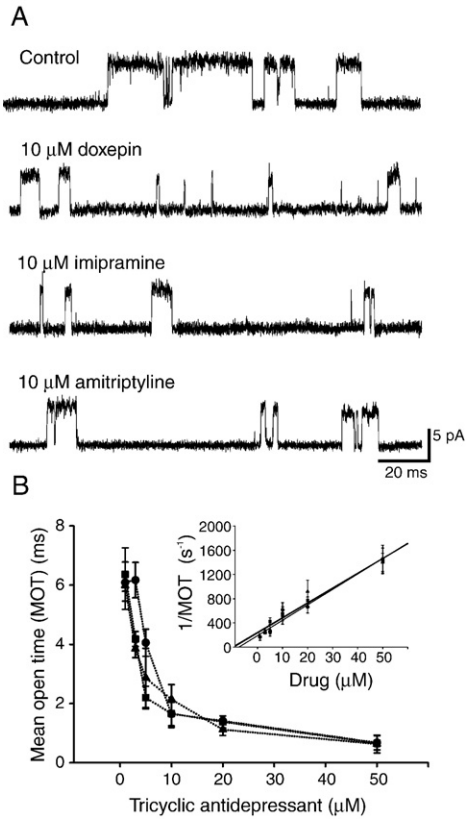


Fig. 1. Effects of tricyclic antidepressants on single α7-5HT$_{3A}$(HC) channel currents. A. Channels were recorded in the cell-attached configuration in the absence and presence of 10 μM doxepin, imipramine, or amitriptyline. Acetylcholine concentration: 1 mM. Membrane potential: −70 mV. Filter 10 kHz. B. Relationship between the mean open time and the concentration of tricyclic antidepressant. The mean open times were obtained from the corresponding open time histograms and correspond to the slowest component. Data are shown as mean ± S.D. of 5–10 patches for each condition. The inset shows the relationship between the inverse of the mean open time and tricyclic antidepressant concentration. The data are fitted by equation (1/mean open time)=$\alpha+k_{+b}$ [tricyclic antidepressant], where $k_{+b}$ is the association rate for channel block and α is the apparent channel closing rate. Symbols correspond to: (●) imipramine, (■) doxepin and (▲) amitriptyline.

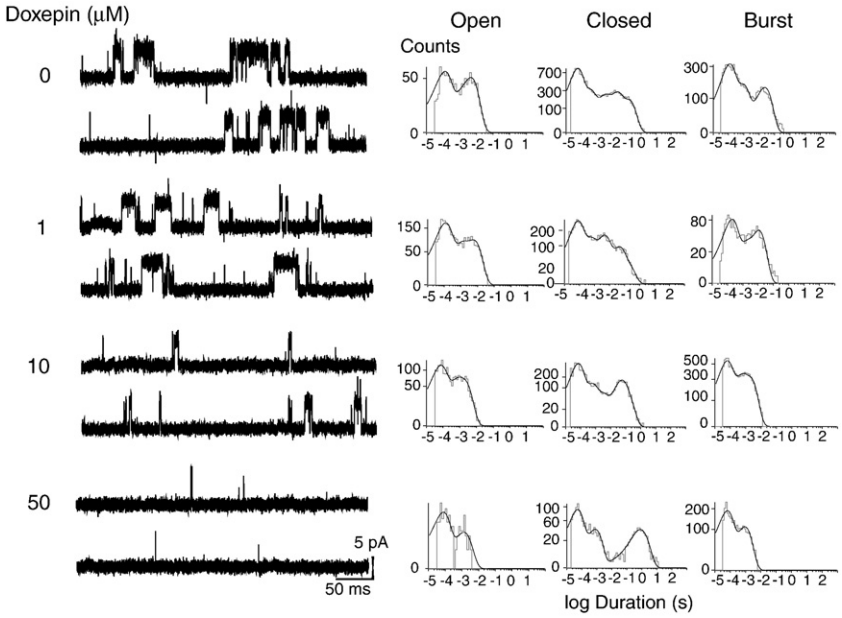


Fig. 2. Concentration-dependent effects of doxepin on single-channel currents of α7-5HT$_{3A}$(HC). Left: channels were recorded in the absence or presence of doxepin in the pipette solution. Acetylcholine concentration: 1 mM. Membrane potential: −70 mV. Filter: 10 kHz. Right: open, closed, and burst duration histograms corresponding to each condition.

amitriptyline, imipramine or doxepin, significantly alter the kinetics but not the amplitude of the channels (Fig. 1A).

A significant concentration-dependent decrease in the open channel duration is observed in the presence of all tricyclic antidepressants (Figs. 1 and 2). The duration of the slowest open component is 10- to 15-fold briefer in the presence of 50 μM tricyclic antidepressant (Figs. 1B and 2). The concentration-dependent decrease in the mean open time is indicative of an open-channel blockade. If we simplify the kinetics of activation of α7-5HT$_{3A}$(HC) receptors (Rayes et al., 2005) to the simplest linear blocking scheme, where in C the channel is closed, O corresponds to the slowest open component and OB to the blocked state, a linear relationship between the reciprocal of the mean open time and tricyclic antidepressant concentration ([B]) is observed (Fig. 1B). The estimated value for the forward rate constant of the blocking reaction ($k_{+b}$ in Scheme 1), given by the slope of the curve, is $25.5\times10^6$, $24.4\times10^6$ and $25.5\times10^6$ $M^{-1}$ $s^{-1}$ for doxepin, amitriptyline, and imipramine, respectively.

Tricyclic antidepressants reduce the duration of bursts of openings (Fig. 2). At 10 μM, doxepin reduces the mean burst duration from 15.6±2.5 ms to 3.2±0.15 ms. As shown in the figure for doxepin, the drugs also displace to longer durations the closed time distributions of the entire recording. Within bursts, closed time distributions of the control chimeric receptor

$$C \underset{k_{-b}}{\overset{k_{+b}\,[B]}{\rightleftharpoons}} O \underset{k_{-b}}{\overset{k_{+b}\,[B]}{\rightleftharpoons}} OB$$

Scheme 1.

are well described by the sum of two exponentials (35±10 μs and 550±210 μs), indicating brief and long closings within each burst (Rayes et al., 2005). The brief component is the predominant one, being its relative area of 0.71±0.04. At 10 μM doxepin, closed time histograms are also fitted by two components, but the major component shows a mean duration of 545±28 μs (area=0.66±0.08) and the minor one corresponds to brief closings (41±8 μs, area=0.34±0.07).

Taken together, the results show that under the continued presence of tricyclic antidepressants, the open and burst durations decrease and the duration of dwells in closed states increases. All this will significantly reduce the probability of finding the channel in the open state.

### 3.2. Tricyclic antidepressants reduce the frequency of a7-5HT$_{3A}$ (HC) opening events

To analyze the effects of antidepressants on the frequency of openings we recorded channels 1 and 8 min after seal formation from cell-attached patches using a pipette in which the tip was filled with 1 mM acetylcholine and the shaft was filled with 1 mM acetylcholine and 10 or 20 μM doxepin. Under these conditions, rapid sealing allowed us to record channel activity in the absence and presence of doxepin on the same patch (Benoit and Changeux, 1993; Spitzmaul et al., 2001). During the first minute, channel activity is similar to that observed in the absence of doxepin (Fig. 3A). After a few minutes, a dramatic decrease in channel activity is observed because of the diffusion of doxepin to the tip of the pipette. The frequency of opening events decreases more than 50% and 90% at 10 and 20 μM doxepin, respectively (Fig. 3B). In contrast, the frequency of openings in control recordings decreases only about 20% (18±13%), probably due to desensitization.

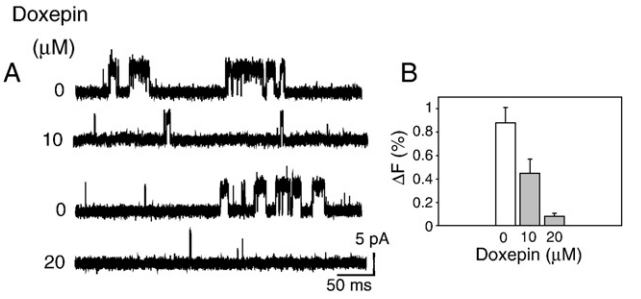


Fig. 3. Effect of tricyclic antidepressants on the frequency of opening events. A. α7-5HT$_{3A}$(HC) channels were recorded using a pipette containing 1 mM acetylcholine in the tip and 1 mM acetylcholine plus 10 or 20 μM doxepin in the shaft. Channels were recorded immediately after seal formation and 8 min later. Membrane potential: −70 mV. Filter 10 kHz. B. Decrease of the frequency of opening events in the presence of doxepin. Δ$F$ is the ratio between the frequency of opening events (events per second) recorded 8 min and 1 min after seal formation. Data are shown as mean±SD.

### 3.3. Effects of antidepressants on macroscopic responses of a7-5HT$_{3A}$(HC)

To further study the mechanism of inhibition of α7-5HT$_{3A}$ (HC) by antidepressants we evaluated the effects of drugs on macroscopic currents activated by 1 mM acetylcholine.

We determined the state-dependence of drug action by using different perfusion protocols (see Methods). Tricyclic antidepressants were applied 2 min before acetylcholine (+/−protocol), together with acetylcholine (−/+protocol), or before and together with acetylcholine (+/+protocol). All currents were referred to those recorded in the same cell in the absence of antidepressants.

To determine if the drugs interact with the resting closed state of α7-5HT$_{3A}$(HC), we recorded currents from cells previously exposed to doxepin (+/−protocol). A concentration-dependent reduction of the peak current is observed (Fig. 4A and C). The IC$_{50}$ calculated for the inhibition is 6.5±1.9 μM (nH=1.1, $n$=4). After less than 30-s wash with bath solution the peak currents can be completely recovered (91%, $n$=17), thus indicating that the inhibition is reversible (Fig. 4A). No changes in the decay time constant occur due to preincubation with doxepin (Fig. 4A).

When doxepin is present only in the acetylcholine solution (−/+protocol), a concentration-dependent decrease in the peak current is also observed (Fig. 4B). The IC$_{50}$, is 13.2±4.4 μM (nH=1.34, $n$=4) (Fig. 4C). Interestingly, the original peak currents cannot be recovered even after a 15-min wash with bath solution. At doxepin concentrations lower than 20 μM, decays are well fitted by a single exponential function and no significant changes in current decay rates are observed (Fig. 4B). However, at higher drug concentrations (50 μM) a 20% decrease in the decay time constant is observed.

Because imipramine has been shown to change the decay rate of macroscopic 5-HT$_{3A}$ currents (Eisensamer et al., 2003), we evaluated if this could also occur in α7-5HT$_{3A}$(HC) currents. The decrease of the peak current produced by imipramine (−/+protocol) is quantitatively similar to that produced by doxepin (43±5% and 51±7% at 10 and 20 μM imipramine, respectively). We determined that 10 and 20 μM imipramine also produce a decrease of the decay time constant of 17±2% and 25±5%, respectively. Therefore, imipramine increases the current decay rate from the open state at lower concentrations than doxepin.

When the patch is continuously exposed to 10 μM doxepin (+/+protocol), the peak inhibition (65%) is greater than that observed with the other protocols. However, the percentage of current inhibition under this protocol is not completely additive with respect to that observed when the drug acts only at the closed (+/−protocol) and open (−/+protocol) states. Recovery of only 50% of the current is achieved, as observed in the −/+protocol (Fig. 4B).

In summary, tricyclic antidepressants act on both open and closed states of α7-5HT$_{3A}$(HC), being the peak reduction greater when the drugs act on the resting channels. The effects on closed resting channels are reversible whereas those on open channels are irreversible.

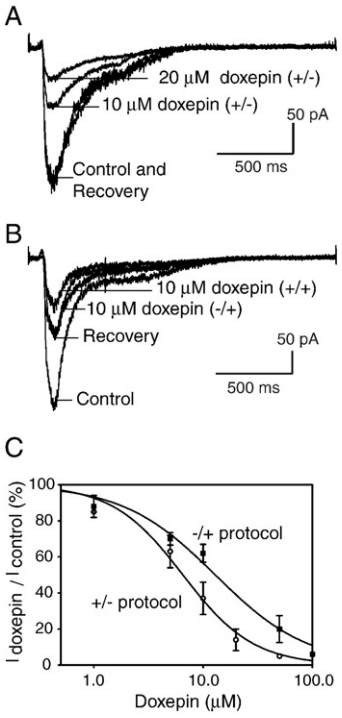


Fig. 4. Effects of doxepin on macroscopic currents from α7-5HT$_{3A}$(HC) receptors. Currents activated by 1 mM acetylcholine were exposed to 10 μM doxepin under different protocols. Membrane potential: −50 mV. Filter 5 kHz. A. Ensemble mean α7-5HT$_{3A}$(HC) currents obtained from whole-cell patches preincubated in the absence (control, −/−protocol) or presence of doxepin (+/−protocol). 90–95% of the control peak currents are recovered after 30-s wash. Each trace represents the average of 5 to 10 applications of agonist. B. Curves correspond to control conditions (−/−protocol), simultaneous acetylcholine/doxepin application without preincubation (−/+protocol) and simultaneous acetylcholine/doxepin application with preincubation with doxepin (+/+protocol). Peak currents cannot be recovered after 15-min wash with buffer. C. Dose–response curves for the inhibition of α7-5HT$_{3A}$(HC) peak currents by doxepin applied under different protocols. 100% corresponds to the peak current in the absence of doxepin. Data are fitted by the Hill equation. Each point corresponds to 3–10 experiments.

### 3.4. Noncompetitive inhibition of a7-5HT$_{3A}$(HC) by tricyclic antidepressants

To explore further the mechanism of inhibition by antidepressants we compared the reduction by doxepin of the peak currents in the open state (−/+protocol) at different acetylcholine concentrations (1 and 2 mM). The reduction of the peak current does not change with the 2-fold increase in agonist concentration (42±11% and 47±7% at 1 mM and 2 mM acetylcholine, respectively). These results show that doxepin act on α7-5HT$_{3A}$(HC) in a manner consistent with a noncompetitive mechanism. This observation is in great agreement with previous studies of the actions of different types of antidepressants on *Torpedo*, muscle and neuronal nicotinic receptors (Eldefrawi et al., 1981; Schofield et al., 1981; García-Colunga et al., 1997; García-Colunga and Miledi, 1999; López-Valdez et al., 2002; Gumilar et al., 2003).

### 3.5. Effects of tricyclic antidepressants on α7-5HT$_{3A}$(HC) as a function of membrane potential

We examined the voltage-dependence of the inhibitory effects of doxepin on α7-5HT$_{3A}$(HC) currents elicited by acetylcholine. As described before, the control current–voltage relationship shows slight inward rectification (Bouzat et al., 2004). In the presence of acetylcholine plus 10 μM doxepin (−/+protocol) the inhibition of the peak current is about 40% at a membrane potential of −50 mV. In contrast, the current is only slightly reduced at positive membrane potentials (15%) (Fig. 5). This voltage dependence supports an open-channel block mechanism. In contrast, when the drug acts on the closed state, evaluated by application of doxepin under the +/−protocol, the voltage dependence is not evident: the decrease of the peak is similar when 10 μM doxepin acts at positive and negative membrane potentials (about 60% in both cases) (Fig. 5B). Similar results were observed for the action of imipramine (data not shown).

The voltage-dependence differences in the effects of tricyclic antidepressants acting at different conformational states suggest that the drug acts at different sites.

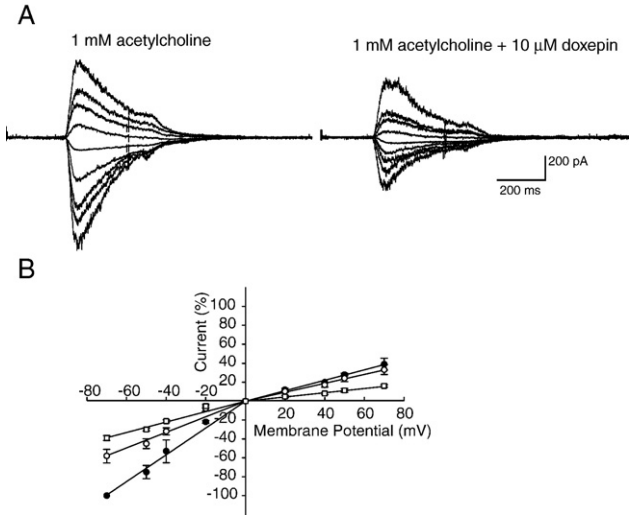


Fig. 5. Effect of membrane potential on the inhibition of α7-5HT$_{3A}$(HC) currents. A. α7-5HT$_{3A}$(HC) currents recorded at various membrane potentials (−90, −70, −50, −40, −20, +20, +40, +50, +70 mV) in the absence (Left) or presence of 10 μM doxepin (Right). Each trace represents the average of 5–10 applications of 1 mM acetylcholine. Protocol application: −/+. B. Current–voltage relationships for α7-5HT$_{3A}$(HC) currents in the absence (●, −/−protocol), or presence of 10 μM doxepin either in the acetylcholine solution (○, −/+protocol) or in the preincubation solution (□, +/−protocol). Currents were normalized to the control current at −70 mV. Data are expressed as mean±S.D. Each point corresponds to the average of 5 experiments.

### 3.6. Effects of antidepressants on 5-HT$_{3A}$(HC) receptors

To elucidate whether the different effects of antidepressants on the closed and open states are mediated by interactions with different domains, we determined their actions at 5-HT$_{3A}$ receptors. To increase 5-HT-activated currents we used the high conductance form of the 5-HT$_{3A}$ receptor (Kelley et al., 2003). As described in Materials and methods, the EC$_{50}$ for activation of 5-HT$_{3A}$(HC) by 5-HT is similar to that reported for wild-type 5-HT$_{3A}$ receptors (Mott et al., 2001). Peak currents of 5-HT$_{3A}$(HC) are reached within tens of milliseconds (20–80% rise time 15±6.8 ms) in response to the rapid application of 10 μM serotonin, as described for wild-type 5-HT$_{3A}$ (Mott et al., 2001). 5-HT$_{3A}$(HC) currents decay with a dual exponential time course whose time constants are: $\tau_{fast}$ 150±30 ms (area 81%) and $\tau_{slow}$ 942±400 ms (Fig. 6A). These values are similar to those reported for wild-type 5-HT$_{3A}$ (Mott et al., 2001). Therefore, activation properties of 5-HT$_{3A}$(HC) are similar to those of wild-type 5-HT$_{3A}$ receptors.

When 10 μM doxepin is applied together with the agonist (−/+protocol), 5-HT$_{3A}$(HC) currents decrease about 40% with no changes in the decay rates ($\tau_{fast}$: 140±30 ms and $\tau_{slow}$: 810±540 ms). The currents reach the peak amplitude in 21.6±6.8 ms, similarly to the control. Fig. 6B shows that doxepin decreases the peak current in a concentration-dependent manner. The IC$_{50}$ calculated under this condition (−/+protocol) is 14.7±2.7 μM. As shown for α7-5HT$_{3A}$ receptors, the original peak current cannot be recovered after the 15-min wash. However, if 150-ms pulses of agonist are applied every 2 min during the wash, partial recovery is achieved (72±8% of the original current, $n$=5). This suggests that, to some extent, the drug is trapped in the pore. However, full recovery of control currents is not achieved.

As described for α7-5HT$_{3A}$(HC), we evaluated if imipramine increases the current decay rate of 5-HT$_{3A}$(HC) receptors. When 10 or 20 μM imipramine is applied together with serotonin a reduction of the peak current is observed, being this reduction similar to that produced by doxepin (46±9% and 61±10% at 10 and 20 μM imipramine, respectively). However, in contrast to doxepin, a significant increase in the decay rate is observed. The decay time constants of both components decreased about 50% with respect to the control. At 10 μM imipramine: $\tau_{fast}$=74±40 ms (area: 85±1%) and $\tau_{slow}$=590±100 ms ($n$=5); and at 20 μM: $\tau_{fast}$=66±36 (area: 83±1%) and $\tau_{slow}$=517±77 ms ($n$=5).

To analyze the action of doxepin on the closed state of the 5-HT$_{3A}$(HC) receptor, we exposed the patch to doxepin

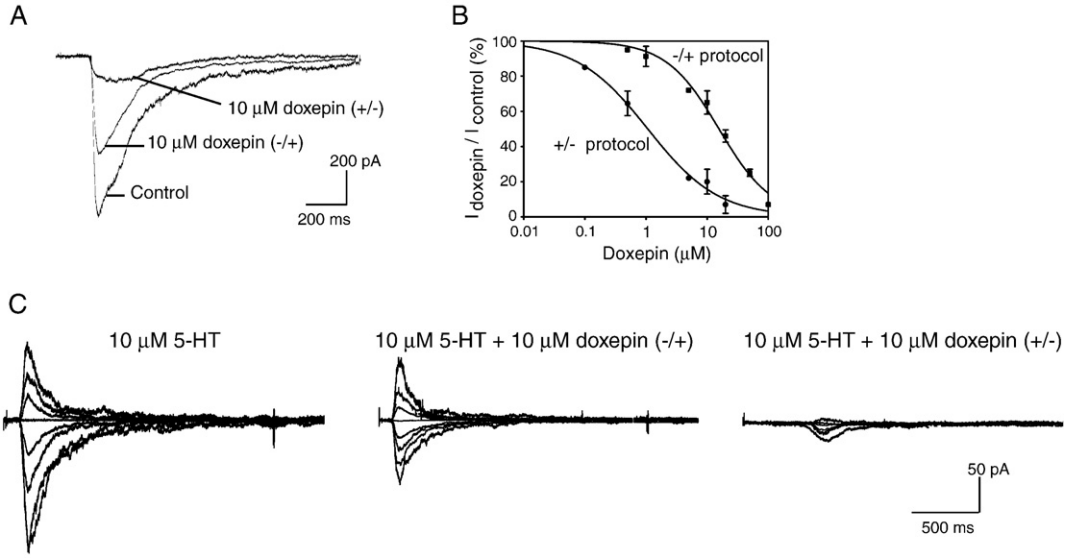


Fig. 6. Effects of doxepin on 5-$HT_{3A}$(HC) currents. Currents recorded from outside-out patches were elicited by 10 μM serotonin and exposed to doxepin using different protocols. A. 10 μM doxepin was applied together with the serotonin solution (−/+protocol) or 2 min before the agonist (+/−protocol). Membrane potential: −50 mV. B. Dose–response curves for the inhibition of 5-$HT_{3A}$(HC) by doxepin. Peak currents were measured in the presence of different doxepin concentrations applied before (+/−protocol) or together with the agonist (−/+protocol). Each point corresponds to 3–10 experiments. C. 5-$HT_{3A}$(HC) currents recorded at different membrane potentials in the absence or presence of 10 μM doxepin. Each trace represents the average of 5–10 responses to 10 μM 5-HT recorded at −70, −50, −40, −20, 0, +20, +50, +70 mV. Left: Control (−/−protocol). Middle: Activation with 5-HT plus 10 μM doxepin (−/+protocol). Right: Preincubation with 10 μM doxepin followed by activation with 10 μM 5-HT (+/−protocol).

during 2 min and then recorded currents activated by 5-HT (+/−protocol). Again, the peak current is reduced as a function of doxepin concentration (Fig. 6A and B). The $IC_{50}$ value determined under this drug-application protocol is 1.1 ± 0.5 μM ($n$ = 5). At 10 μM doxepin the reduction is 80 ± 7.0% and full recovery of the current is achieved after a 30-s wash, showing that the inhibition is reversible. Interestingly, the peak current is reached significantly later than in the absence of drug (rise time = 102 ± 11.8 ms), suggesting that channel opening is impaired (Fig. 6A). To further corroborate that channel opening is impaired, we determined the $EC_{50}$ for 5-HT activation in outside-out patches incubated with 1 μM doxepin before application of the agonist (+/− protocol). The agonist response curve is shifted to the right (data not shown), and the $EC_{50}$ for 5-HT increases from 2.7 μM in the absence of doxepin to 10.7 μM for receptors preincubated with doxepin.

To determine if doxepin is acting at the binding site, which could result in an increase of the rise time, we increased 10-fold serotonin concentration (100 μM) and recorded currents after preincubation with 10 μM doxepin. No changes in the rise time ($t_{20-80}$ = 95.6 ± 9.8 ms) nor in the reduction of the peak current (85 ± 6.0%) were observed with respect to currents activated by 10 μM 5-HT from patches previously exposed to 10 μM doxepin. Also, 10 μM doxepin applied together with the agonist (−/+protocol), produces the same inhibition of currents elicited by 10 or 100 μM 5-HT (46 ± 9% and 44 ± 7% at 10 μM and 100 μM 5-HT, respectively). Therefore, as reported for tricyclic antidepressants on 5-$HT_{3A}$ (Eisensamer et al., 2003), the inhibition of 5-$HT_{3A}$(HC) receptors by doxepin and other tricyclic antidepressants is not due to competitive antagonism.

### *3.7. Effects of tricyclic antidepressants on 5-$HT_{3A}$(HC) as a function of membrane potential*

We determined the effects of doxepin on 5-$HT_{3A}$(HC) currents as a function of membrane potential. In the presence of 5-HT plus 10 μM doxepin (−/+protocol) the reduction of the peak current is about 40% at a membrane potential of −50 mV but only 10% at +50 mV (Fig. 6C). As described for α7-5$HT_{3A}$ (HC), this voltage-dependence supports an open-channel block mechanism. In contrast, when tricyclic antidepressant acts on the closed state, which is evaluated by the application of doxepin under the +/−protocol, the effects are independent of membrane potential. The decrease of the peak current at 10 μM doxepin is similar at +50 mV and −50 mV (about 80% in both cases) (Fig. 6C). Similar results were observed for the action of imipramine (data not shown). Again, the differences in voltage dependence may indicate that tricyclic antidepressants act by two different mechanisms on the different conformational states.

## 4. Discussion

Several antidepressant drugs have been shown to act as noncompetitive inhibitors of nicotinic receptors and 5-$HT_{3A}$ receptors. Although the results of this modulation are not completely understood, new lines of evidence give support to the role of Cys-loop receptors in depression and antidepressant actions (Shytle et al., 2002; Caldarone et al., 2004; Dremencov et al., 2006). Therefore, elucidating the molecular mechanisms of their modulation by antidepressants is of great importance. We used the high conductance forms of α7-5$HT_{3A}$ and 5-$HT_{3A}$ as models of homomeric Cys-loop receptors to gain new insights

into the mechanisms of action of tricyclic antidepressants. The introduction of mutations at the intracellular domain to increase channel conductance does not affect significantly the activation properties of α7-5HT$_{3A}$ chimeric receptors (Rayes et al., 2005) and, as shown in this study, of 5-HT$_{3A}$ receptors.

We here show that tricyclic antidepressants inhibit channel activity of homomeric α7-5HT$_{3A}$(HC) and 5-HT$_{3A}$(HC) receptors in a noncompetitive manner. Tricyclic antidepressants act on these receptors at two different domains in open and closed states, and the interaction with each domain mediates reduction of channel activity by different molecular mechanisms.

The actions of tricyclic antidepressants at the open state support an open-channel block mechanism. The effects are similar for both receptors: they decrease the peak current with similar potencies and in a voltage-dependent manner. Given that the receptors share the transmembrane region, this similarities strongly suggest that tricyclic antidepressants interact with this domain, which would be expected if they block the 5-HT$_{3A}$ pore. In agreement with this, doxepin, imipramine and amitriptyline predominate in their positively charged form at physiological pH, and a mechanism of open-channel block includes voltage dependence. Although in a fast open-channel blockade mechanism a two-component decay time course is commonly observed (Dilger et al., 1997; Forman 1999), a decrease in peak amplitudes may be observed if the drug association rate is similar compared to the rate for channel opening, and therefore current block develops when the channels begin to open. The reduction of the peak current is not fully reversible, thus suggesting that the drug dissociates slowly from the pore. The fact that partial recovery of 5-HT$_{3A}$ currents is achieved when the channel is allowed to open during the wash shows that the drug is indeed trapped in the pore.

In addition to the decrease of the peak current, doxepin increases the decay rate of macroscopic currents but this effect is observed at concentrations higher than 50 μM. For imipramine, concentrations higher than 10 μM produce a clear decrease of the decay time constant of both α7-5HT$_{3A}$(HC) and 5-HT$_{3A}$(HC) receptors. The increase in the current decay rates may be explained by both open channel block with a slow unblocking rate or increase desensitization (Papke and Oswald, 1989; Gumilar et al., 2003). Distinguishing between these two mechanisms is very difficult. A decrease of different magnitude in the decay time constants of 5-HT$_{3A}$ currents was reported in the presence of imipramine, desipramine, trimipramine, and fluoxetine (Choi et al., 2003; Eisensamer et al., 2003). The authors explained the mechanism of the acceleration of current decay by an increase of desensitization rate. For the muscle nicotinic receptor, we have previously determined that doxepin increases the decay rate in a concentration-dependent manner, and that this effect is compatible with increase desensitization or slow open channel block (Gumilar et al., 2003).

The potency for inhibition of the peak current is greater when tricyclic antidepressants act on the closed state than in the open state. A similar finding was reported for the inhibition of 5-HT$_3$-currents from NCB-20 cells by fluoxetine (Choi et al., 2003). By acting at the closed state, tricyclic antidepressants reduce the peak currents of both receptors with distinct potencies. The inhibition is 6-fold higher for 5-HT$_{3A}$(HC) than for α7-5HT$_{3A}$(HC). This result supports that the effects on the closed state are mediated by a domain which is not shared by both receptors, thus evidencing that this binding site may be located at the extracellular domain. In agreement with this, the current inhibition produced by preincubation with doxepin is not voltage dependent, thus discarding a transmembrane region as the site of interaction. The inhibition by tricyclic antidepressants acting at the closed state may arise from block of closed channels, increase in the desensitization of resting channels or from a change in the equilibrium between resting and desensitized unliganded states by selective binding to the latter state. Any of these mechanisms will result in reduced currents. The significant increase in the rise time of 5-HT$_{3A}$(HC) receptors together with the increase in the EC$_{50}$ for 5-HT activation confirm that channel opening is clearly impaired. Because the current is rapidly recovered, it is probable that desensitization is not taking place.

The use of the high conductance form of α7-5HT$_{3A}$ allowed us to provide detailed information about the molecular actions of drugs (Bartos et al., 2006). Because in the cell-attached mode channels are exposed to the continued presence of tricyclic antidepressant, the effects at the single-channel will correspond to the combined effects on both the open and closed states. Tricyclic antidepressants reduce in a concentration-dependent manner the duration of the open state without any apparent flickering or change in channel conductance. Such changes can be explained by a slow channel block. We have previously determined that α7-5HT$_{3A}$(HC) activation is best described by a cyclic scheme (Rayes et al., 2005) and therefore Scheme 1 is a simplification that accounts only for the main open component. Nevertheless, the value for the forward rate constant of the blocking process estimated for α7-5HT$_{3A}$(HC) on the basis of the simplest scheme is in the range of that determined for other blocking drugs at the nicotinic receptor (Arias et al., 2006). However, the decrease in burst duration as a function of tricyclic antidepressant concentration is not compatible with a classical open channel blocking mechanism, as that described in Scheme 1, because the model predicts that the duration of the bursts should increase linearly with blocker concentration. The lack of increase of burst duration may be due to the fact that the channel enters long-lived closed states. Due to this, we propose that once blocked by tricyclic antidepressant, α7-5HT$_{3A}$(HC) is able to close. This mechanism, in turn, agrees with the macroscopic recordings that show that the effect is not fully reversible when the drug acts at the open state. Closure of blocked nicotinic receptor channels has been suggested for the actions of other noncompetitive blockers (Bouzat and Barrantes, 1996; Dilger et al., 1997; Arias et al., 2006).

In addition to the decreased open duration, a dramatic decrease of the frequency of opening events is observed at the single-channel level. The decrease is probably mediated by the drugs acting on both open and closed states. On the open state, tricyclic antidepressants may stabilize the receptor in a closed-blocked state, from which reopening is inhibited. On the closed state, tricyclic antidepressants may inhibit channels in the resting state, preventing them from opening. The fact that the effects are

qualitatively similar for both receptors suggests that the mechanism of inhibition is conserved among members of the Cys-loop receptor superfamily.

There are uncertainties regarding clinically available concentrations of antidepressants in the CNS. Therapeutic concentrations of tricyclic antidepressants attainable in blood are in order of 1 μM (Clarke, 1986) but concentrations of antidepressants and/or their metabolites in the brain can be as much as 20-fold higher (Schmalzing, 1988; Renshaw et al., 1992). These concentrations would be high enough to alter function of homomeric Cys-loop receptors.

The elucidation of the molecular mechanisms of the effects of tricyclic antidepressants on Cys-loop receptors may eventually contribute to the development of novel therapeutic agents for treating depression or understanding secondary effects in patients treated with these drugs.

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