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Effects of clomipramine on neuronal nicotinic acetylcholine receptors

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Abstract

The action of the tricyclic antidepressant clomipramine on membrane currents elicited by acetylcholine was studied in *Xenopus* oocytes expressing neuronal $\alpha 2\beta 4$ nicotinic acetylcholine receptors. Clomipramine inhibited the acetylcholine responses rapidly and reversibly, with a similar IC_{50} when the oocytes were preincubated with clomipramine (1.3 ± 0.2 μM) or when they were exposed simultaneously with acetylcholine and clomipramine (1.5 ± 0.3 μM). The EC_{50} was 39.9 ± 2.1 μM for acetylcholine alone and 65.7 ± 3.6 μM for acetylcholine in the presence of 2 μM clomipramine. The inhibitory effect of clomipramine was weakly voltage-dependent, with an electric distance of ~ 0.14. Moreover, clomipramine increased the rate of decay of currents elicited by acetylcholine. From all of these, we conclude that clomipramine reversibly and noncompetitively regulates neuronal $\alpha 2\beta 4$ nicotinic acetylcholine receptors by blocking the open receptor–channel complex at a site close to the extracellular vestibule of the channel. The actions of clomipramine on neuronal nicotinic acetylcholine receptors may play an important role in the treatment of mental depression and other mood disorders.

Keywords: Clomipramine; Cholinergic–serotonergic interaction; Nicotinic receptor modulation; *Xenopus* oocyte; Mental depression

1. Introduction

In addition to its well-known inhibitory action on neuronal monoamine reuptake, clomipramine, a tricyclic antidepressant and an antiobsessional drug, has the ability to act upon various neurotransmitter receptors including muscarinic acetylcholine receptors, histamine H_1 receptors, α_1- and α_2-adrenoceptors and dopamine D2 receptors (Richelson and Nelson, 1984; Richelson, 1994).

Nicotinic acetylcholine receptors are diverse members of the neurotransmitter-gated ionic channel superfamily that includes the γ-aminobutyric acid types A- and C-, glycine- and 5-hydroxytryptamine (5-HT) type 3 receptors (Karlin and Akabas, 1995; Lukas, 1998). They are composed of various subunits coded by different genes (Paterson and Nordberg, 2000); each receptor is presumed to have a pentameric structure (Cooper et al., 1991) that consists of five subunits delimiting a central ionic pathway (Unwin, 1996). Nicotinic acetylcholine receptors are activated after binding the endogenous neurotransmitter acetylcholine and they mediate fast signal transmission across the vertebrate neuromuscular junction and some central and peripheral synapses (Role and Berg, 1996; Lukas et al., 1999).

A great deal of evidence indicates that the most common action of serotonergic compounds, both agonists and antagonists of 5-HT receptors as well as inhibitors of 5-HT transporters, on nicotinic acetylcholine receptors is a noncompetitive inhibition (García-Colunga and Miledi, 1994, 1995, 1996, 1999a; García-Colunga et al., 1997; Arias, 1998; Maggi et al., 1998; Fryer and Lukas, 1999a,b; Hennings et al., 1999; Blanton et al., 2000). Recently, we reported that imipramine, a tricyclic antidepressant with a molecular structure similar to that of clomipramine, also blocks neuronal nicotinic acetylcholine receptors in a noncompetitive manner (López-Valdés and García-Colunga, 2001). Moreover, the effects of clomipramine on nicotinic acetylcholine receptors had only been studied briefly (Aronstam and Narayanan, 1981), without exploring its mechanisms of action. Therefore, it was of interest to study the interactions of clomipramine with the neuronal $\alpha 2\beta 4$ nicotinic acetylcholine receptors.

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2. Methods

The experiments were performed on *Xenopus laevis* oocytes expressing rat neuronal α2β4 nicotinic acetylcholine receptors (García-Colunga and Miledi, 1995). Briefly, cloned DNAs encoding rat neuronal α2 or β4 nicotinic acetylcholine receptor subunits were transcribed in vitro, and equal quantities (0.5–5 ng) of each subunit cRNA were mixed and injected into individual oocytes in 50 nl of water. The follicle-enclosed oocytes were dissected from the ovary and maintained at 16–17 °C in Barth's solution containing (in mM): 88 NaCl, 1 KCl, 0.33 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 0.82 $MgSO_4$, 2.4 $NaHCO_3$, 5 HEPES and 0.1 mg/ml gentamicin sulfate; pH 7.4 with NaOH. Two days later they were treated with collagenase (140 units/ml; Sigma, type I) for 0.5–1 h in order to remove the ovarian epithelial and follicular cells (Miledi and Woodward, 1989). Membrane currents were recorded, at room temperature (20–23 °C), 3–9 days after injection, using a voltage clamp with two microelectrodes filled with 3 M KCl (Miledi, 1982). The oocytes were continuously superfused at a rate of 7–10 ml/min (chamber volume ~ 0.1 ml) with frog Ringer solution containing (in mM): 115 NaCl, 2 KCl, 1.8 $CaCl_2$, 5 HEPES; pH 7.0 with NaOH. Unless otherwise indicated, the oocyte membrane potential was maintained at −60 mV. Membrane current values are given as the mean ± standard error (S.E.).

The concentration–response relationships were fitted with the Hill equation to obtain the IC_{50} of clomipramine and the EC_{50} of acetylcholine in the absence or presence of clomipramine (López-Valdés and García-Colunga, 2001).

Drugs used were acetylcholine and clomipramine (RBI, Natick, MA) that were diluted in Ringer's solution and applied onto the oocytes by superfusion.

3. Results

3.1. The inhibition of acetylcholine response by clomipramine

When clomipramine was applied alone (at concentrations up to 10 μM) to non-injected oocytes, or to oocytes expressing neuronal α2β4 nicotinic acetylcholine receptors, no measurable membrane currents were observed. In contrast, when the current was elicited with acetylcholine alone and clomipramine was added after the peak current was reached, the current was rapidly inhibited (Fig. 1A). The

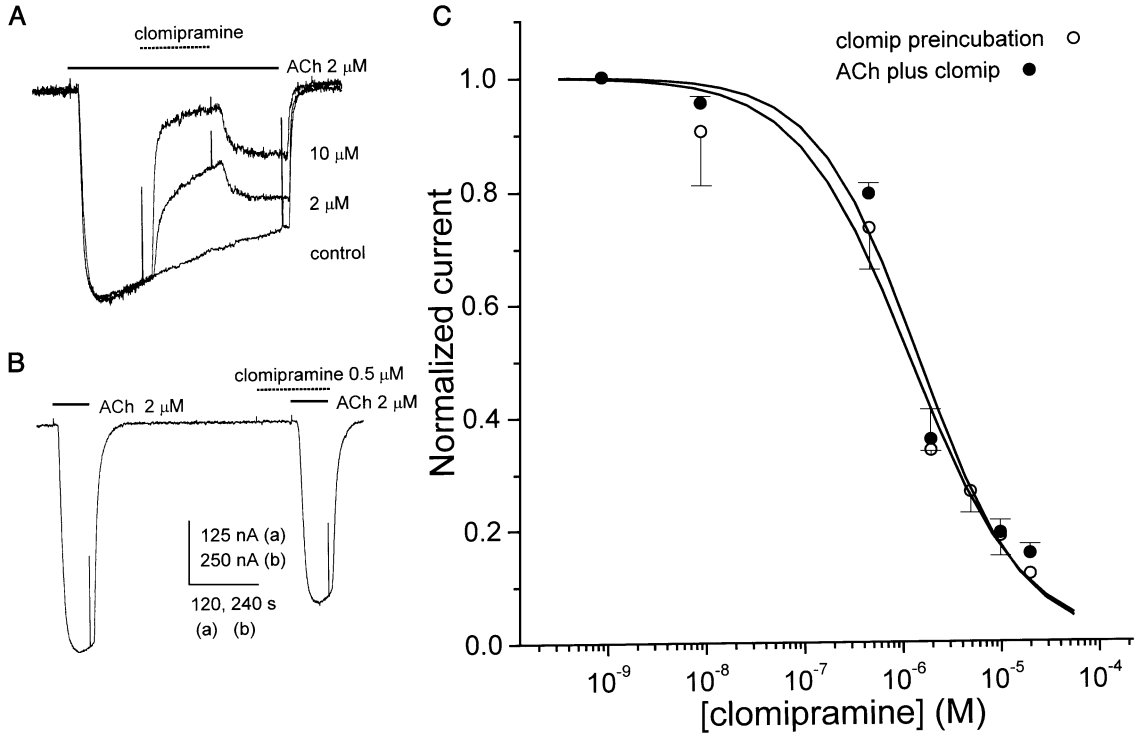

Fig. 1. The inhibition of acetylcholine response by clomipramine. (A) Superimposed records of the control current elicited by acetylcholine (ACh) and those in the presence of clomipramine at the indicated concentrations. (B) Representative records of the control current and that obtained by preincubating an oocyte with clomipramine and then coapplying acetylcholine and clomipramine. (C) Clomipramine concentration/current response relationships when clomipramine (clomip) was applied after the current reached the peak (●) or when the oocyte was preincubated with clomipramine (○). Membrane currents in the presence of clomipramine were normalized to the control current. Data were fitted with the Hill equation (—). In this and subsequent figures, the bars above the records indicate the time that acetylcholine and clomipramine were applied, and was also marked with 10 mV pulses to monitor membrane conductance. Data were from three oocytes.

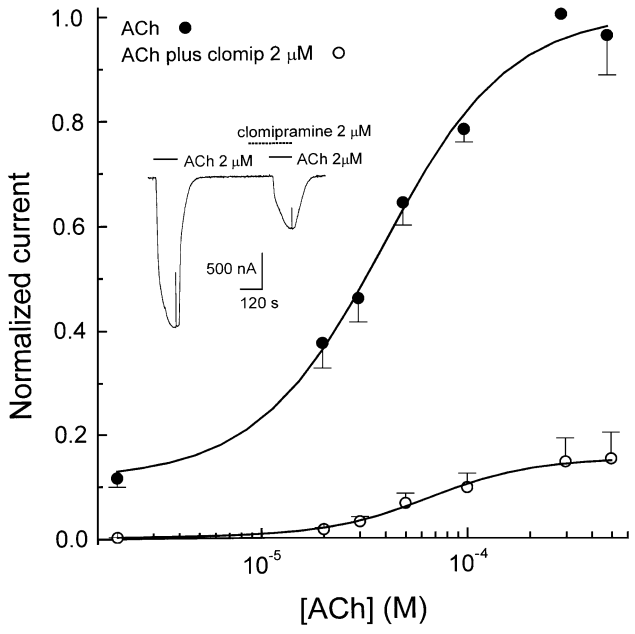

Fig. 2. Acetylcholine concentration/current response relation. The plots are concentration–response relationships for acetylcholine alone and acetylcholine plus clomipramine. The amplitudes of membrane currents were normalized to the maximal control current and fitted with the Hill equation (—). *Inset*: representative records of control current and that in the presence of clomipramine. Data were from three oocytes.

degree of inhibition and the level of recovery of the current depended on the concentration of clomipramine; at high concentrations, the recovery was slower and incomplete.

The half-maximal inhibitory concentration, IC_{50}, for clomipramine on membrane current elicited by 2 μM acetylcholine was 1.5 ± 0.3 μM ($n = 3$), with a Hill coefficient of 0.86 ± 0.12 (Fig. 1C). Similarly, when oocytes were first preincubated with clomipramine and then exposed to acetylcholine plus clomipramine (Fig. 1B), the IC_{50} was 1.3 ± 0.2 μM ($n = 3$), and the Hill coefficient 0.78 ± 0.11 (Fig. 1C). These results indicate that clomipramine interacts with α2β4 nicotinic acetylcholine receptors at a single site, and that, to exert the inhibitory effect, clomipramine acts on the activated receptor.

3.2. Noncompetitive inhibition of nicotinic acetylcholine receptors by clomipramine

In order to explore further the mechanism of inhibition of the acetylcholine response by clomipramine, we determined full acetylcholine concentration/current response relationships for acetylcholine alone and for acetylcholine in the presence of clomipramine. The oocytes were first preincubated (2 min) with clomipramine (2 μM) and then acetylcholine was added (2–500 μM, cf. Fig. 2). The results were well fitted by the Hill equation, allowing the estimation of both the half-maximal effective concentration, EC_{50}, of acetylcholine and the Hill coefficient, nH. These parameters were 39.9 ± 2.1 μM and 1.40 ± 0.25 ($n = 3$) for acetylcholine alone, values which are similar to those previously reported (López-Valdés and García-Colunga, 2001); in the presence of clomipramine, these values were 65.7 ± 3.6 μM and 1.76 ± 0.16. Additionally, the inhibition of acetylcho-

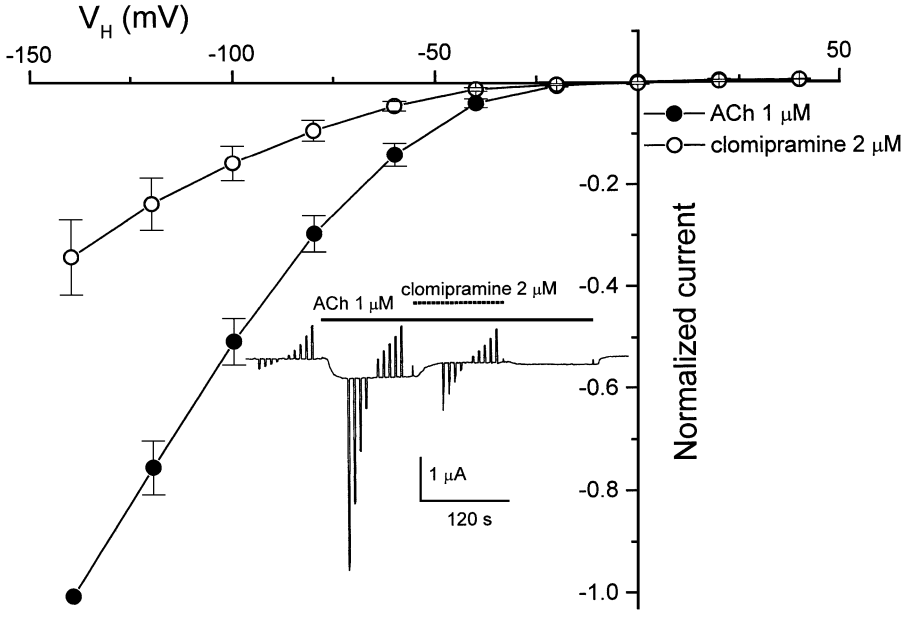

Fig. 3. Voltage dependence of the inhibition of acetylcholine response. Current/voltage relationships in the absence and presence of clomipramine. The membrane potential was held at −60 mV and voltage pulses (20 mV) were applied from −140 to 40 mV in normal Ringer, during acetylcholine and in acetylcholine plus clomipramine. The magnitudes of membrane currents were normalized to the control current at −140 mV and were plotted against membrane potential. *Inset*: representative current induced by acetylcholine alone and acetylcholine plus clomipramine. Voltage pulses were applied as described above. Data were from six oocytes.

line response by clomipramine was insurmountable by increasing the acetylcholine concentration (Fig. 2), suggesting a noncompetitive inhibition.

3.3. Voltage-dependent inhibition of nicotinic acetylcholine receptors by clomipramine

Studies of the effects of a substance on a receptor while the membrane potential is held at different levels provide some information on the site and mechanism of action of the substance (Woodhull, 1973; García-Colunga and Miledi, 1996). Therefore, we examined the inhibitory effects of clomipramine on currents elicited by acetylcholine as a function of membrane potential. A typical experiment is illustrated in the inset of Fig. 3. Acetylcholine was applied to an oocyte expressing $\alpha2\beta4$ nicotinic acetylcholine receptors, while the membrane potential was held at -60 mV; voltage pulses to different levels (from -140 to 40 mV in 20-mV steps) were applied before acetylcholine, during acetylcholine and in clomipramine plus acetylcholine superfusion.

The control current to voltage relationship (Fig. 3, ●) shows an inward rectification (García-Colunga and Miledi, 1999b). At positive voltages, the magnitude of the current was near zero with no clear-cut reversal of the current. In the presence of clomipramine, the current was slightly more inhibited at hyperpolarized potentials than at depolarized potentials.

To evaluate the voltage dependence of the inhibition of acetylcholine response by clomipramine, a comparison was made of the membrane current in the presence and absence of clomipramine at each membrane potential; the results were analyzed using a simple one-site blocking model (Woodhull, 1973; García-Colunga and Miledi, 1996) that allows estimation of the electrical distance, which corresponds to the fraction of the electrical field sensed at the binding site of clomipramine within the ion channel. The calculated electrical distance was 0.14 ± 0.06 ($n = 6$), indicating that clomipramine interacts with the $\alpha2\beta4$ nicotinic acetylcholine receptor within the pore of the receptor–channel complex, close to the extracellular end of the ionic channel. The concentration of clomipramine for the half-maximal inhibition at 0 mV, $IC_{50}(0)$, was 1.6 μM. These values are similar to those found for imipramine (an electrical distance of 0.10 and an $IC_{50}(0)$ of 1.2 μM) in the same subtype of nicotinic acetylcholine receptor (López-Valdés and García-Colunga, 2001). All of these suggest the same site and mechanism of action for both substances.

3.4. Effect of clomipramine on the decay of membrane current

A fairly general property of neurotransmitter receptors is that the membrane current generated by a prolonged exposure to an agonist is not maintained, but decays even in the continuous presence of the agonist, presumably because the receptor undergoes a conformational change from an active state to a desensitized form (Katz and Thesleff, 1957).

The control membrane current elicited by acetylcholine (100 μM) reached its peak and then declined in the continuous presence of acetylcholine (Fig. 4A). When the oocyte was simultaneously superfused with acetylcholine and clomipramine, the peak current was substantially reduced and the current decay was accelerated (Fig. 4B).

The decay of membrane currents in the absence or in the presence of clomipramine was compared in two ways. First, we measured the fraction of the current that was inhibited after 5-min exposure to acetylcholine alone or acetylcholine plus clomipramine, compared with their respective peak currents. In the presence of clomipramine, this value was higher than for the control current (Table 1).

In the second method, the decay of membrane currents was fitted to exponential functions. Control currents decayed during the acetylcholine application, and were

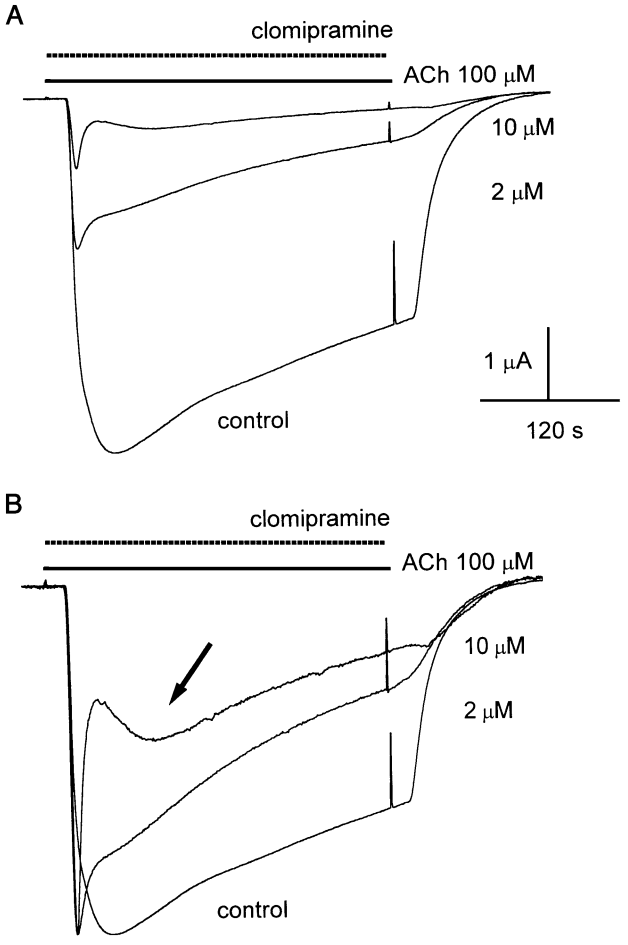

Fig. 4. Increase of the current decay by clomipramine. (A) Representative superimposed records of the control current and those in the presence of clomipramine. (B) Normalized records (cf. a). The time course of decay of the control current was fitted by one exponential function and the two phases of decay of the current in the presence of clomipramine were fitted by two exponential functions. The arrow illustrates a transient increase of the current in the presence of clomipramine (see text).

Table 1
Amplitudes and time constants of the decay components of membrane currents

Parameters[a]	ACh 100 μM (8)	ACh 100 μM + 2 μM clomipramine (4)	ACh 100 μM + 10 μM clomipramine (4)
D_{ft}	0.60 ± 0.09	0.74 ± 0.05	0.94 ± 0.03
I_o	0.34 ± 0.05	0.11 ± 0.04	0.03 ± 0.02
A_f	0.66 ± 0.05	0.37 ± 0.11	0.66 ± 0.13
τ_f (s)	219.7 ± 13.6	3.7 ± 0.5	4.1 ± 0.6
A_s	–	0.54 ± 0.13	0.31 ± 0.13
τ_s (s)	–	167.2 ± 41.9	142.8 ± 32.9
A_s/A_f	–	1.46	0.46

[a] D_{ft} is the fraction of the current that was inhibited after 5-min exposure to acetylcholine (ACh) alone or acetylcholine plus clomipramine, compared with their respective peak currents. Amplitudes of the fast (A_f) and slow components (A_s) and the asymptotic component (I_o) were normalized to the total current amplitude. The application of acetylcholine and clomipramine was simultaneous. Each value represents mean ± S.E. and the number of oocytes studied is in parentheses.

fitted with a single time constant and a steady-state component. The decay of membrane currents in the presence of clomipramine was very fast, followed markedly by a transient increase of the current (Fig. 4B, arrow) and then continued to decay with a slower time course. This effect was more pronounced as the concentration of clomipramine was increased (Fig. 4B). The first phase of decay was fitted with a fast time constant, τ_f. The second phase of decay was fitted with a slow time constant, τ_s, and a steady-state component, I_o.

At high clomipramine concentration, the fast amplitude component, A_f, contributed more to the membrane current than the slow amplitude component, A_s. Both time constants (τ_f and τ_s) of current decay in the presence of clomipramine were faster than the time constant of the control current decay (Table 1 and Fig. 4). In addition, the two time constants of decay were similar for the two concentrations of clomipramine tested.

4. Discussion

The present study shows that clomipramine inhibits neuronal α2β4 nicotinic acetylcholine receptors in a non-competitive manner, similarly to its effects on nicotinic acetylcholine receptors from the electric organ of *Torpedo* (Aronstam and Narayanan, 1981). Moreover, other substances such as imipramine, a tricyclic antidepressant with a structure very similar to clomipramine, desipramine, fluoxetine, an inhibitor of serotonin uptake, amitriptyline, nortriptyline, and several serotonergic agents, including 5-HT, 8-hydroxy-2-(di-*n*-propylamino)tetralin, spiperone, methysergide and ketanserin, also regulate *Torpedo*, muscle and neuronal nicotinic acetylcholine receptors (Aronstam, 1981; Aronstam and Narayanan, 1981; Eldefrawi et al., 1981; Schofield et al., 1981; Connolly et al., 1992; García-Colunga et al., 1997; Maggi et al., 1998; García-Colunga and Miledi, 1999a; López-Valdés and García-Colunga, 2001).

Because of the high diversity of nicotinic acetylcholine receptor subtypes are widely distributed throughout the peripheral and central nervous systems and are involved in a diversity of brain functions and malfunctions (Clementi et al., 2000; Paterson and Nordberg, 2000; Picciotto et al., 2000), we decided to continue with the pharmacological characterization of the rat neuronal α2β4 nicotinic acetylcholine receptors (García-Colunga and Miledi, 1995, 1997, 1999b; García-Colunga et al., 1997, 2001; López-Valdés and García-Colunga, 2001). Particularly, the mRNA encoding for these subunits are located in the cortex and the mesocorticolimbic system (Clementi et al., 2000), brain structures that are altered during mental depression (Drevets, 2000).

The inhibition of acetylcholine response by clomipramine slightly depended on the membrane potential, probably at negative potentials clomipramine, is stronger drawn into the ion channel, accounting for the increased rate decay and the slow recovery of the current. Taking into account the electric distances for the actions of imipramine (0.12), fluoxetine (0.23) and other serotonergic agents (~ 0.20) on nicotinic acetylcholine receptors (García-Colunga and Miledi, 1995, 1999a; López-Valdés and García-Colunga, 2001) and that obtained for clomipramine in this work (0.14), it is possible that these substances all act within the ion channel of the receptor, but involve two or more sites. Thus, the open-channel blockage of nicotinic acetylcholine receptors by antidepressants and other serotonergic agents seems to be the most common mechanism of inhibition (Arias, 1998).

The variable inhibitory potency of different antidepressants on nicotinic acetylcholine receptors may depend on molecular differences in the various subunits that make up the heteromultimeric nicotinic acetylcholine receptors (Le Novère and Changeux, 1999). This is exemplified by muscle nicotinic acetylcholine receptors, in which the omission of the δ-subunit abolishes its inhibition by 5-HT (García-Colunga and Miledi, 1996). Given the very wide diversity of neuronal nicotinic acetylcholine receptors and the many behavioral effects associated with them (Picciotto et al., 2000), more detailed studies of clomipramine and other antidepressants on the different types of nicotinic acetylcholine receptors are needed.

On the other hand, the total concentration of clomipramine in blood sera of human patients undergoing treatment for panic disorder is about 0.3 μM (Marcourakis et al., 1999; Herrera et al., 2000), and the IC_{50} obtained in this work for neuronal α2β4 nicotinic acetylcholine receptors was ~ 1.5 μM. Moreover, the total concentration within the brain can be ~ 13-fold higher than in blood serum (Weigmann et al., 2000). Therefore, the effects of clomipramine on nicotinic acetylcholine receptors appear to be clinically important because, at therapeutic doses, it targets at least the 5-HT transporter and nicotinic acetylcholine receptors.

Interestingly, desmethylclomipramine, the major metabolite of clomipramine, which is twice as concentrated than clomipramine in blood sera (Marcourakis et al., 1999; Herrera et al., 2000) and whose concentration in the brain can be approximately eightfold higher than in blood sera (Weigmann et al., 2000), inhibits *Torpedo* nicotinic acetylcholine receptors with similar potency to that of clomipramine (Aronstam and Narayanan, 1981). Thus, it will be important also to examine the effects of desmethylclomipramine on neuronal nicotinic acetylcholine receptors.

It now seems clear that a common characteristic of most antidepressants is that they do not only inhibit the reuptake of neurotransmitters; but they inhibit also different neurotransmitter receptors (Richelson and Nelson, 1984; Cusack et al., 1994). Therefore, in the treatment of depression with strong inhibitors of 5-HT reuptake, such as imipramine, clomipramine or fluoxetine, it is possible that additional inhibitory effects on nicotinic acetylcholine receptors and other membrane proteins may occur (García-Colunga et al., 1997; Ni and Miledi, 1997; Maggi et al., 1998; Fryer and Lukas, 1999a,b; García-Colunga and Miledi, 1999a; Hennings et al., 1999; López-Valdés and García-Colunga, 2001). Moreover, when the 5-HT transporter is inhibited, the extracellular levels of 5-HT are increased in which case 5-HT itself might inhibit nicotinic acetylcholine receptors (Grassi et al., 1993; García-Colunga and Miledi, 1994, 1995, 1996, 1999a). It is possible that the actions of antidepressants on various types of neurotransmitter receptors and ionic channels contribute to their beneficial effects and or to secondary undesirable actions.

Finally, it may also be relevant to examine the effects of inhibition of nicotinic acetylcholine receptors by clomipramine in therapies for smoking cessation because antidepressants, such as bupropion, fluoxetine, nortriptyline, and the noncompetitive blocking agent of nicotinic acetylcholine receptors mecamylamine, are effective for smoking cessation. The mechanisms of action are not well understood, but a proposal is that these antidepressants participate by modulating the levels of norepinephrine and dopamine (Leischow and Cook, 1999). Considering that clomipramine shows similar inhibitory characteristics on monoamine reuptake to those drugs effective for smoking cessation (Richelson, 1994), and that clomipramine also inhibits nicotinic acetylcholine receptors as other antidepressants do (Schofield et al., 1981; Connolly et al., 1992; Fryer and Lukas, 1999b; García-Colunga et al., 1997; Maggi et al., 1998), it is justifiable to presume that clomipramine may facilitate smoking cessation.

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