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Molecular mechanisms of inhibition of nicotinic acetylcholine receptors by tricyclic antidepressants

Fernanda Gumilar [a], Hugo R. Arias [b], Guillermo Spitzmaul [a], Cecilia Bouzat [a,*]

[a] *Instituto de Investigaciones Bioquímicas de Bahía Blanca, Universidad Nacional del Sur, CONICET, Camino La Carrindanga Km 7, B8000FWB Bahía Blanca, Argentina*
[b] *Department of Pharmaceutical Sciences, Western University of Health Sciences, Pomona, CA, USA*

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Abstract

In addition to their well known actions on monoamine reuptake, tricyclic antidepressants have been shown to modulate ligand-gated ion channels (LGICs). Since the muscle nicotinic acetylcholine receptor (AChR) has been the model for studying structure–function relationships of LGICs, we analyzed the action of tricyclic antidepressants on this type of AChR at both single-channel and macroscopic current levels. We also determined their effects on ACh equilibrium binding and their interactions with the different conformational states of the AChR.

Antidepressants produce a significant concentration-dependent decrease in the duration of clusters of single-channels (eight fold at 20 μM). They also decrease the peak amplitude and increase the decay rate of currents elicited by rapid perfusion of ACh to outside-out patches. In equilibrium binding assays, antidepressants promote the typical high-affinity desensitized state and inhibit binding of [piperidyl-3,4-^{3}H (*N*)]-(*N*-(1-(2-thienyl)cyclohexyl)-3,4-piperidine ([^{3}H]TCP) to its locus in resting and desensitized AChRs. The results indicate that tricyclic antidepressants: (i) interact with resting (closed), open, and desensitized channels; (ii) do not affect significantly channel opening and closing rates; (iii) do not act as fast open-channel blockers; (iv) inhibit activation of resting channels; and (v) may increase the rate of long-lived desensitization from the open state.

Keywords: Acetylcholine; Tricyclic antidepressant; Nicotinic receptor; Noncompetitive inhibitor

1. Introduction

The nicotinic acetylcholine receptor (AChR) is the paradigm of the ligand-gated ion channel superfamily. The muscle-type AChR is a pentamer of homologous subunits with composition $\alpha_2\beta\varepsilon\delta$ (in adult muscle) or $\alpha_2\beta\gamma\delta$ (in embryonic muscle and *Torpedo* electric organ). The AChR exists in at least three different functional states: resting (closed but activatable), active (open), and desensitized (closed). The transitions between these states are affected by both agonists and competitive antagonists, acting at the neurotransmitter binding sites, as well as by a broad class of compounds named noncompetitive inhibitors (NCIs). NCIs bind to different sites within the ion channel (luminal sites) or to distinct nonluminal domains (reviewed in Arias, 1998; Arias et al., 2002a). In general, NCIs decrease the probability of channel opening by different mechanisms: (1) steric blockade of the ion pore; (2) allosteric inhibition; and/or (3) enhancement of desensitization. The relationships between the chemical structures of NCIs and their sites of interaction and mechanisms of action are not yet fully understood.

Early studies have shown that tricyclic antidepressants, such as imipramine (Arita et al., 1987), amitriptyline (Schofield et al., 1981), and desipramine (Rana et al., 1993) act as NCIs of AChRs. García-Colunga et al. (1997) reported that fluoxetine, a selective serotonin reuptake inhibitor (SSRI), inhibits membrane currents elicited by activation of both embryonic muscle and neuronal AChRs in a noncompetitive manner, either by increasing the rate of desensitization and/or by inducing channel blockade. More recently, several SSRIs have also been shown to block $^{86}Rb^+$ efflux elicited by muscle

* Corresponding author. Fax: +54-291-4861200.
E-mail address: inbouzat@criba.edu.ar (C. Bouzat).

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and neuronal AChR activation (Fryer and Lukas, 1999). These results suggest possible roles for neuronal AChRs as targets for the clinical use of antidepressants.

Here, we investigate the mechanistic bases for the noncompetitive action of three members that are representative of the tricyclic antidepressant group: amitriptyline, imipramine and doxepin. Together with their *N*-demethylated derivatives, they are the most common tricyclic antidepressants used today for the treatment of major depression. These antidepressants share a common scaffold composed of a tricyclic core, a side chain, and a tertiary amine. However, they show slight differences in their structures, in their potencies to inhibit the reuptake of serotonin and noradrenaline and in their anticholinergic potencies (Baldessarini, 1996). Muscle-type AChRs have two features that make them a good model for the study of the mechanism of action of antidepressants: these receptors can be expressed at high density on mammalian cells, and they have relatively slower desensitization kinetics with respect to neuronal AChRs. The use of the muscle-type AChR allowed us to describe, for the first time, the kinetic changes at the single-channel level. In addition, it was possible to examine in detail the kinetic properties of macroscopic currents elicited by rapid perfusion of acetylcholine (ACh) to outside-out patches in the presence of antidepressants. The interaction of antidepressants with different conformational states of the AChR was investigated by determining the inhibition of [piperidyl-3,4-^{3}H (*N*)]-(*N*-(1-(2-thienyl) cyclohexyl)-3,4-piperidine) ([^{3}H]TCP) binding to *Torpedo* AChR native membranes in both resting and desensitized states. [^{3}H]TCP is a structural and functional analog of the high-affinity NCI phencyclidine (PCP), and binds with high-affinity to both desensitized (Pagán et al., 2001) and resting AChRs (Arias et al., 2002a,b).

We conclude that the inhibitory action of tricyclic antidepressants on the AChR is exerted through both closed and open channels and it is mediated by the enhancement of desensitization and/or by a slow channel blockade.

2. Materials and methods

2.1. Materials

[Piperidyl-3, 4-^{3}H(*N*)]-(*N*-(1-(2 thienyl)cyclohexyl)-3,4-piperidine) ([^{3}H]TCP; 41.8 Ci/mmol) and [^{125}I]α-bungarotoxin ([^{125}I]α-BTX) were obtained from New England Nuclear Research Products (Boston, MA). Imipramine hydrochloride, amitriptyline hydrochloride, doxepin hydrochloride, suberyldicholine dichloride, acetylcholine chloride, carbamylcholine chloride (CCh), tetracaine hydrochloride, and chlorpromazine chloride (CPZ) were purchased from Sigma Chemical Co. (St. Louis, MO). [1-(Dimethylamino) napthalene-5-sulfonamido] ethyltrimethylammonium perchlorate (dansyltrimethylamine) was obtained from Pierce Chemical Co. (Rockford, IL). Other organic chemicals were of the highest purity available.

2.2. Expression of AChR

Human embryonic kidney cells (HEK293 cells) were transfected with mouse α,β,δ, and ε cDNA subunits using calcium phosphate precipitation at a subunit ratio of 2:1:1:1 for α:β:δ:ε, respectively, essentially as previously described (Bouzat et al., 1994, 1998). For transfections, cells at 40–50% confluence were incubated for 8–12 h at 37 °C with the calcium phosphate precipitate containing the cDNAs in DMEM plus 10% fetal bovine serum. Cells were used for single-channel measurements 1 or 2 days after transfection.

2.3. Preparation of AChR native membranes

AChR native membranes were prepared from frozen *Torpedo californica* electric organs obtained from Aquatic Research Consultants (San Pedro, CA) by differential and sucrose density gradient centrifugation, as described previously (Pedersen et al., 1986). Specific activities of these membrane preparations were determined by the decrease in dansyltrimethylamine (6.6 μM) fluorescence produced by the titration of suberyldicholine into receptor suspensions (0.3 mg/ml) in the presence of 100 μM CPZ and ranged from 0.9 to 1.2 nmol of suberyldicholine binding sites/mg total protein (0.45–0.60 nmol AChR/mg protein). Dansyltrimethylamine excitation and emission wavelengths were 280 and 546 nm, respectively. To reduce stray-light effects, a 530-nm cutoff filter was placed in the path of the dansyltrimethylamine emission beam. The AChR membrane preparations were stored at −80 °C.

2.4. 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 142 mM KCl, 5.4 mM NaCl, 1.8 mM $CaCl_2$, 1.7 mM $MgCl_2$ and 10 mM HEPES (pH 7.4). Patch pipettes were pulled from 7052 capillary tubes (Garner Glass, CA) and coated with Sylgard (Dow Corning, Midland MI). Pipette resistance ranged from 5 to 7 MΩ. Acetylcholine and amitriptyline, imipramine, or doxepin were added to the pipette solution at final concentrations ranging from 1 to 20 μM. Channels were typically recorded at a membrane potential of −70 mV. For studying the voltage dependence of the effect of antidepressants, channels were also recorded at a membrane potential of –40, –90, and +70 mV. Single-channel currents were recorded using an Axopatch 200 B patch-clamp amplifier (Axon Instru-

ments, Inc., CA), 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 a final bandwidth of 10 kHz. 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 TACFit program (Bruxton Corporation, Seattle, WA). Clusters of openings corresponding to a single-channel were identified as a series of closely spaced events preceded and followed by closed intervals greater than a specified duration (τ_{crit}). This duration was taken as the point of intersection of the predominant closed component and the succeeding one in the closed time histogram. Cluster duration histograms were constructed setting the burst resolution time to that corresponding to τ_{crit}.

For outside-out patch recordings, the pipette solution contained 140 mM KCl, 5 mM EGTA, 5 mM $MgCl_2$ and 10 mM HEPES, pH 7.3. Extracellular solution (ECS) contained 150 mM NaCl, 5.6 mM KCl, 1.8 mM $CaCl_2$, 5 mM $MgCl_2$ and 10 mM HEPES pH 7.3. The patch was excised in this configuration and moved into position at the outflow of a perfusion system. The perfusion system consisted of solution reservoirs, manual switching valves, a solenoid-driven pinch valve and two tubes (id = 0.3 mm) oriented at 90° inserted into the culture dish (modified from Liu and Dilger, 1991; Spitzmaul et al., 2001). The perfusion system allows for a rapid (0.1–1 ms) exchange of the solution bathing the patch. A series of applications of 300 μM ACh were applied to the patch. We then recorded the responses of the patch to applications of 300 μM of ACh (200 ms pulse) during continuous exposure to different concentrations of antidepressants. After a series of applications of a given antidepressant solution, drug-free solutions were applied again to assess loss of channel activity. Macroscopic currents were filtered at 5 kHz, digitized at 20 kHz and stored on the hard disk. Data analysis was performed using the IgorPro software (WaveMetrics Inc., Lake Oswego, OR). The ensemble mean current was calculated for 10 individual current traces. Mean currents were fitted by a single exponential function: $I_{(t)} = I_0 \exp(-t/\tau_d) + I_\infty$ where I_0 and I_∞ are the peak and the steady state current values, respectively, and τ_d is the decay time constant that measures the current decay due to desensitization. Current records were aligned with each other at the point where the current had risen to 50% of its maximum level. Peak currents correspond to the value obtained by extrapolation of the decay current to this point.

2.4.1. Simulation of macroscopic currents

Macroscopic currents were simulated using the SIMU program from the QuB Suite (State University of New York, Buffalo) on the basis of Scheme 2 (see Results). For the simulations, the concentrations of ACh and doxepin were 300 and 10 μM, respectively. We used the agonist association and dissociation, opening, closing, and desensitization rate constants determined in previous studies under similar experimental conditions (Bouzat et al., 2000, 2002; Spitzmaul et al., 2001). The values are: k_{+1} = 300 $\mu M^{-1}s^{-1}$; k_{-1} = 27,000 s^{-1}; k_{+2} = 150 $\mu M^{-1}s^{-1}$; k_{-2} = 54,000 s^{-1}; β = 50,000 s^{-1}; α = 1500 s^{-1}; d_+ = 35 s^{-1}; d_- = 0.3 s^{-1}. The simulated currents were analyzed with the IGOR Pro software.

2.5. Ligand binding measurements

2.5.1. Effect of tricyclic antidepressants on equilibrium binding of ACh

Binding of ACh was measured by competition against the initial rate of [^{125}I]α-BTX binding as described previously (Sine and Taylor, 1982; Sine et al., 1994) and compared to binding in the presence of doxepin. HEK cells expressing adult AChRs were resuspended in high potassium Ringer's solution in the absence or in the presence of antidepressant, and divided into aliquots for ligand binding measurements. Cells were first incubated for 30 min with different concentrations of ACh, [^{125}I]α-BTX was subsequently added to a final concentration of 5 nM, and the cells were incubated for an additional 20 min to allow occupancy of at most 50% of the binding sites by α-BTX (Sine et al., 1994; Spitzmaul et al., 2001). The total number of binding sites was determined by incubating cells with 5 nM [^{125}I]α-BTX for 2 h in the absence of ACh. Binding was terminated by the addition of potassium Ringer's solution containing 20 mM CCh. Nonspecific binding was determined in the presence of 20 mM CCh. Rates of α-BTX binding in the absence and presence of competing ligand were calculated from binding measured at 20 and 120 min. These rates are related to ligand occupancy by $k_{obs} = k_{max}(1-Y)$, where k_{obs} is the rate of toxin binding in the presence of a specified concentration of competing ligand, k_{max} is the rate in the absence of competing ligand, and Y is the occupancy function for the competing ligand, given by the Hill equation. Fractional occupancy by ACh was fitted by the Hill equation:

$$1 - \text{fractional occupancy} = 1/(1 + ([\text{ACh}]/K_d)^{n_H} \quad (1)$$

where K_d is the apparent dissociation constant, and n_H is the Hill coefficient.

2.5.2. Effect of tricyclic antidepressants on [^{3}H]TCP binding to the AChR in the resting or desensitized state

The effect of imipramine, amitriptyline, and doxepin on [^{3}H]TCP binding to either the resting or desensitized AChR was examined. For the experiments in the desensitized state, *Torpedo* AChR native membranes (0.2 μM AChR) were suspended in 50 mM Tris–HCl, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4, with 6.2 nM [^{3}H]TCP in the presence of 1 mM CCh, and preincubated for 1 h at room temperature (RT). This suspension was then incubated for an additional 2 h at RT with increasing concentrations of the drug under study (the concentration ranged between 0.001 and 500 μM). Nonspecific binding was determined in the presence of 100 μM CPZ. For the experiments in the resting state, an initial concentration of 8.2 nM [^{3}H]TCP was used, and the nonspecific binding was determined in the presence of 100 μM tetracaine. At this initial concentration of [^{3}H]TCP, the AChR does not become desensitized (Arias et al., 2002b). AChR-bound [^{3}H]TCP was then separated from free ligand by filtration assay using a 48-sample harvester system (Brandel, Gaithersburg, MD) with GF/B Whatman filters (previously soaked with 0.5% polyethylenimine for 45 min). The radioactivity of the membrane-containing filters was determined using a Beckman LS 7000 scintillation counter (Beckman Coulter, Inc., Fullerton, CA).

For the binding experiments described above, the concentration–response data were curve-fitted by nonlinear least squares analysis using the Prism program (GraphPad) and the corresponding IC_{50} values calculated. Taking into account that the AChR presents one binding site for TCP in the desensitized (Pagán et al., 2001) or in the resting state (Arias et al., 2002b), the observed IC_{50} values from the competition experiments were transformed into K_i values using the Cheng–Prusoff relationship (Cheng and Prusoff, 1973):

$$K_i = IC_{50}/\{1 + ([TCP]/K_d^{TCP})\} \quad (2)$$

where [TCP] is the initial concentration of [^{3}H]TCP and K_d^{TCP} is the dissociation constant for [^{3}H]TCP (0.83 μM in the resting state (Arias et al., 2002b) and 0.25 μM in the desensitized state (Pagán et al., 2001)).

Experimental data are shown as mean ± SD. Statistical comparisons were done using the unpaired Student's *t*-test. A level of $p < 0.05$ is considered significant.

3. Results

3.1. Effects of antidepressants on single-channel currents

3.1.1. The main effect of tricyclic antidepressants on AChR channels is a decrease in the cluster duration

In order to evaluate the influence of antidepressants on AChR activation, channels were activated by 30 μM ACh in the absence and the presence of antidepressants. A concentration of 30 μM was chosen because it is close to the EC_{50} for the adult muscle AChR and therefore it is sensitive to changes in activation parameters. At this ACh concentration, opening events occur in clusters, each cluster reflecting the activity of a single AChR (Sakmann et al., 1980). A cluster starts when one AChR recovers from desensitization and continues with the receptor undergoing cycles of agonist association/dissociation and channel gating. During a cluster, it is possible to measure the probability of channel open, and the distribution of closed and open states. The duration of the cluster provides information on the rate at which the long-lived desensitized state develops (Auerbach and Akk, 1998).

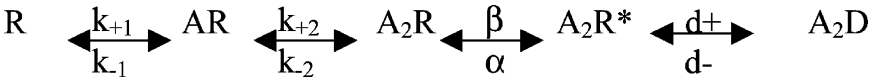
Scheme 1 shows a general model for activation where two agonists (A) bind to the receptor (R) in the resting state. Receptors occupied by two agonist molecules (A_2R) open with rate β and close with rate α. The model shows that under control conditions desensitization proceeds mainly from the doubly liganded open state (Dilger and Liu, 1992; Auerbach and Akk, 1998).

Fig. 1 shows clusters recorded from cell-attached patches in the presence of 30 μM ACh and 10 μM antidepressant in the pipette solution. As observed in the figure, amitriptyline, imipramine, and doxepin, significantly reduce the duration of the activation periods. Antidepressants do not affect the channel amplitude (see Fig. 1). The calculated amplitudes for AChR channels recorded at a membrane potential of –70 mV were 5.2 ± 0.2 pA (control), 5.3 ± 0.2 pA (10 μM imipramine), 5.1 ± 0.3 pA (10 μM amitriptyline), and 5.2 ± 0.3 pA (10 μM doxepin).

To describe the kinetic changes, we first compared dwells in the closed and open states within clusters in the absence and presence of antidepressants.

In the absence of antidepressants, closed time histograms of AChRs activated by 30 μM ACh can be well fitted with three or four components (Fig. 2). Typically, there is a fast component (20−60 μs), a major intermediate component of about 1 ms which is sensitive to ACh concentration, and one or two small variable slow components associated with periods between independent activation episodes and dependent on the number of channels in the patch (Bouzat et al., 2000, 2002). Closed time distributions are similarly affected by the three antidepressants tested here. The duration of the main component, corresponding to closings within clusters, remains constant in the presence of 10 μM antidepressant. The mean closed time of the main closed component for channels activated by 30 μM ACh is: control = 1.34 ± 0.40 ms (n = 10); amitriptyline = 1.74 ± 0.50 ms (n = 5); imipramine = 1.78 ± 0.30 ms (n = 5); doxepin = 1.12 ± 0.25 ms (n = 6).

Although the duration of the main component does not change in the presence of antidepressants, its relative



Scheme 1. Kinetic model for AChR activation.

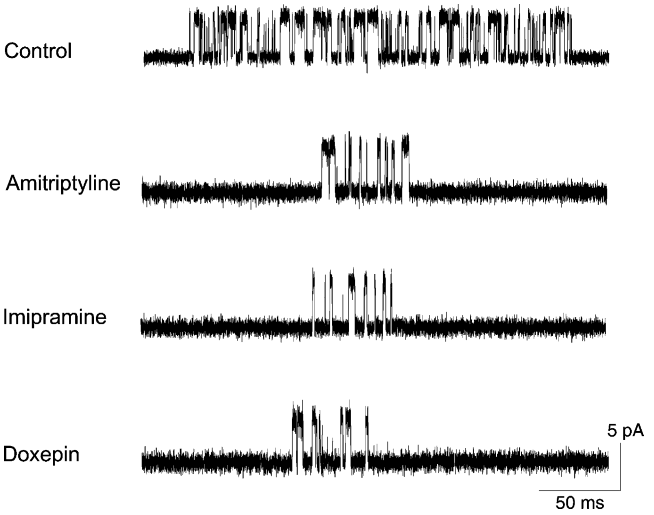


Fig. 1. Clusters of single-channel currents in the presence of tricyclic antidepressants. Channel traces were recorded in the cell-attached configuration in the absence and presence of 10 μM amitriptyline, imipramine and doxepin in the pipette solution. ACh: 30 μM. Membrane potential: −70 mV. Filter: 10 kHz.

area is significantly reduced. The relative areas of the main closed components mentioned before are: control = 0.74 ± 0.07; amitriptyline = 0.61 ± 0.10 ($p < 0.025$); imipramine = 0.56 ± 0.09 ($p < 0.005$); doxepin = 0.48 ± 0.08 ($p < 0.001$).

Taken together, the data suggest that antidepressants have little effect on the channel opening rate, and on ACh association and dissociation, as well as that they do not produce a fast open-channel blockade.

Open time distributions of AChRs recorded in the presence of 10 μM of amitriptyline, imipramine, or doxepin are similar to control histograms (Fig. 2). At 30 μM ACh and in the absence of antidepressants open time distributions show a major component of 1.02 ± 0.26 ms with a relative amplitude larger than 0.8 in all recordings (Fig. 2) (Bouzat et al., 1994, 2002). The duration of this component remains constant in the presence of 10 μM antidepressants (0.94 ± 0.24, 0.96 ± 0.11, and 0.90 ± 0.10 ms for AChRs recorded in the presence of 10 μM amitriptyline, imipramine, and doxepin, respectively). These results suggest that antidepressants do not increase the rate of channel closing and do not produce a fast open-channel blockade.

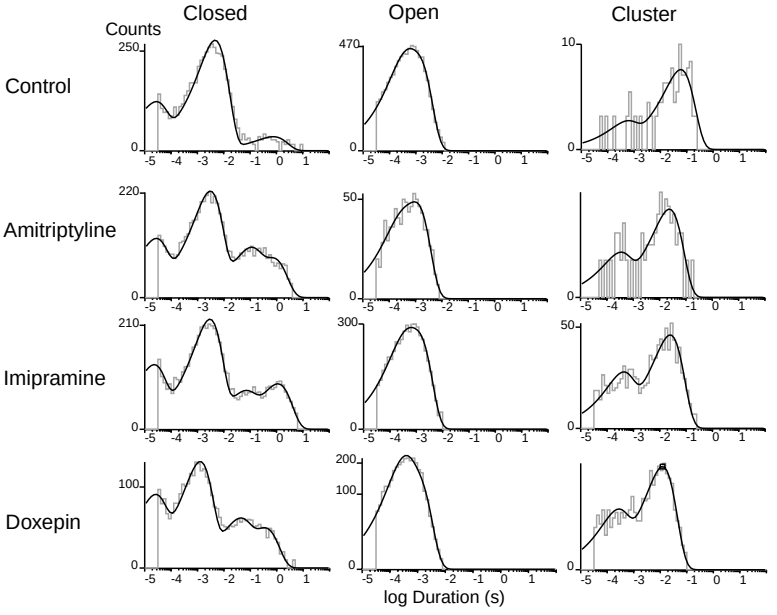


Fig. 2. Closed, open and cluster duration histograms in the presence of antidepressants. AChR channels were activated by 30 μM ACh in the absence and presence of 10 μM amitriptyline, imipramine and doxepin in the pipette solution. Clusters of openings corresponding to a single-channel were identified as a series of closely spaced events preceded and followed by closed intervals greater than a specified duration (τ_{crit}); this duration was taken as the point of intersection of the predominant closed component and the succeeding one in the closed time histogram. Cluster duration histograms were constructed setting the burst resolution time to that corresponding to τ_{crit}.

Typically, cluster duration histograms from control recordings at 30 μM ACh show a main component of 69.0 ± 5.5 ms whose relative area is larger than 0.9 (Fig. 2). A minor component is present in all the histograms and corresponds to isolated openings. As shown in Fig. 2, significant changes in the cluster duration histograms are observed in the presence of antidepressants. The mean cluster durations decrease to 16 ± 3, 17 ± 4, and 11 ± 2 ms, in the presence of 10 μM amitriptyline, imipramine, and doxepin, respectively.

3.1.2. Dependence of mean closed time, mean open time, mean cluster duration and openings per cluster on antidepressant concentration

The dependence of channel kinetics on the concentration of antidepressants was evaluated from the recordings performed in the presence of 30 μM ACh and 1–20 μM amitriptyline, imipramine or doxepin (Fig. 3).

For the three antidepressants tested here, the durations of the main components of both closed-time and open time histograms remain constant in the range of concentrations 1–20 μM (Figs. 3a,b and 4). Statistically insignificant differences are observed between the lowest and highest concentrations.

As described before, the main effect of antidepressants is a dose-dependent reduction in the duration of clusters, such reduction being about six fold at 20 μM imipramine and amitriptyline, and about eight fold at 20 μM doxepin (Figs. 3c and 4). The IC_{50} values calculated for the decrease in the cluster duration are 8.1, 7.7 and 3.7 μM for amitriptyline, imipramine, and doxepin, respectively. The decrease in the number of openings per cluster parallels that of cluster duration for all the antidepressants (Fig. 3d), indicating that the decrease in the duration of the activation periods is mainly due to a decrease in the number of opening events.

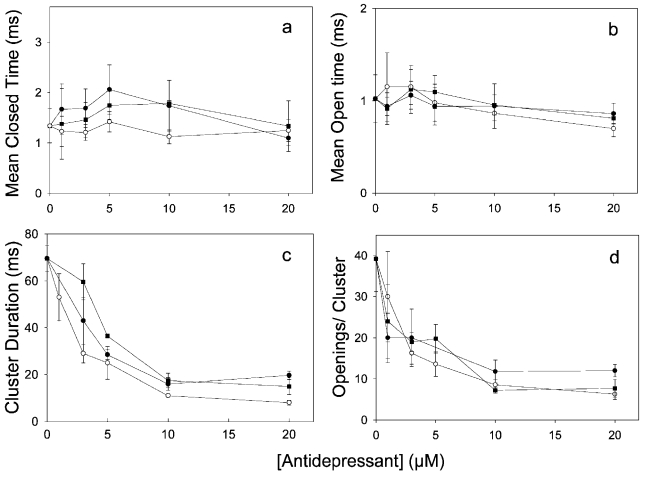


Fig. 3. Dependence of mean closed time (a) mean open time (b) mean cluster duration (c) and number of openings per cluster (d) on tricyclic antidepressant concentration. The closed time corresponds to the main closed component of the closed time histogram. The mean open time was obtained from the corresponding open time histogram. Cluster duration histograms were constructed setting the burst resolution time to that corresponding to τ_{crit}. The curves correspond to: (■) imipramine; (●) amitriptyline and (○) doxepin.

3.1.3. Effects of antidepressants on clusters activated by high ACh concentrations

At 300 μM ACh, sojourns in open and closed states within clusters correspond mostly to the closing and reopening of the biliganded AChR ($A_2R \leftrightarrow A_2R*$, see Scheme 1). We compared cluster properties in the absence and presence of 10 μM doxepin. Cluster duration histograms of control recordings obtained at 300 μM ACh show a main component of 32 ± 2 ms whose relative area is larger than 0.8 (n = 3) and the number of openings per cluster is 29 ± 9. In the presence of 10 μM doxepin, the mean cluster duration is reduced to 5.5 ± 0.3 ms and the number of openings per cluster to 5.7 ± 0.3. The decrease in the cluster duration observed at 300 μM ACh is quantitatively similar to that observed at 30 μM ACh. Because 30 μM ACh is below the half maximal effective concentration of ACh (see below and Fig. 5a), the channel is more likely to be in the closed than in the open state at this concentration, whereas it is mostly in the open state at 300 μM ACh. The fact that the clusters are affected similarly by both ACh concentrations suggests that antidepressants act on both open and closed states.

Again, the mean open and closed times within clusters are not significantly affected in the presence of antidepressants. The mean values obtained from the corresponding distributions are: mean open-channel duration: 0.97 ± 0.12 and 1.02 ± 0.05 ms for control and doxepin, respectively; mean closed duration: 0.095 ± 0.012 and 0.090 ± 0.004 ms for control and doxepin, respectively.

3.1.4. Open probability within clusters in the presence of antidepressants

To determine if doxepin affects the open probability, we recorded AChRs activated by different concentrations of ACh in the presence of 10 μM doxepin and calculated the probability that the channel is open within a cluster (P_{open}). For wild-type AChRs, P_{open} increases with increasing ACh concentration, showing an EC_{50} of about 38 μM (Fig. 5a and Bouzat et al., 2000). A similar P_{open} curve was obtained in the presence of 10 μM doxepin (Fig. 5a). The calculated value for the EC_{50} was 41 μM. Thus, although the cluster duration decreases, P_{open} values do not change in the presence of doxepin.

Auerbach and Akk (1998) demonstrated that the value of $(\tau_c P_{open})^{-1}$, where τ_c is the mean cluster duration and P_{open} is the probability of being open within a cluster, is a direct measure of the desensitization rate constant. For channels activated by 300 μM ACh and in the absence of antidepressants, the calculated value for $(\tau_c P_{open})^{-1}$ = 32 ± 4 s^{-1}. This value agrees with desensitization rates

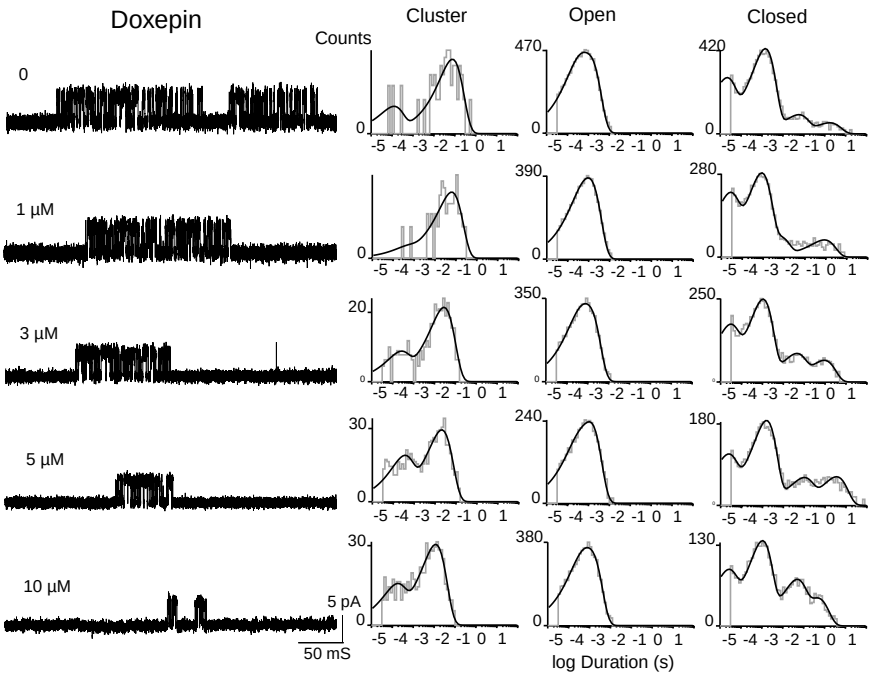


Fig. 4. AChR clusters in the presence of different concentrations of doxepin. Channel traces were recorded from cell-attached patches in the absence and presence of 1, 3, 5 and 10 μM doxepin in the pipette solution. ACh: 30 μM. Membrane potential: −70 mV. Filter: 10 kHz. Left: openings are shown as upward deflections. Right: cluster-, open- and closed-duration histograms corresponding to the different conditions.

calculated from the decay of macroscopic currents (Dilger and Liu, 1992; Spitzmaul et al., 2001; see below). The value of $(\tau_c P_{open})^{-1}$ increases to 200 ± 9 s^{-1} in the presence of 10 μM doxepin.

3.1.5. *Effects of tricyclic antidepressants on AChR as a function of membrane potential*

To investigate whether the action of antidepressants on the AChR is voltage dependent, we recorded AChR channels activated by 30 μM ACh at different membrane potentials. Since the main effect of antidepressants is the reduction of cluster duration, we compared the relationship between membrane potential and cluster duration in the absence and presence of doxepin. Fig. 5b shows that in the presence of doxepin, this relationship parallels that of the control, suggesting that the action of doxepin is not significantly voltage dependent.

3.2. *Effects of antidepressants on macroscopic AChR currents*

3.2.1. *Doxepin increases the rate of current decay due to desensitization*

To determine the overall consequences of antidepressants on AChR activation, we studied the effect of doxepin on outside-out patches rapidly perfused with 300 μM ACh. Fig. 6a shows ensemble currents obtained from a single outside-out patch exposed to brief applications of ACh alone (control) and together with different concentrations of doxepin applied after a 2 min preincubation with doxepin-containing buffer. In control data, the current reaches the peak after 0.1–1 ms and then decays with a time constant (τ_d) of about 20–30 ms due to desensitization (Fig. 6a). This τ_d corresponds to a desensitization rate of 37 ± 8 s^{-1} ($1/\tau_d$). When doxepin is present in both ACh-free and ACh-containing solutions, the peak currents are reduced, reaching values of about 50% at 20 μM doxepin (Fig. 6a and Table 1). The reduction in the peak current suggests that the drug affects closed (resting) channels. In addition, a concentration-dependent increase in the decay rate ($1/\tau_d$) is observed in the presence of doxepin (Fig. 6a and Table 1). At all drug concentrations, decays are well fitted by a single exponential function. This observation discards a fast open-channel blockade mechanism, since a two-component decay time course should have been observed instead (Dilger et al., 1997; Forman, 1999), and suggests an increase in the desensitization rate. However, the observed monoexponential decay might also result from a channel blockade with a slow unblocking reaction.

Assuming that channel blockade is the mechanism responsible for the increase in the decay rate, it is possible to provide information on how slow the unblocking rate would have to be to lead to a monoexponential decay of the macroscopic current. To this end, we simulated macroscopic currents incorporating a blocked state into Scheme 1 (Scheme 2).

This model assumes that the macroscopic currents activated by ACh decay by means of two mechanisms:

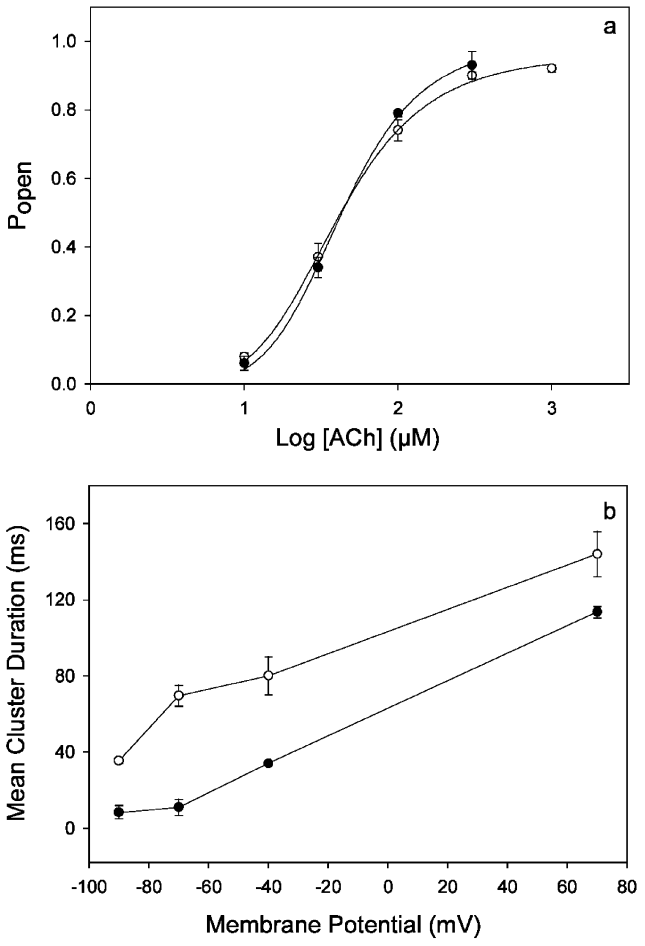


Fig. 5. (a) Agonist concentration dependence of the channel open probability. The mean fraction of time the channel is open during a cluster (P_{open}) was experimentally determined at the indicated concentrations of ACh. Each point corresponds to the mean ± SD of three patches. The symbols correspond to the following conditions: ○, control; ●, 10 μM doxepin. (b) Relationship between membrane potential and cluster duration in the presence of doxepin. AChRs activated by 30 μM ACh were recorded in the absence (○) or presence of 10 μM doxepin (●) at different membrane potentials. Data were obtained from the cluster duration histograms and are expressed as the mean ± SD of 3–5 different patches.

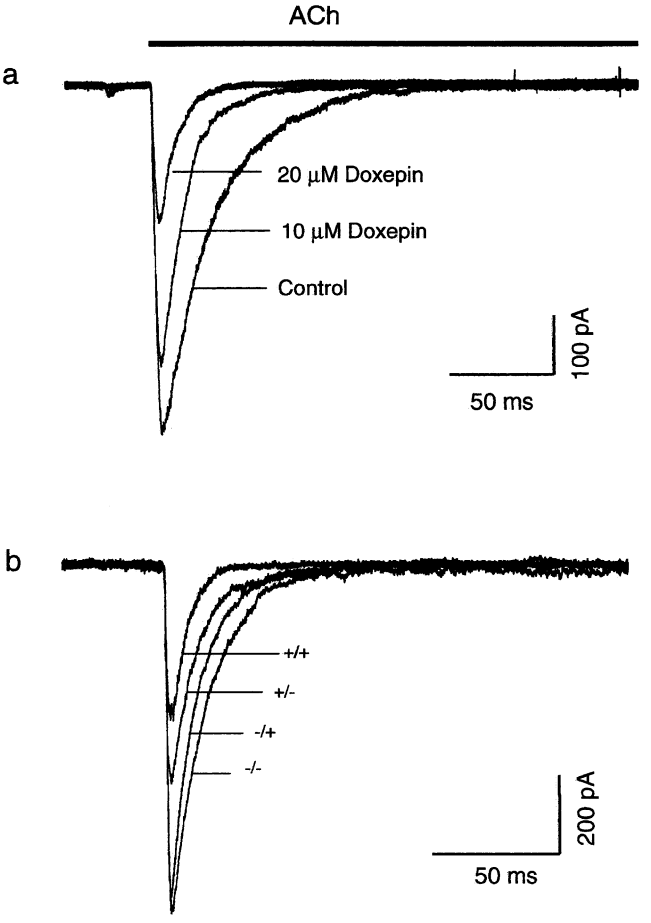


Fig. 6. Effect of doxepin on the decay of macroscopic currents activated by 300 μM ACh. (a) Ensembled mean currents obtained from outside-out patches preincubated in the absence (control) or presence of doxepin and activated by 300 μM ACh in the absence (control) and presence of doxepin. Each trace represents the average of 6–10 applications of agonist. Curves from right to the left correspond to: control, 10 and 20 μM doxepin. The calculated decay time constants (τ_d) are 25 ms for control curves and 10 and 7.5 ms for 10 and 20 μM doxepin, respectively. Membrane potential: −50 mV. (b) Effects of doxepin application protocol on macroscopic currents. Superimposed current responses to 300 μM of ACh and 10 μM doxepin applied using different protocols. From right to left curves correspond to control conditions (−/− protocol; τ_d = 24 ms), simultaneous ACh/doxepin application without preincubation with doxepin (−/+ protocol; τ_d = 17 ms; peak current = 92% of the control), ACh application following 2 min preincubation with doxepin (+/− protocol; τ_d = 13 ms; peak current = 81% of the control) and simultaneous ACh/doxepin application with preincubation with doxepin (+/+ protocol; τ_d = 8.5 ms; peak current = 70% of the control).

desensitization (A_2D) and blocking (A_2B). For the simulations, the concentrations of ACh and doxepin were 300 and 10 μM, respectively. We used the agonist association and dissociation, opening, closing and desensitization rate constants determined in previous studies under similar experimental conditions (see Materials and methods) (Bouzat et al., 2000, 2002; Spitzmaul et al., 2001). The value for the forward blocking rate constant (k_{+b}) was estimated by the slope of the curve of the reciprocal of the cluster duration as a function of doxepin concentration (5×10^6 M^{-1} s^{-1}). We systematically varied k_{-b} from 0.1 to 100 s^{-1} and simulated the currents by using the SIMU program from the QuB Suite (State University of New York, Buffalo). Control curves (simulated without the blocked state of Scheme 2) were similar to the experimental curves, showing a τ_d = 32 ms. Current decays were well fitted with one exponential when k_{-b} values varied from 0.1 to 15 s^{-1}. The decay rate constant of the simulated currents in the presence of doxepin was 11 ms, a value that is similar to that obtained experimentally. In contrast, when k_{-b} = 20 s^{-1}, a double exponential function was necessary for a good fit. In summary, the simulation results suggest that the increase in the decay rate observed in the presence

Table 1
Influence of doxepin on the decay rate and amplitude of macroscopic currents

Protocol	τ_d (ms)	Peak current (%)
Control (−/−)	24.7 ± 8.6	100
+/+10 μM	9.1 ± 2	71 ± 10
+/+20 μM	8.1 ± 0.5	50 ± 6
+/−10 μM	13.7 ± 3	79 ± 6
−/+10 μM	18.0 ± 1.0	91 ± 3

The table shows the decay time constants (τ_d) of currents obtained from outside-out patches exposed to 300 μM ACh and doxepin. Values are expressed as the mean ± SD of at least three different experiments for each condition. Application protocols of exposure to doxepin are: −/− (control); simultaneous ACh/doxepin application with previous incubation with 10 or 20 μM doxepin (+/+); ACh application following 2 min preincubation with doxepin (+/−); and simultaneous ACh/doxepin application without preincubation with doxepin (−/+). Membrane potential −50 mV. Peak currents are related to control conditions of the same experiment.

of doxepin could be explained, not only by the increase in the desensitization rate, but also by a channel blockade with an off rate slower than 15 s^{-1}.

3.2.2. *Effects of the application of doxepin before or after channel opening on macroscopic currents*

In the macroscopic current experiments described so far, patches were equilibrated with doxepin prior to application of the ACh plus doxepin solution (+/+ protocol). When doxepin is omitted from the preincubation solution (−/+ protocol), the effect of the antidepressant on desensitization is less pronounced (Fig. 6b and Table 1). In addition, no changes on the peak currents are observed. When the patch is preincubated with doxepin followed by application of ACh alone (+/− protocol), the effects on both desensitization rate and peak current are intermediate between those observed in the −/+ and +/+ protocols (Table 1). Thus, it appears that prior incubation of the patch with doxepin is necessary for its maximal pharmacological action. To determine if preincubation times longer than 2 min resulted in more pronounced effects, we measured the decrease in the time constant of current decay (τ_d) and in the peak current at different preincubation times, which varied from 2 to 12 min (+/− protocol). No differences in the decrease in either τ_d or peak current were observed between the briefest and longest preincubation times, thus confirming that the maximal effect is achieved at 2 min of exposure to doxepin (data not shown).

In all these experiments, after each application protocol, drug-free solutions were applied again and then a new control current was elicited by activation with 300 μM ACh (−/− protocol) to ensure that both the peak current and time constant of the decay remained unchanged.

3.3. *Effects of antidepressants on equilibrium binding of ACh*

To determine whether antidepressants introduce changes in equilibrium agonist binding, we studied if the inhibition of [^{125}I]α-BTX binding by ACh was affected by doxepin. Cells were preincubated for 15 min in the absence or presence of 10 μM doxepin before the binding assay. As shown in Fig. 7, the curve is displaced to the left in the presence of doxepin. The apparent K_ds are 1.6 and 0.5 μM for control and doxepin-treated AChRs, respectively. The calculated values for control conditions are in good agreement with those reported previously (Sine et al., 1994; Spitzmaul et al., 2001). The decrease

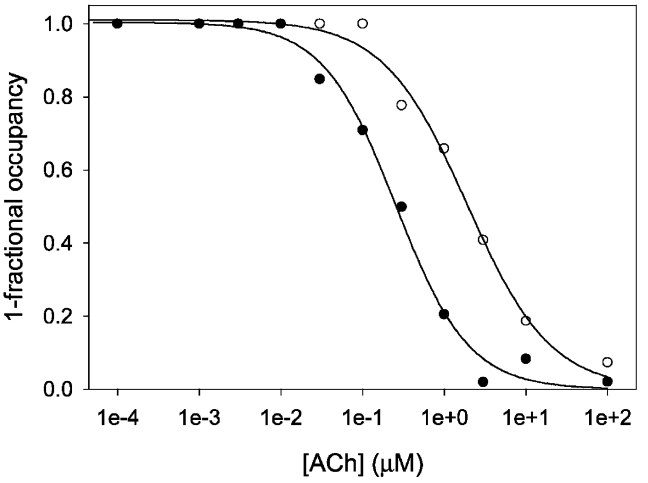
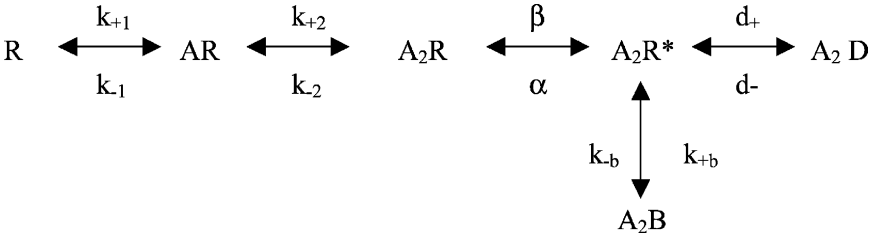


Fig. 7. Effects of doxepin on equilibrium binding of ACh. ACh binding was measured by competition against the initial rate of [^{125}I]α-BTX binding in the absence (○) or presence of 10 μM doxepin (●). The curves are fits to the Hill equation (see Materials and methods).



Scheme 2. Kinetic model for AChR activation, desensitization and blockade.

in K_d is in agreement with previous work showing that some NCIs, such as proadifen, convert the AChR to a state whose affinity coincides with that of the desensitized state (Sine and Taylor, 1982; Prince and Sine, 1998; Spitzmaul et al., 2001).

3.4. Effects of antidepressants on equilibrium binding of the NCI, TCP

3.4.1. Inhibition of [^{3}H]TCP binding to either the resting or the desensitized AChR by tricyclic antidepressants

In an attempt to identify the site of interaction of antidepressants and to determine the relative association properties of tricyclic antidepressants in different conformational states of the AChR, the antidepressant-induced inhibition of [^{3}H]TCP binding to *Topredo* AChRs was studied. The high concentration of *Torpedo* AChRs in the membrane allows a high level of specific [^{3}H]TCP binding (~90%) which cannot be obtained with entire cells transiently expressing AChRs. Experiments were carried out in the absence and presence of 1 mM carbamylcholine. In the absence of CCh, only a fraction of receptors (<20%) are desensitized, whereas in the presence of CCh, all receptors are desensitized (Arias and Blanton, 2002).

The results show that each tricyclic antidepressant completely eliminated specific [^{3}H]TCP binding to the AChR in a concentration-dependent fashion in either the absence (resting AChR) or presence of CCh (desensitized AChR) (Fig. 8). From these experiments, we obtained the respective antidepressant IC_{50} (nonabsolute) values, which were transformed to absolute K_i values according to the Cheng–Prusoff equation (Eq. (2), see Materials and methods). In the desensitized state, the K_i values ranged from 0.7 to 5.3 μM (see Table 2), indicating that the relative potencies for binding to the TCP site are: imipramine~amitriptyline > doxepin. In the resting state, the same rank order was obtained (Table 2). Nevertheless, the K_i values in the resting state were 9.1- (imipramine), 4.3- (amitriptyline), and 3.0-fold (doxepin) higher than those in the desensitized state. Therefore, it seems likely that antidepressants bind with higher affinity to the desensitized than to the resting state.

From these results and considering that the n_H values are close to 1 (Table 2), we can conclude that tricyclic antidepressants displace [^{3}H]TCP from its high-affinity binding site in a mutually exclusive (steric) manner when the receptor is in either the desensitized or resting state. Thus, it is likely that the locus for tricyclic antidepressants overlaps, at least partially, the TCP binding site in both states.

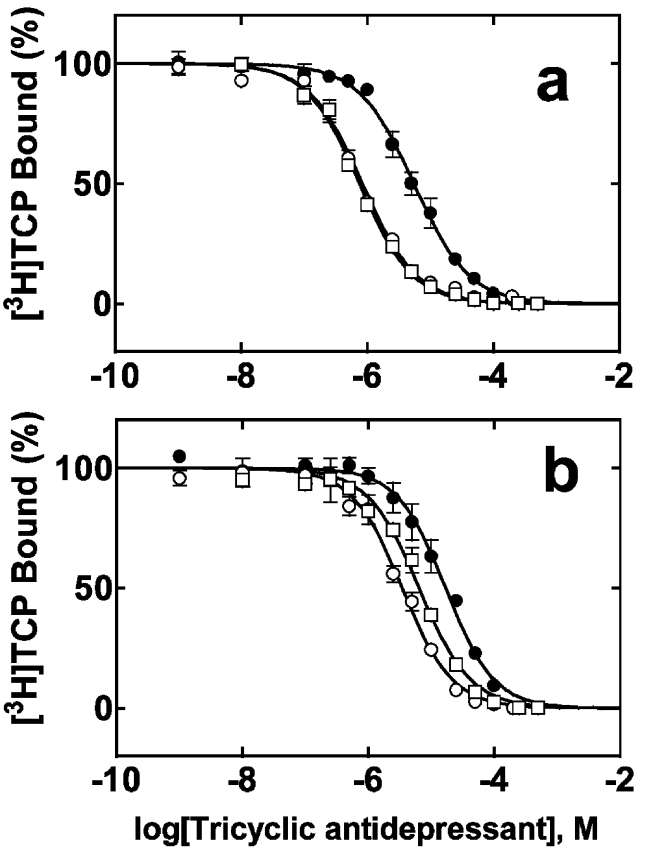


Fig. 8. Inhibition of [^{3}H]TCP binding to either the desensitized (a) or resting (b) AChR by tricyclic antidepressants. AChR native membranes (0.2 μM) were equilibrated (1 h) with [^{3}H]TCP (6.2 (a) or 8.2 nM (b)) in the absence (b) or in the presence (a) of 1 mM CCh. Then, the suspension was incubated for an additional 2 h in the presence of increasing concentrations of the antidepressant (the concentration ranged between 0.001 and 500 μM): imipramine (□), amitriptyline (○), and doxepin (●). Nonspecific binding was determined in the presence of 100 μM CPZ (a) or 100 μM tetracaine (b). Each plot is the average of three different experiments. The IC_{50} values were calculated by nonlinear least-squares fit for a single binding site. The K_i values were calculated using these IC_{50} values according to Eq. (2) (see Materials and methods) and are summarized in Table 2.

Table 2
Affinities of tricyclic antidepressants for AChRs in resting and desensitized states

Tricyclic antidepressant	Resting		Desensitized	
	K_i (μM)	n_H	K_i (μM)	n_H
Imipramine	6.4 ± 0.4	1.06 ± 0.06	0.7 ± 0.1	1.03 ± 0.05
Amitriptyline	3.4 ± 0.3	1.09 ± 0.08	0.8 ± 0.1	1.01 ± 0.03
Doxepin	15.8 ± 1.4	1.08 ± 0.09	5.3 ± 0.3	0.99 ± 0.06

The inhibition constants (K_i) were calculated using the IC_{50} values obtained from Fig. 8 according to Eq. (2). n_H, Hill coefficient.

4. Discussion

We have identified the mechanistic bases for the noncompetitive action of antidepressants on AChRs as well as their interactions at NCI sites on conformationally different AChRs.

Cluster analysis showed that the main effect produced by antidepressants is a concentration-dependent decrease in the cluster duration, which in turn is due to a decrease in the number of successive openings within a cluster. No changes in the P_{open} and duration of closed and open periods within clusters are observed. The early termination of clusters supports an increase in the desensitization rate (Auerbach and Akk, 1998). This conclusion is in good agreement with that deduced from macroscopic current experiments which show an increase in the decay rate of currents activated by 300 μM ACh in the presence of doxepin. At this ACh concentration, the decay rate equals the rate of desensitization from the double liganded open state (Spitzmaul et al., 2001). The current decay was adequately fitted using a single exponential function, which discards the possibility of a fast open-channel blockade mechanism.

Although the data suggest that antidepressants increase the desensitization rate of agonist-activated AChRs, the occurrence of slow channel blockade, which has been described for other noncompetitive inhibitors (Papke and Ostwald, 1989), may result in similar changes in both clusters and macroscopic currents. If the antidepressant blocked the AChR and its unblocking reaction were quite slow, distinguishing between this process and the increase in the desensitization rate would be problematic. By simulating currents, we demonstrated that if doxepin produced blockade, the unblocking rate constant would be slower than 15 s^{-1}. Although we cannot discard a slow channel blockade, several lines of evidence presented in this paper point towards an increase in desensitization as the mechanism of antidepressant inhibition. First, our binding studies with ACh demonstrate that doxepin produces the increase in ACh affinity expected for a desensitizing agent. In this regard, it has been demonstrated that tacrine, which acts as both an open and closed channel blocker of the muscle AChR, does not affect ACh affinity (Prince et al., 2002). Second, the action of doxepin is not voltage dependent, a property which would have been expected for an open-channel blocker. Third, in [^{3}H]TCP binding experiments, all tricyclic antidepressants show high-affinity for the desensitized state of the AChR.

Macroscopic current recordings also show a decrease in the peak current after preincubation of the resting channels with the antidepressant. Thus, together with the acceleration of desensitization from the open state, antidepressants inhibit the opening of resting channels. The latter effect might be due to either an increase in desensitization or a blockade of unliganded channels. In either case, after a 2 min preincubation, the maximal effect of doxepin on both current and decay rate is achieved.

The results from [^{3}H]TCP binding experiments suggest that tricyclic antidepressants specifically displace TCP from its high-affinity site in a mutually exclusive manner in either the resting or desensitized state. The structural and functional analog of PCP, TCP, binds with high-affinity to both desensitized (Pagán et al., 2001) and resting AChRs (Arias et al., 2002b). The exact location for the PCP (or TCP) site is still not firmly established (reviewed in Arias et al., 2002a). Many lines of indirect evidence support single sites for PCP (one on each conformational state) within the ion channel of muscle-type AChRs (Eterovic et al., 1999; Eaton et al., 2000; Arias et al., 2002b). However, the pharmacological effects of antidepressants are strikingly different from those produced by other NCIs that interact with M2, such as the local anesthetic QX-222 (Leonard et al., 1991; Bouzat and Barrantes, 1996), general anaesthetics and alcohols (Dilger et al., 1991; Forman, 1997). In agreement with the different effects of antidepressants, the PCP (TCP) site has been reported to be located more extracellularly than the sites of the above mentioned NCIs. In the resting AChR, the PCP site has been located between the valine ring (position 13′) and the extracellular ring (position 20′) (Arias et al., 2002b; Arias and Blanton, 2002).

The present work provides experimental evidence supporting the fact that tertiary-amine tricyclic antidepressant compounds exhibit similar mechanisms of action at AChRs. Structural differences at the tricyclic core and side chain lead to subtle differences in their inhibitory potencies. Antidepressants inhibit [^{3}H]TCP binding in either the desensitized or resting state with the following rank order: imipramine~amitriptyline > doxepin. However, single-channel experiments show that the decrease in cluster duration is similar for imipramine and amitriptyline but is slightly higher for doxepin. This apparent inconsistency could be due to different relationships between binding affinity and efficacy of the different antidepressants. An alternative explanation could be that antidepressant affinities are different between *Torpedo* and mammalian muscle AChRs (Eterovic et al., 1999).

From our data, we can conclude that tricyclic antidepressants: (i) affect both open and closed channels; (ii) do not act as fast open-channel blockers; (iii) do not affect channel opening and closing rates; (iv) inhibit channel opening upon binding to the resting state; and finally, (v) induce a slow process which shortens cluster activity by probably increasing the rate of desensitization or by producing a slow off-rate blockade from the open state.

A more detailed analysis at both single-channel and macroscopic current levels can be achieved with the muscle rather than with the neuronal AChR. Since the

muscle AChR has been the prototype for the study of all members of the LGIC superfamily, it may be possible that antidepressants inhibit neuronal AChRs by similar mechanisms to the ones described here for the muscle AChR. Supporting this hypothesis, studies of ACh-currents generated by rat neuronal $\alpha_2\beta_4$ and mouse muscle AChRs expressed in *Xenopus* laevis oocytes showed that imipramine inhibits both types of AChRs with similar potency and through similar mechanisms (López-Valdés and García-Colunga, 2001).

Therapeutic concentrations of antidepressants attainable in blood are in the order of 1 μM (Clarke, 1986). In the brain, however, antidepressants can reach concentrations as high as 20 μM (Besret et al., 1996). These concentrations would be high enough to alter AChRs. Therefore, AChRs in the brain might play a role in the therapeutic and/or secondary effects of antidepressants. In this regard, it has been recently reported that hypercholinergic neurotransmission, which is associated with depressed mood states, may be mediated through excessive neuronal nicotinic receptor activation and that the therapeutic actions of many antidepressants may be in part mediated through inhibition of AChRs (Shytle et al., 2002). Moreover, it has been recently reported that desensitization of neuronal AChRs, which, as shown in the present work is enhanced in the presence of antidepressants, could be important in normal information processing as well as in various diseased states (Quick and Lester, 2002).

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