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Inhibition of neuronal nicotinic acetylcholine receptors by La^{3+}

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Abstract

A study was made of the effects of La^{3+} on neuronal $\alpha2\beta4$ nicotinic acetylcholine receptors expressed in *Xenopus* oocytes. La^{3+} by itself (up to 10 μM) did not elicit significant membrane currents. However, La^{3+} reversibly inhibited the ionic currents induced by acetylcholine ($IC_{50} = 13.5 \pm 4.3$ μM). When La^{3+} and acetylcholine were simultaneously applied onto an oocyte, the level of inhibition of the acetylcholine response was the same as when the oocyte was first preincubated with La^{3+} and then exposed to acetylcholine plus La^{3+}. In the presence of La^{3+}, the EC_{50} decreased from 43.8 ± 6.4 to 26.5 ± 5.1 μM, suggesting a small increase in the affinity of acetylcholine for the receptors through a noncompetitive mechanism. The inhibition of acetylcholine response was independent of the membrane potential. From these results we conclude that La^{3+} regulates nicotinic receptors, reversibly and noncompetitively, presumably by inhibiting allosterically the receptor through interactions at an external domain of the receptor complex.

Keywords: Noncompetitive inhibition; Nicotinic receptor modulation; *Xenopus* oocyte; Metal ion

1. Introduction

Neuronal nicotinic acetylcholine receptors are neurotransmitter-gated channels that belong to a gene superfamily that includes γ-aminobutyric acid (GABA) types A- and C-, glycine- and 5-hydroxytryptamine (5-HT) type 3 receptors. These receptors are made up by combinations of multiple homologous subunits arranged around a central ionic channel (Lukas et al., 1999; Clementi et al., 2000). Structurally, most types of neuronal nicotinic acetylcholine receptors are *heteromeric*, made up of two different kinds of subunits: at least two α subunits and three β subunits, with a presumed pentameric stoichiometry (McGehee and Role, 1995). In contrast, the neuronal α7, α8 and α9 subunits have the ability to form functional *homomeric* nicotinic receptors (McGehee and Role, 1995; Lukas et al., 1999). Functionally, nicotinic acetylcholine receptors are activated by the neurotransmitter acetylcholine and are regulated by a wide variety of endogenous and exogenous agents (nicotinic receptor agonists and antagonists, local anesthetics, serotonergic compounds, polyvalent ions, etc.) with very diverse and unrelated structures (Lukas and Bencherif, 1992; Grassi et al., 1993; García-Colunga and Miledi, 1995, 1996, 1999; Palma et al., 1997; Arias, 1998; Maggi et al., 1998).

It is known that lanthanides interact with many cellular components such as nucleoproteins, amino acids, phospholipids, enzymes and intermediary metabolites (Das et al., 1988). In particular, La^{3+} is a substitute of Ca^{2+} for some cellular processes (Sun and Petersheim, 1990). Furthermore, it is also known that La^{3+} affects the process of synaptic transmission at various levels. For instance, La^{3+} causes a very large increase in the frequency of spontaneous release of transmitter quanta (Heuser and Miledi, 1971; Miledi, 1971; Miledi et al., 1983), but abolishes the release of acetylcholine caused by nerve impulses by blocking the influx of Ca^{2+} into the nerve terminals (Miledi, 1966, 1971). In bovine chromaffin cells La^{3+}, which directly triggers the release of noradrenaline, is more effective than Ca^{2+} (Powis et al., 1994). Moreover, La^{3+} also has strong effects on a wide variety of membrane proteins, including voltage- and ligand-gated ionic channels (Miledi, 1971; Nathan et al., 1988; Calvo et al., 1994; Powis et al., 1994; García-Colunga and Miledi, 1997b). Of special interest is the fact that La^{3+} augments the responses of heteromeric $GABA_A$ and homomeric $GABA_C$ receptors (Calvo et al., 1994; Narahashi et al., 1994). Furthermore, La^{3+} exerts opposite effects on different

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types of nicotinic receptors: enhancing the acetylcholine response in muscle nicotinic receptors whilst inhibiting the acetylcholine responses elicited by several types of neuronal nicotinic receptors (García-Colunga and Miledi, 1997b). Notwithstanding this wealth of information, a more detailed characterization of the effects of La^{3+} on nicotinic receptors is still needed. The present work is an effort in that direction.

2. Materials and methods

The methods used were essentially as previously described (Miledi, 1982; García-Colunga and Miledi, 1995). Briefly, plasmids containing cDNA clones encoding rat neuronal α2 or β4 nicotinic acetylcholine receptor subunits were used to obtain cRNAs, which were resuspended in RNase-free water at a concentration of 1 μg/μl. A mixture was made with equal quantities of each subunit cRNA and stored at −80 °C for subsequent injection into *Xenopus* oocytes.

Xenopus laevis follicles (Xenopus I or Nasco) were manually isolated from the ovary, and maintained at 16–18 °C in Barth's solution (88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 5 mM HEPES, pH adjusted to 7.4 with NaOH and supplemented with 0.1 mg/ml gentamicin sulfate). One day later, each oocyte was injected with 0.05–50 ng of the cRNA mixture; and 2 days after injection, the oocytes were treated with collagenase in normal Ringer (140 units/ml, type I, Sigma) for 0.5–1 h to remove the enveloping epithelial and follicular cells (Miledi and Woodward, 1989).

Membrane currents were recorded 3 to 9 days after cRNA injection, using a voltage-clamp with two microelectrodes filled with 3 M KCl. The oocytes were placed in a recording chamber continuously perfused (7–10 ml/min), at room temperature (20–23 °C), with normal frog Ringer's solution (115 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 5 mM HEPES, pH adjusted to 7.0 with NaOH). Ionic currents were recorded with a digital oscilloscope (Nicolet 310) and stored for subsequent analyses using a program written by Rico Miledi. The acetylcholine and $LaCl_3$, made daily from concentrated stocks, were diluted in normal Ringer's solution and applied in the superfusion system. Unless otherwise indicated, the membrane potential was held at −60 mV. Data are presented as the mean ± standard error (S.E.).

3. Results

3.1. Inhibition of acetylcholine responses by La^{3+}

When La^{3+} was applied alone (at concentrations up to 10 μM), to either injected or noninjected oocytes, no obvious membrane currents were observed. Concentrations between 0.1 and 100 nM La^{3+} did not alter the acetylcholine response; whilst at concentrations above 100 nM La^{3+} reversibly inhibited the acetylcholine responses (Fig. 1). The extent of the inhibition, and the recovery of the acetylcholine response, depended on the concentration of La^{3+}; with the inhibition being greater and the recovery being slower as the concentration of La^{3+} increased (Fig. 1). Concentrations above 1 mM La^{3+} abolished completely the acetylcholine responses. The inhibiting effect of La^{3+} was assessed as the ratio between the current elicited by acetylcholine plus La^{3+} over the control acetylcholine response value at the end of the 2-min La^{3+} application, thus giving the fraction of the acetylcholine response that remained at that time (Fig. 2). The half-inhibitory La^{3+} concentration (IC_{50}), obtained by fitting the data with the Hill equation (Hill, 1909), was 13.5±4.3 μM ($n=13$) and the Hill coefficient was 0.45±0.06.

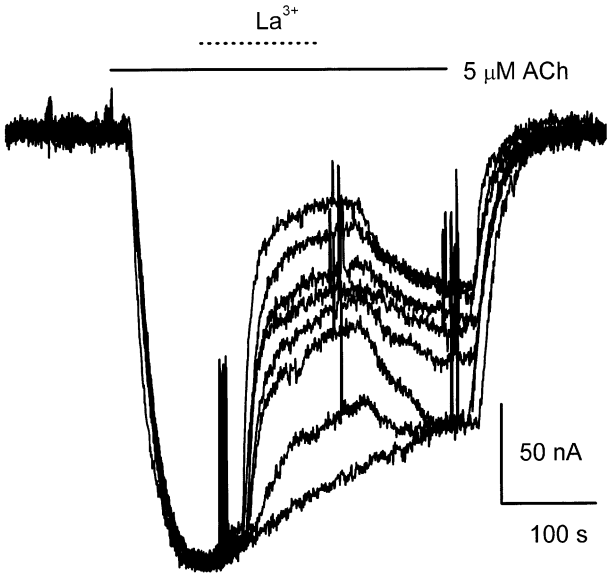

Fig. 1. Inhibition of acetylcholine responses by La^{3+}. Superimposed currents elicited by acetylcholine in one oocyte expressing neuronal α2β4 nicotinic receptors. Acetylcholine was applied at ~10-min intervals and after the current had reached its peak, La^{3+} was added at the following concentrations (from below): 2, 10, 20, 50, 100, 200 and 400 μM La^{3+}. In this and subsequent figures, the timing of acetylcholine and La^{3+} application are indicated by bars above the records, and by 10-mV depolarizing pulses used to monitor membrane conductance.

3.2. Effects of La^{3+} applied in different ways

To learn more about the actions of La^{3+}, this was applied in various ways. First: a control acetylcholine response was obtained and after a 10-min recovery the oocyte was exposed simultaneously to the same concentration of acetylcholine plus 10 μM La^{3+} (Fig. 3A,B). The percent inhibition of the peak current was 45 ± 4% when acetylcholine was 5 μM and 39 ± 4% when it was 100 μM ($n=5$ oocytes). Second: after a control current the oocyte was preincubated with La^{3+} alone and then exposed simultaneously to acetylcholine plus La^{3+} (Fig. 3C,D). The inhibition of the peak acetylcholine response was 51 ± 2% for

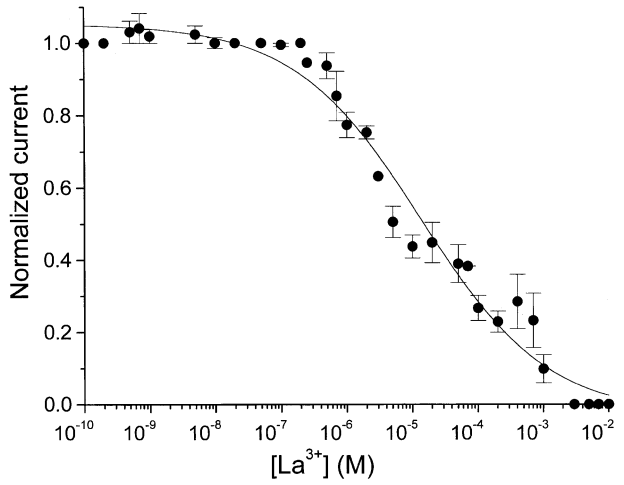

Fig. 2. La^{3+} concentration/acetylcholine response inhibition relationship. Current amplitudes in the presence of La^{3+} were normalized to the acetylcholine response in the absence of La^{3+} (c.f. Fig. 1) and plotted as a function of La^{3+} concentration. Data obtained from 13 independent experiments were fitted with the Hill equation (continuous line).

currents elicited by 5 μM acetylcholine and 58 ± 3% for those elicited by 100 μM acetylcholine ($n = 5$ oocytes). In a third form of application La^{3+} was applied after the acetylcholine response had reached its peak (Fig. 3E,F). At the end of La^{3+} application, the inhibition of acetylcholine response was 49 ± 4% and 62 ± 5% for 5 and 100 μM acetylcholine, respectively ($n = 5$ oocytes).

It is important to note that the percentage of acetylcholine response inhibition by La^{3+} was different for the peak currents than for the currents remaining at the end of 2-min application of acetylcholine. For example, when acetylcholine and La^{3+} were simultaneously applied, the acetylcholine response decrease was smaller at the peak of the current than at the end of La^{3+} application (45 ± 4% against 51 ± 6% with 5 μM acetylcholine and 39 ± 4 % against 57 ± 3% with 100 μM acetylcholine). However, when the oocyte was first preincubated with La^{3+} and then exposed simultaneously to acetylcholine plus La^{3+}, the level of inhibition was the same at the peak of the current and at the end of La^{3+} application (51 ± 2% against 51 ± 2% with 5 μM acetylcholine and 58 ± 3% against 61 ± 4% with 100 μM acetylcholine). All data are the mean ± S.E. from 11 oocytes. Moreover, the results show that the inhibition of acetylcholine response by La^{3+} was stronger when the current was elicited with 100 μM than with 5 μM acetylcholine. These results are opposite to those expected if La^{3+} ions were interacting at the acetylcholine binding sites, indicating noncompetitive inhibition.

3.3. Effect of La^{3+} on the acetylcholine-concentration/acetylcholine-response relationship

To examine in more detail the inhibitory mechanism of nicotinic receptors by La^{3+}, we determined the full acetylcholine-concentration/response relationship for acetylcholine alone and for acetylcholine in the presence of La^{3+}. The inhibitory effect was measured at the end of La^{3+} application, ensuring that the system approached drug equilibrium.

As already well known, the current amplitude increased with increasing acetylcholine concentrations. With 10 μM

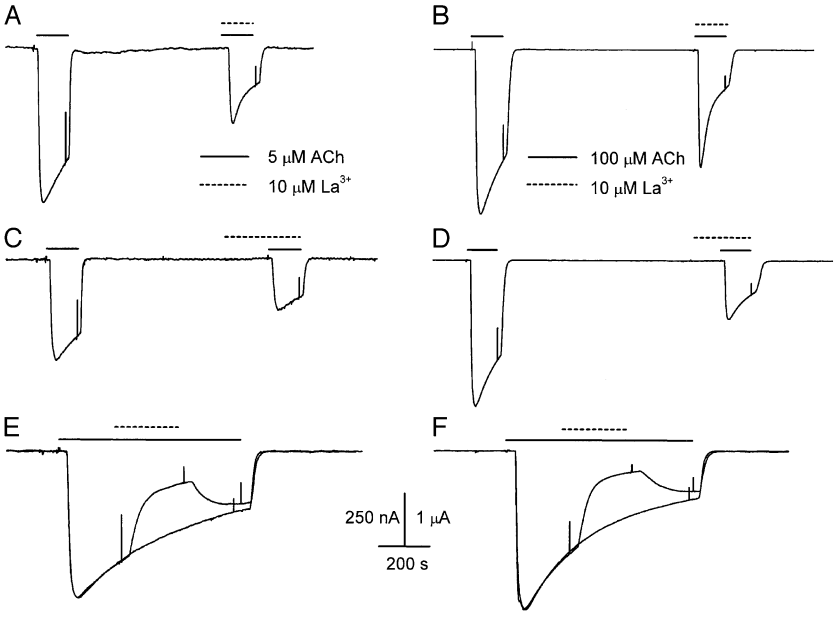

Fig. 3. Inhibition of acetylcholine response using different forms of La^{3+} application. Control currents were elicited with 5 (A, C, E) or 100 (B, D, F) μM acetylcholine alone, or together with 10 μM La^{3+} applied in different ways: simultaneously with acetylcholine (A, B); before the current was elicited by acetylcholine and in the presence of La^{3+} (C, D); and in the continuing presence of acetylcholine after the peak control current was reached (E, F; the control acetylcholine responses are superimposed to show the current trend in the absence of La^{3+}). All the records were obtained from the same oocyte expressing neuronal α2β4 nicotinic receptors.

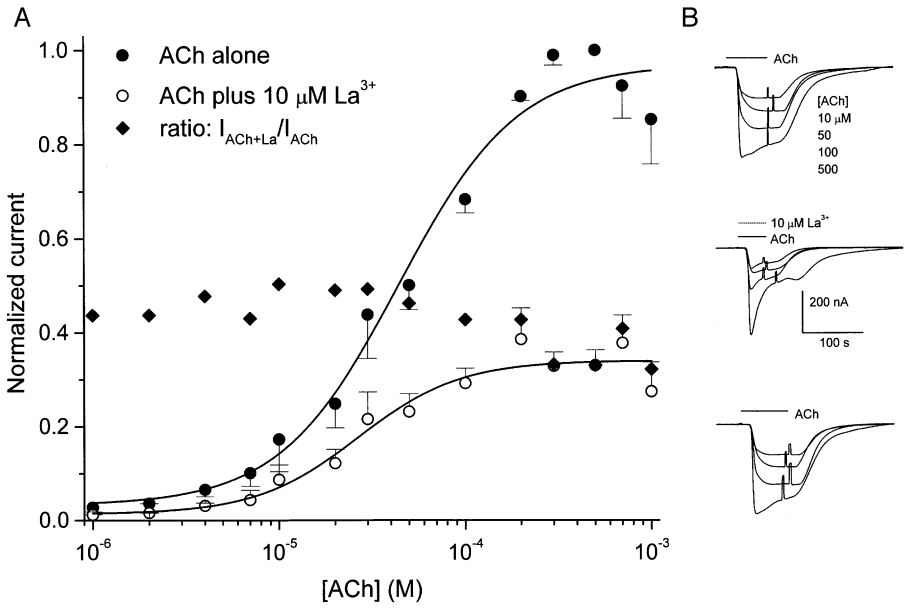

Fig. 4. Acetylcholine-concentration/acetylcholine-response relationships. (A) For acetylcholine alone (filled circles) and in the presence of La^{3+} (open circles). Data from five oocytes were normalized to the maximal control acetylcholine response and fitted to the Hill equation (continuous lines). Filled diamonds show the ratio between acetylcholine responses in the presence of La^{3+} and the control acetylcholine responses. (B) Representative and superimposed records of the control (top records), inhibited by La^{3+} (middle records) and recovered acetylcholine responses (bottom records), elicited by the acetylcholine concentrations indicated.

La^{3+}, the acetylcholine responses were reduced with equal potency, 0.43 ± 0.02 ($n = 5$ oocytes) of the control current when the acetylcholine concentration was 1–50 μM, and ~ 0.3 at higher acetylcholine concentrations (Fig. 4, filled diamonds). The acetylcholine-concentration/response relationships, in the absence or presence of La^{3+}, fitted with the Hill equation, yielded EC_{50} of 43.8 ± 6.4 μM in the absence of La^{3+} and 26.5 ± 5.1 μM in the presence of La^{3+}; whilst the Hill coefficients were 1.4 ± 0.3 and 1.5 ± 0.5, respectively. Thus, the slight decrease in EC_{50} caused by La^{3+} suggests that the affinity of neuronal $\alpha 2\beta 4$ nicotinic receptors for acetylcholine may be slightly increased by La^{3+}, probably through an allosteric interaction, whilst maintaining the same degree of cooperativity.

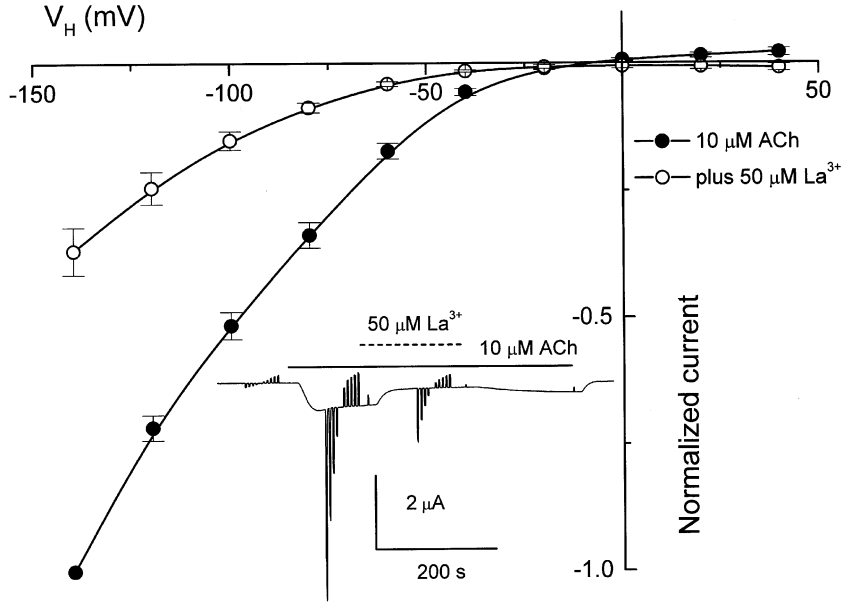

Fig. 5. Inhibition of acetylcholine response by La^{3+} as a function of membrane potential. Current to voltage relationships are shown in the absence and in the presence of La^{3+}. The results from five oocytes were normalized to the control acetylcholine responses at −140 mV. Inset: A representative current elicited by acetylcholine and its inhibition by La^{3+}. The membrane potential was held at −60- and 20-mV voltage pulses were applied from −140 to 40 mV, in normal Ringer solution, during acetylcholine alone and during co-application of acetylcholine and La^{3+}.

At acetylcholine concentrations higher than about 400 μM, the control current amplitude decreased, probably because acetylcholine itself blocks the ion-channel (Katz and Miledi, 1978; Sine and Steinbach, 1987).

3.4. La^{3+} inhibition as a function of membrane potential

The results described above indicate that La^{3+} does not compete with acetylcholine for the same site(s) but could interact with the nicotinic receptor at some other extracellular region of the protein, perhaps within the ionic channel, similar to curare or serotonergic substances (Katz and Miledi, 1978; García-Colunga and Miledi, 1996, 1999; García-Colunga et al., 1997; López-Valdés and García-Colunga, 2001). To discriminate between these possibilities, we studied the inhibition of acetylcholine responses by La^{3+} at different membrane potentials. For these experiments, the acetylcholine response was elicited and after it had reached its peak La^{3+} was added to the superfusing solution. The membrane potential was held at −60 mV and voltage pulses, from −140 to 40 mV in steps of 20 mV, were applied before and during acetylcholine application, as well as in the presence of La^{3+} plus acetylcholine (Fig. 5, inset).

The current to voltage ($I–V$) relationship was reduced by La^{3+} to about 30%, for membrane potentials between −140 and −40 mV. We then analyzed the control and inhibited acetylcholine responses at each membrane potential using a simple one-site blocking model (Woodhull, 1973; García-Colunga and Miledi, 1996), to calculate the 'electrical distance', which corresponds to the fraction of the electrical field sensed at a possible binding site for La^{3+} within the ionic channel. As already mentioned, the inhibition of acetylcholine responses by La^{3+} was independent of the membrane potential. This feature was also evident using the one-site blocking model, which gave an average electrical distance of 0.07 ($n=5$). All this indicates that La^{3+} acts outside the pore of the receptor-channel complex.

4. Discussion

Our results extend the notion that nicotinic acetylcholine receptors contain sites that recognize cations whose binding modifies receptor function (Miledi, 1966; Heuser and Miledi, 1971; García-Colunga and Miledi, 1997a,b; Palma et al., 1998; García-Colunga et al., 2001).

The rare-earth metal lanthanum is a member of the lanthanide group of metal ions, which are trivalent and somewhat similar in their chemical and biological properties to the divalent alkaline earth elements (Ca^{2+}, Mg^{2+}, Ba^{2+}, Ra^{2+}, etc.). Lanthanides have been used in dentistry (Vardimon et al., 1991); and in medicine as anti-cancer, anti-inflammatory and antiviral agents (Sedmak et al., 1986; Anghileri et al., 1987; Yamage and Evans, 1989; Canada and Carpentier, 1991). On the other hand, lanthanides are also used industrially in color TV, lasers, photographic cameras, semiconductors, binoculars and movie films. In view of the increasing and continued use of these elements, it is important to obtain detailed information on their cellular pharmacology and toxicology.

In contrast to its actions on $GABA_A$ and $GABA_C$ receptors (Calvo et al., 1994; Narahashi et al., 1994), La^{3+} itself did not generate significant membrane currents in neuronal α2β4 nicotinic receptors. However, La^{3+} reversibly and noncompetitively inhibited, in a voltage-independent manner, the acetylcholine responses gated by α2β4 nicotinic receptors. All these results suggest that La^{3+} binds to an extracellular site of the receptor. Nevertheless, La^{3+} does not compete with acetylcholine for its binding site because the acetylcholine response in the presence of 10 μM La^{3+} was reduced to 0.3–0.4 of the control current for all acetylcholine concentrations tested.

With simultaneous applications of acetylcholine and La^{3+}, when probably the drugs have not yet reached their equilibrium concentrations, the inhibition of acetylcholine response is less for the peak current than at the end of La^{3+} application. This may be due to differences in the diffusion and kinetics of acetylcholine and La^{3+} actions, and partly also to the blocking of open nicotinic receptors and acceleration of their desensitization. In contrast, by preincubating nicotinic receptors with La^{3+} the inhibition of acetylcholine response was the same for the peak current and at the end of La^{3+} application. Thus, in near equilibrium conditions, the degree of inhibition of the acetylcholine response was approximately the same with the three forms of La^{3+}application.

Based on the similarity of action among various lanthanides, it is very likely that they share the same binding site(s) (Narahashi et al., 1994). Nevertheless, it is clear that La^{3+} acts by different mechanisms depending on the type and on the molecular structure of the receptors. For example, in contrast to the inhibitory effect of La^{3+} on neuronal α3β4 and α2β4 nicotinic receptors, a potentiating effect has been observed on muscle nicotinic receptors (Heuser and Miledi, 1971; García-Colunga and Miledi, 1997a,b) and on $GABA_A$ and $GABA_C$ receptors (Calvo et al., 1994; Narahashi et al., 1994). On the other hand, the affinity of La^{3+} for different classes of ligand-gated ion channels extends over a wide range. The apparent dissociation constant of La^{3+} on $GABA_A$ receptors is 231 μM (Narahashi et al., 1994) and 135 μM for $GABA_C$ receptors (Calvo et al., 1994), whereas the half-inhibitory concentration of La^{3+} on α2β4 nicotinic receptors obtained here is 13.5 μM.

The fact that both the La^{3+} potentiation of the currents gated by $GABA_A$-, $GABA_C$- and muscle nicotinic-receptors, and the La^{3+} inhibition of α3β4 and α2β4 nicotinic receptor gated currents are independent of the membrane potential (Calvo et al., 1994; Narahashi et al., 1994; García-Colunga and Miledi, 1997b), indicates that the binding site for La^{3+}, although apparently located externally in all cases, is not the same for all ligand-gated ion channels; and indicates also that the mechanisms of action are not identical. The cations that

potentiate the responses of neurotransmitter receptors (Miledi, 1966; Heuser and Miledi, 1971; Ma et al., 1994; García-Colunga and Miledi, 1997a,b; García-Colunga et al., 2001) may have similar forms of action: increasing the channel open-time or increasing agonist affinity (Ma et al., 1994).

Further studies, including some at the single-channel level are required to determine the mechanisms whereby La^{3+} alters the properties of nicotinic receptors. It is apposite to mention here that lanthanides have been used in luminescence studies of metal-protein interactions, revealing that lanthanides can bind to proteins close to an aromatic acid, particularly in aromatic chains of tryptophan, tyrosine and phenylalanine (Sun and Petersheim, 1990). Altogether, these studies open the possibility that various lanthanide interaction sites are present on the same neurotransmitter receptor; a possibility that could be explored by using luminescence as well as electrophysiological probes. All these studies may help to understand the function and regulation of nicotinic acetylcholine receptors, which are widely distributed in the brain and which play very important roles in many physiological and behavioral processes (Lukas et al., 1999; Paterson and Nordberg, 2000).

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