Subunit-Dependent Modulation of Neuronal Nicotinic Receptors by Zinc

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We examined the effect of zinc on rat neuronal nicotinic acetylcholine receptors (nAChRs) expressed in Xenopus oocytes as simple heteromers of α2, α3, or α4 and β2 or β4. Coapplication of zinc with low concentrations of acetylcholine (≤EC10) resulted in differential effects depending on receptor subunit composition. The α2β2, α2β4, α3β4, α4β4, and α4β4 receptors exhibited biphasic modulation by zinc, with potentiation of the acetylcholine response occurring at 1–100 μM zinc and inhibition occurring at higher zinc concentrations. In contrast, α3β2 receptors were only inhibited by zinc (IC50 = 97 ± 16 μM). The greatest potentiating effect of zinc was seen with α4β4 receptors that were potentiated to 560 ± 17% of the response to ACh alone, with an EC50 of 22 ± 4 μM zinc. Cadmium, but not nickel, was also able to potentiate α4β4 receptors. Both zinc potentiation of α4β4 receptors and zinc inhibition of α3β2 receptors were voltage independent. The sensitivity of zinc potentiation of α4β4 to diethylpyrocarbonate treatment and alterations in pH suggested the involvement of histidine residues. Zinc continued to inhibit α4β4 and α3β2 after diethylpyrocarbonate treatment. Application of a potentiating zinc concentration increased the response of α4β2 and α4β4 receptors to saturating ACh concentrations. The rate of ACh-induced desensitization of these receptors was unaffected by zinc. Our results reveal zinc potentiation as a new mode of neuronal nAChR modulation.

Key words: neuronal nicotinic receptors; zinc; potentiation; inhibition; modulation; acetylcholine

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels found at the neuromuscular junction and throughout the CNS and PNS. Neuronal nAChRs are similar to muscle nAChRs: they are formed as pentameric assemblies of subunits (Anand et al., 1991; Cooper et al., 1991). To date, the neuronal nAChR subunit family consists of nine α subunits (α2–α10) and three β subunits (β2–β4) (Corringer et al., 2000). These subunits can assemble, in exogenous expression systems, in various combinations to form receptors with varying functional and pharmacological properties (Role, 1992). In the nervous system, neuronal nAChRs can form as pentameric homomers (such as α7 receptors) (Chen and Patrick, 1997; Drisdel and Green, 2000), as simple heteromers composed of a single type of α subunit and a single type of β subunit (such as α2β2 receptors) (Whiting et al., 1991; Flores et al., 1992), or as complex heteromers of three or more subunits (such as α3β4α7 receptors) (Conroy and Berg, 1995).

Ionic zinc has been found in neurons throughout the brain, with highest concentrations in the cerebral cortex and limbic areas (Frederickson et al., 2000). Zinc is localized to small, clear vesicles in synaptic terminals and is released in a calcium-dependent manner (Assaf and Chung, 1984; Howell et al., 1984). The extracellular concentration of zinc is estimated to reach concentrations as high as 300 μM (Assaf and Chung, 1984). Zinc modulates the functions of members of several ligand-gated ion channel families, including glutamate, GABA, glycine, and ATP receptors (Mayer et al., 1989; Draguhn et al., 1990; Rassendren et al., 1990; Cloues et al., 1993; Bloomenthal et al., 1994; Paoletti et al., 1997; Krishk et al., 1998; Harvey et al., 1999; Xiong et al., 1999; Laube et al., 2000). Both potentiation and inhibition of agonist-induced responses have been observed. The ability to modulate ligand-gated ion channel function suggests that zinc may be an important modulator of synaptic activity.

Relatively few studies have examined the effect of zinc on neuronal nAChRs. Zinc was shown to block ACh responses of rat intracardiac parasympathetic neurons (Nutter and Adams, 1995). Zinc also attenuates the ACh-induced response of homomeric α7 nAChRs exogenously expressed in Xenopus oocytes (Palma et al., 1998). We have now examined the effect of zinc on the several neuronal nAChRs that can be formed by pairwise expression of the α2, α3, or α4 subunits with the β2 or β4 subunits in Xenopus oocytes. We find that although all subunit combinations are inhibited by high concentrations of zinc, some subunit combinations are potentiated by low zinc concentrations (≤100 μM). Moreover, the extent of potentiation by zinc varies markedly depending on receptor subunit composition. Potentiation by zinc represents a new mode of neuronal nAChR modulation.

MATERIALS AND METHODS

Materials. Xenopus laevis frogs were purchased from Nasco (Fort Atkinson, WI). The care and use of X. laevis frogs in this study were approved by the University of Miami Animal Research Committee and meet the guidelines of the National Institutes of Health. RNA transcription kits were from Ambion (Austin, TX). Collagenase B was from Boehringer Mannheim (Indianapolis, IN). All other reagents were from Sigma (St. Louis, MO).

Neuronal nAChR expression in X. laevis oocytes. m7G(5′)ppp(5′)G-capped cRNA transcripts encoding nAChR subunits were prepared by in vitro transcription from linearized template DNA encoding the α2, α3,
Zinc solutions used in all experiments shown in this study were prepared from zinc acetate stock solutions. To rule out the effect of the acetate, zinc concentration effect curves for the α3β2, α4β2, and α4β4 receptors were replicated using ZnCl2 with similar results (data not shown). In the absence of ACh, zinc concentrations <70 μM had no effect on α4β4-expressing, α3β2-expressing, or un.injected oocytes. Zinc concentrations >70 μM elicited small, slow, variable (inward and outward) currents. Because these currents were always <5% of ACh responses, they were disregarded in our analysis.

**Diethylpyrocarbonate treatment of nAChRs.** Diethylpyrocarbonate (DEPC) was diluted in perfusion solution immediately before application. Preliminary experiments determined that a 10 min incubation achieved a maximal DEPC effect (data not shown). During incubation, the DEPC solution was exchanged twice at regular intervals. After incubation, the DEPC was washed out of the chamber for an additional 10 min with perfusion solution. Electrophysiological measurements were taken before DEPC application and immediately after the 10 min wash-out period.

**Data analysis.** Data from metal concentration–response experiments for α2β2, α3β4, α3β4, α4β2, and α4β4 receptors were analyzed as follows. Concentration–potentiation curves were fit to the following equation for concentrations up to and including those concentrations of metals that achieved maximal potentiation: \( I = I_{\text{max}}/(1 + IC_{50}) \), where \( I \) represents the current response at a given metal concentration, \( X; I_{\text{max}} \) is the maximal current; \( IC_{50} \) is the concentration of metal yielding half-maximal potentiation; \( n \) is the Hill coefficient. Concentration–inhibition curves were fit according to the following equation for concentrations of metals at or above those concentrations that achieved maximal potentiation: \( I = I_{\text{max}}(1 + (EC_{50}/Y)^n) \), where \( I \) represents the current response at a given metal concentration, \( X; I_{\text{max}} \) is the maximal current; \( EC_{50} \) is the concentrations of metal yielding half-maximal inhibition; \( n \) is the Hill coefficient. Because no potentiation was apparent for the α3β2 receptor, the entire data set was fit with the concentration–inhibition equation.

The data presented in Figure 2 suggest that at some concentrations zinc may be exerting both potentiating and inhibiting effects on neuronal nAChRs. If this is true, then the maximal potentiation that we observe may be an underestimate. To examine this issue, we fit the zinc concentration–effect data to a more complex equation that included both a potentiating and an inhibitory site: \( I = I_{\text{max}} + \left( I_{\text{inhib}} - I_{\text{max}} \right) \left[1/(1 + (EC_{50}/Y)^n) - 1/(1 + (IC_{50}/Y)^n)\right] \), where \( I \) represents the current response at a given metal concentration, \( X; I_{\text{max}} \) is the minimal current; \( I_{\text{inhib}} \) is the maximal current; \( EC_{50} \) and \( IC_{50} \) are the concentrations of metal yielding half-maximal potentiation and inhibition, respectively; \( n \) and \( m \) are the Hill coefficients for potentiation and inhibition, respectively (Harvey et al., 1999). Results of this analysis suggest that the maximal potentiation that we observe is indeed a combination of potentiation and inhibition. Fitting to this equation suggests that the true maximal potentiation is severalfold greater than what we observe. However, because the measured data covers only the lower portion of the putative full curve, we have reported only the results of fitting the potentiating and inhibiting data sets separately.

ACh concentration–response curves in Figure 8 were fit to the following equation: \( I = \left( I_{\text{max}}(1 + (EC_{50}/Y)^n) \right) \), where \( I \) represents the current response at a given ACh concentration, \( X; I_{\text{max}} \) is the maximal current; \( EC_{50} \) is the concentration of ACh yielding half-maximal response; \( n \) is the Hill coefficient.

Plasmid software (GraphPad, San Diego, CA) was used to fit the data and to assess statistical significance using a two-tailed unpaired t test.
RESULTS

Zn$^{2+}$ modulates neuronal nAChRs

Simple heteromeric neuronal nAChRs consisting of one type of $\alpha$ subunit ($\alpha 2$, $\alpha 3$, or $\alpha 4$) and one type of $\beta$ subunit ($\beta 2$ or $\beta 4$) were expressed in Xenopus oocytes. Current responses were recorded under two-electrode voltage clamp on application of ACh (at or below the EC$_{50}$ for each receptor) in the absence or presence of various concentrations of Zn$^{2+}$. The effect of Zn$^{2+}$ varied depending on the subunit composition of the receptor.

Zn$^{2+}$ application had a biphasic effect on ACh responses of $\alpha 4\beta 2$-expressing oocytes. Zn$^{2+}$ concentrations in the range of 1–100 $\mu$M increased the inward current elicited by 10 $\mu$M ACh (Figs. 1A, top trace, 2C). A maximal potentiation of 260 ± 17% was achieved with 70 $\mu$M Zn$^{2+}$, with an EC$_{50}$ for potentiation of 16 ± 4 $\mu$M Zn$^{2+}$. At higher Zn$^{2+}$ concentrations, the degree of potentiation by Zn$^{2+}$ was diminished until at 1 mM Zn$^{2+}$, steady current in response to ACh + Zn$^{2+}$ was less than current in response to ACh alone (Figs. 1A, bottom trace, 2C). This apparent inhibition of $\alpha 4\beta 2$ receptors by Zn$^{2+}$ had an IC$_{50}$ of 440 ± 140 $\mu$M.

In contrast to the biphasic effect of Zn$^{2+}$ on $\alpha 4\beta 2$ nAChRs, $\alpha 3\beta 2$ receptors exhibited only inhibition by Zn$^{2+}$ (Fig. 1B, 2B). Reduction in the current elicited by 4 $\mu$M ACh occurred with Zn$^{2+}$ concentrations ranging from 1 $\mu$M to 3 mM, with an IC$_{50}$ of 97 ± 16 $\mu$M. Zn$^{2+}$ concentrations ≥1 mM almost completely blocked the response to ACh. The loss of Zn$^{2+}$ potentiation on changing the $\alpha$ subunit from $\alpha 4$ to $\alpha 3$ supports a role for the $\alpha$ subunit in mediating potentiation.

To determine whether $\beta$ subunits also play a role in Zn$^{2+}$ potentiation of nAChRs, we examined the effect of Zn$^{2+}$ on the $\alpha 5\beta 4$ receptor (Fig. 2B). Current in response to 17 $\mu$M ACh was potentiated to a maximum of 140 ± 2% at 200 $\mu$M Zn$^{2+}$, with an EC$_{50}$ of 47 ± 9 $\mu$M Zn$^{2+}$. Similar to our results with $\alpha 4\beta 2$
receptors, high concentrations of Zn$^{2+}$ inhibited the $\alpha_3\beta_4$ receptor (IC$_{50} = 3200 \pm 1400 \mu M$ Zn$^{2+}$).

The ability of $\alpha_4\beta_2$ and $\alpha_3\beta_4$, and the failure of $\alpha_3\beta_2$ nAChRs, to be potentiated by Zn$^{2+}$ suggests that the $\alpha_4$ and $\beta_4$ subunits are each capable of supporting Zn$^{2+}$ potentiation when they are present in a receptor. When both of these subunits were present in the same receptor ($\alpha_4\beta_4$), the effect of Zn$^{2+}$ was dramatic (Figs. 1C, 2C). The $\alpha_4\beta_4$ receptor was potentiated to a maximum of 560 ± 17% at 100 $\mu M$ Zn$^{2+}$, with an EC$_{50}$ of 22 ± 4 $\mu M$ Zn$^{2+}$. Again, higher concentrations of Zn$^{2+}$ were found to be inhibitory, with an IC$_{50}$ of 510 ± 37 $\mu M$ Zn$^{2+}$.

The $\alpha_2$ subunit is highly homologous to the $\alpha_4$ subunit. As might be expected, $\alpha_2\beta_2$ and $\alpha_2\beta_4$ receptors were also potentiated by low Zn$^{2+}$ concentrations and inhibited by high Zn$^{2+}$ concentrations (Fig. 2A; Table 1).

When high concentrations of Zn$^{2+}$ are coapplied with ACh to some subunit combinations, the current responses can be complex. A good example can be seen when 3 mM Zn$^{2+}$ is coapplied to the $\alpha_4\beta_4$ receptor (Fig. 1C, bottom trace). On coaplication of Zn$^{2+}$, a rapid transient potentiation is seen, followed by a rapid decrease in the response. On removal of Zn$^{2+}$, there is again a rapid potentiation of the current followed by a slow decline toward the response amplitude seen with ACh alone. These potentiation transients can be explained by considering the relatively slow fluid exchange rate in the perfusion chamber. When a high concentration of Zn$^{2+}$ is applied, the concentration of Zn$^{2+}$ in the chamber will pass through a concentration range that potentiates but does not inhibit the receptors, before reaching a final concentration that both potentiates and inhibits the receptors. Similarly, when Zn$^{2+}$ is withdrawn, the declining concentration in the chamber will again pass through a potentiating, but not inhibiting, concentration range. To determine whether this explanation is valid, we used a revised protocol in which Zn$^{2+}$ alone was applied to $\alpha_4\beta_4$ receptors with a maximal potentiation of 250 ± 26% and an EC$_{50}$ of 45 ± 24 $\mu M$ Cd$^{2+}$. At 1 mM Cd$^{2+}$ the potentiation was diminished, and at 3 mM Cd$^{2+}$ the Ach-induced response was almost completely inhibited. In contrast to Zn$^{2+}$ and Cd$^{2+}$, Ni$^{2+}$ had almost no ability to potentiate the ACh response. The maximal potentiation by Ni$^{2+}$ was only to 112 ± 5% of the response to ACh alone. Concentrations of Ni$^{2+}$ at or above 1 mM inhibited the ACh response.

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**Table 1. Potentiation and inhibition of neuronal nAChRs by Zn$^{2+}$**

<table>
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<tr>
<th>Receptor</th>
<th>Potentiation</th>
<th>Inhibition</th>
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<tr>
<td></td>
<td>Maximum (%)</td>
<td>EC$_{50}$ (\mu M)</td>
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<tr>
<td>$\alpha_2\beta_2$</td>
<td>140 ± 4</td>
<td>13 ± 7</td>
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<tr>
<td>$\alpha_2\beta_4$</td>
<td>200 ± 5</td>
<td>45 ± 6</td>
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<tr>
<td>$\alpha_3\beta_2$</td>
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<tr>
<td>$\alpha_3\beta_4$</td>
<td>140 ± 2</td>
<td>47 ± 9</td>
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<tr>
<td>$\alpha_4\beta_2$</td>
<td>260 ± 17</td>
<td>16 ± 4</td>
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<tr>
<td>$\alpha_4\beta_4$</td>
<td>560 ± 17</td>
<td>22 ± 4</td>
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<tr>
<td>$\alpha_3$-216-$\alpha_4\beta_4$</td>
<td>170 ± 5</td>
<td>9 ± 3</td>
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<tr>
<td>$\alpha_4$-216-$\alpha_3\beta_4$</td>
<td>250 ± 10</td>
<td>26 ± 4</td>
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Maximum ACh-induced current in the presence of Zn$^{2+}$ is presented as a percentage of the control response to ACh alone (see Materials and Methods). For potentiation, EC$_{50}$ and Hill coefficient (nH) values were determined by fitting to a Hill equation, and for inhibition, IC$_{50}$ and Hill coefficient (nH) values were determined by fitting to a separate Hill equation (see Materials and Methods). All values are the mean ± SEM of three to six oocytes.

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**Figure 3.** Zn$^{2+}$ preincubation eliminates potentiation transients seen with high Zn$^{2+}$ concentrations. A, Current responses of an $\alpha_4\beta_4$-expressing oocyte preincubated with 0, 100 $\mu M$, and 1 mM Zn$^{2+}$ for 20–30 sec before coaplication of 1 $\mu M$ ACh. Calibration: 50 nA, 10 sec. B, Current responses during coaplication of various concentrations of Zn$^{2+}$ and 1 $\mu M$ ACh were plotted as a percentage of the response to 1 $\mu M$ ACh alone recorded immediately before Zn$^{2+}$ preincubation (mean ± SEM of three oocytes).
Potential involvement of histidine residues in mediating Zn\textsuperscript{2+} potentiation

Histidine residues are often involved in coordinating Zn\textsuperscript{2+} at Zn\textsuperscript{2+}-binding sites. DEPC, under some conditions (pH \(\geq 6\), low millimolar concentrations), can selectively modify the imidazole ring of histidine, eliminating its ability to coordinate Zn\textsuperscript{2+} ions. We examined the effects of DEPC treatment on the ability of Zn\textsuperscript{2+} to modulate \(\alpha 4\beta 4\) and \(\alpha 3\beta 2\) receptors (Fig. 5). A 10 min incubation with DEPC concentrations ranging from 100 \(\mu\)M to 3 \(\mu\)M had little effect on the response of \(\alpha 4\beta 4\) receptors to ACh. Although treatment with 100 \(\mu\)M DEPC had a minimal effect on potentiation by 100 \(\mu\)M Zn\textsuperscript{2+} (Fig. 5B), treatment with 1 or 3 \(\mu\)M DEPC abolished the ability of Zn\textsuperscript{2+} to potentiate the \(\alpha 4\beta 4\) receptor (Fig. 5A,B). This result suggests the involvement of at least one histidine residue in mediating the potentiating effects of Zn\textsuperscript{2+} on neuronal nAChRs. We examined the effect of 3 \(\mu\)M DEPC on potentiation by 100 \(\mu\)M Zn\textsuperscript{2+} (Fig. 5C). Although potentiation was eliminated, Zn\textsuperscript{2+} continued to inhibit \(\alpha 4\beta 4\) receptors after DEPC treatment. DEPC (3 \(\mu\)M) also failed to affect Zn\textsuperscript{2+} inhibition of \(\alpha 3\beta 2\) receptors (Fig. 5D). The ability of Zn\textsuperscript{2+} to inhibit \(\alpha 4\beta 4\) or \(\alpha 3\beta 2\) after DEPC treatment suggests that the inhibitory site on these receptors may not involve histidine residues.

To provide further evidence that histidine residues are involved in mediating Zn\textsuperscript{2+} potentiation of \(\alpha 4\beta 4\), we examined the effect of altering the pH (Fig. 6). At pH 5.5, the extent of potentiation by 100 \(\mu\)M Zn\textsuperscript{2+} was significantly reduced (180 ± 9%) as compared with potentiation at pH 7.2 (our standard conditions). Increasing the pH to 8.0 resulted in a significant increase in the magnitude of the Zn\textsuperscript{2+} effect (770 ± 70%). These results are consistent with a role for one or more histidine residues in mediating the potentiating effect of Zn\textsuperscript{2+}.

Zn\textsuperscript{2+}-mediated potentiation and inhibition of neuronal nAChRs are voltage independent

To assess the proximity of the Zn\textsuperscript{2+} binding sites to the electrical field of the membrane, we examined potentiation of \(\alpha 4\beta 4\) and inhibition of \(\alpha 3\beta 2\) receptors over a range of holding potentials. Zn\textsuperscript{2+} potentiation of the ACh response of \(\alpha 4\beta 4\) receptors by 50 \(\mu\)M Zn\textsuperscript{2+} was examined at several holding potentials ranging from −90 mV to −40 mV (Fig. 7B). This Zn\textsuperscript{2+} concentration was chosen to minimize any influence from the inhibition seen at higher Zn\textsuperscript{2+} concentrations. The extent of potentiation was similar at all holding potentials tested (Fig. 7A,B). The degree of inhibition of \(\alpha 3\beta 2\) nAChR current responses by 100 \(\mu\)M Zn\textsuperscript{2+} was also independent of the holding potential from −90 mV to −40 mV (Fig. 7B).

Zn\textsuperscript{2+} potentiates the response of neuronal nAChRs to saturating acetylcholine concentrations

We examined the effect of Zn\textsuperscript{2+} on the ACh concentration–response relationships of \(\alpha 4\beta 4\) and \(\alpha 3\beta 2\) receptors (see Materials and Methods). Again, 50 \(\mu\)M Zn\textsuperscript{2+} was chosen to provide potentiation with minimal inhibition. Zn\textsuperscript{2+} applications significantly increased the response of \(\alpha 4\beta 4\) to saturating ACh concentrations (160 ± 11% of the response to ACh alone; \(p < 0.02\)) and significantly decreased the EC\textsubscript{50} for ACh activation from 74 ± 22...
Figure 6. Zn$^{2+}$ potentiation of α4β4 is sensitive to alterations in pH. Potentiation of the response to 1 μM ACh by 100 μM Zn$^{2+}$ at pH 5.5, 7.2, and 8.0 is plotted as a percentage of the response to ACh alone (mean ± SEM of 3 oocytes; significant differences from pH 7.2: *p < 0.0001; **p < 0.005).

Figure 7. Zn$^{2+}$ potentiation of α4β4 receptors and inhibition of α3β2 receptors are voltage independent. A. Current responses of an α4β4-expressing oocyte to 1 μM ACh before, during, and after coapplication of 50 μM Zn$^{2+}$ at membrane holding potentials of −40 mV (left trace, calibration: 50 nA, 20 sec) and −90 mV (right trace, calibration: 200 nA, 20 sec). The traces were normalized for comparison. B. Current responses of α4β4-expressing oocytes to 1 μM ACh in the presence of 50 μM Zn$^{2+}$ were recorded at various holding potentials and plotted as a percentage of the response to ACh alone (mean ± SEM of 3 oocytes). Current responses of α3β2 expressing oocytes to 4 μM ACh in the presence of 100 μM Zn$^{2+}$ were recorded at various holding potentials and plotted as a percentage of the response to ACh alone (mean ± SEM of 3 oocytes).

μM in the absence of Zn$^{2+}$ to 23 ± 8 μM in the presence of Zn$^{2+}$ (p < 0.05) (Fig. 8A). Zn$^{2+}$ coapplication also significantly increased the response of α4β2 to saturating ACh concentrations (140 ± 14% of the response to ACh alone; p < 0.02) but had no significant effect on the apparent ACh affinity of α4β2 (Fig. 8B).

Zn$^{2+}$ does not alter receptor desensitization rate

One possible mechanism for Zn$^{2+}$ potentiation is through an effect on receptor desensitization. If Zn$^{2+}$ were to slow the desensitization rate of a receptor, an apparent potentiation of the agonist response would result. This possible mechanism seems unlikely to account for Zn$^{2+}$ potentiation of α4β4, which can be dramatically potentiated even when no appreciable desensitization is evident (Fig. 1C). However, to examine this issue in more detail, we measured the desensitization rate of α4β4 and α4β2 receptors when exposed to an EC$_{50}$ concentration of ACh in the absence and presence of 50 μM Zn$^{2+}$. Oocytes were held at −40 mV and Ba$^{2+}$ was substituted for Ca$^{2+}$ in all solutions to minimize the contribution of the Ca$^{2+}$-activated Cl$^{-}$ channel (see Materials and Methods). For α4β4, there was no difference between desensitization rate for ACh alone (τ = 88 ± 13 sec) and the desensitization rate in the presence of Zn$^{2+}$ (τ = 77 ± 17 sec) (n = 6). For α4β2, there was no difference between the desensitization rate for ACh alone (τ = 42 ± 3 sec) and the desensitization rate in the presence of Zn$^{2+}$ (τ = 43 ± 7 sec) (n = 5). We conclude that Zn$^{2+}$ does not affect the rate of ACh-induced desensitization for these receptors.

Determinants of Zn$^{2+}$ potentiation are only partially localized to the N-terminal extracellular domain

We used chimeras of the α3 and α4 subunits to provide preliminary information regarding the location of amino acid residues involved in mediating Zn$^{2+}$ potentiation (Fig. 9). The α4–216–α3 and α3–216–α4 subunits each consist of the N-terminal extracellular domain of one subunit joined to the remainder of the other subunit. Each of the chimeras was coexpressed with the β4 subunit, and the resulting receptors were then examined for sensitivity to a range of Zn$^{2+}$ concentrations using our standard protocol (see Materials and Methods). Receptors in which the N-terminal extracellular domain of α4 has been replaced with the α3 sequence (α3–216–α4 β4) showed a dramatic loss in sensitivity to Zn$^{2+}$ potentiation. However, 170 ± 5% potentiation at 100 μM Zn$^{2+}$ was still significantly greater than the potentiation seen with α3β4. Receptors in which the region of α4 containing the transmembrane and cytoplasmic domains has been replaced with the α3 sequence (α4–216–α3 β4) also showed a loss in sensitivity to Zn$^{2+}$ potentiation when compared with α4β4. However, with a maximal potentiation of 250 ± 10% at 100 μM Zn$^{2+}$, the α4–216–α3 β4 receptors were potentiated to a greater degree than α3–216–α4 β4 or α3β4 receptors. These results suggest that although the most critical determinants of Zn$^{2+}$ potentiation are located in the N-terminal extracellular domain of α4, important residues also reside in the remainder of the subunit.
Neuronal nAChRs have previously been shown to be potentiated by extracellular calcium (Mulle et al., 1992; Vernino et al., 1996). Calcium exerts its potentiating effect on neuronal nAChRs by increasing the probability of channel opening (Amador and Dani, 1995). Calcium appears to be bound by a series of EF-hand binding domains, and two critical glutamate residues on the α7 subunit have been identified (Galzi et al., 1996). These glutamate residues, as well as other components of the putative EF-hand structures, are conserved in the α and β subunits used in our study. We think it is unlikely that Zn$^{2+}$ potentiation operates through these Ca$^{2+}$ binding sites for the following reasons. First, both α7 and α3β2 receptors are potentiated by Ca$^{2+}$ and contain the putative EF-hand structures (Vernino et al., 1992; Galzi et al., 1996) but are not potentiated by Zn$^{2+}$ (Palma et al., 1998) (Fig. 2B). Second, the ability of DEPC and pH changes to reduce Zn$^{2+}$ potentiation (Figs. 5, 6) suggests the involvement of histidine residues in coordinating Zn$^{2+}$. Histidines are not generally found in EF-hand Ca$^{2+}$ binding structures and are not present in the putative EF-hand structures in any of the subunits used in our study.

Neuronal nAChRs have also been shown to be modulated by various other agents. A group of compounds typified by phystostigmine and galanthamine can activate exogenously expressed α4β2 receptors and α7-containing receptors expressed in hippocampal neurons by interacting with an allosteric site distinct from the ACh binding site (Pereira et al., 1993, 1994; Schrattenholz et al., 1996). Lead inhibits α3β4 and αβ2 receptors at submicromolar concentrations and potentiates α3β2 receptors at concentrations >100 μM (Zwart et al., 1995). Low concentrations (1–10 μM) of (+)-tubocurarine potentiate β4-containing receptors (α2β4 and α3β4) while inhibiting β2-containing receptors (α2β2 and α3β2) (Cachelin and Rust, 1994). Atropine potentiates α2β2 and α4β4 receptors (but not α2β2, αβ2β, or α3β4 receptors) responding to low (1 μM) ACh concentrations while inhibiting responses to high (1 mM) ACh concentrations (Zwart and Vijverberg, 1997). The pattern of potentiation and inhibition of the various receptor subtypes seen with these agents varies markedly from what we have found with Zn$^{2+}$, suggesting that Zn$^{2+}$ modulation of neuronal nAChRs is unrelated to modulation caused by these other agents.

We used DEPC to test for the involvement of histidine residues in Zn$^{2+}$ potentiation of nAChRs. Reaction of DEPC with histidine results in modification of the imidazole ring, rendering it incapable of coordinating Zn$^{2+}$ (Miles, 1977; Lundblad and Noyes, 1984). A role for histidine residues in Zn$^{2+}$ modulation of glycine receptors has been revealed using this technique (Harvey et al., 1999). DEPC can also affect arginine, lysine, cysteine, serine, and tyrosine residues when used under conditions of high concentration (≥10 mM) and low pH (pH 4). However, under our conditions (≤3 mM, pH 7.2), DEPC should be selective for histidine residues (Miles, 1977). We found that Zn$^{2+}$ potentiation of neuronal nAChRs could be abolished by DEPC treatment. In contrast, Zn$^{2+}$ continued to inhibit α4β4 and α3β2 after DEPC treatment.

Histidine residues are also prevented from coordinating Zn$^{2+}$ by protonation of both imidazole nitrogens. The first pKₐ ranges from 6.0 to 6.5, depending on the local environment. If we assume a pKₐ of 6.25, then at pH 7.2 (our standard conditions), 89% of the imidazole rings would have an unprotonated nitrogen capable of coordinating Zn$^{2+}$. Shifting the pH below the pKₐ would reduce the fraction of unprotonated histidines and should reduce histidine coordination of Zn$^{2+}$. At pH 5.5, at which only 18% of histidines would be unprotonated, potentiation of α4β4 was reduced to only 180 ± 9% of ACh alone. At pH 8.0, at which 98% of histidine residues would be unprotonated, potentiation of α4β4 increased to 770 ± 70% of ACh alone. The sensitivity of Zn$^{2+}$ potentiation to both DEPC treatment and pH changes strongly
suggested a role for histidine residues. These histidine residues may be involved in directly coordinating the Zn$^{2+}$ ion. However, we cannot rule out the possibility that DEPC and changes in pH are affecting a histidine within an allosteric pathway through which Zn$^{2+}$ might exert its potentiating effects.

To approximate the location of the Zn$^{2+}$ binding sites in relation to the electric field of the membrane, we examined Zn$^{2+}$ potentiation and inhibition at several holding potentials (Fig. 7). The degree of potentiation of αβ4 receptors and inhibition of αβ2 receptors remained constant across a range of holding potentials. This result suggests that potentiation and inhibition are not under the influence of the electric field of the membrane, and thus the relevant binding sites are not likely to be closely associated with the transmembrane domains of the receptor. We also examined the Zn$^{2+}$ sensitivity of receptors formed by chimeras of the α3 and α4 subunits (Fig. 9). Results of these experiments suggest that determinants of Zn$^{2+}$ potentiation are located within the N-terminal extracellular domain, as well as in the remainder of the protein.

It is clear that both α and β subunits make contributions to Zn$^{2+}$ potentiation of neuronal nAChRs. Possible explanations for this observation include a Zn$^{2+}$ binding site on each individual subunit yielding five binding sites, or Zn$^{2+}$ binding sites formed at the interface between α and β subunits yielding at least two binding sites. The Hill coefficient values between 1.0 and 2.0 that we have observed for Zn$^{2+}$ potentiation (Table 1) suggest that a neuronal nAChR may have one or two Zn$^{2+}$ potentiation sites. If nAChRs have a single site, it might be similar to the Ni$^{2+}$ binding site of retinal cyclic nucleotide-gated (CNG) channels (Shammat and Gordon, 1999). At least two subunits contribute a histidine residue to form a single Ni$^{2+}$ site during the open state of the CNG channel (Gordon and Zagotta, 1995a,b). If neuronal nAChRs have two Zn$^{2+}$ potentiation sites, the sites might be formed similarly to agonist binding sites (at the interface between two subunits). In either case, stabilization of the open state would explain our observation that Zn$^{2+}$ increases the response to saturating ACh concentrations.

Knowledge regarding the effect of Zn$^{2+}$ on neuronal nAChRs expressed in a neuronal context is limited. Nutter and Adams (1995) reported inhibition of ACh-evoked currents in cultured rat parasympathetic neurons. Although the neuronal nAChR subunit expression in these neurons is heterogeneous, the predominant subunits are α3, α7, and a varying ratio of β2 and β4 (Poht et al., 1996, 1997). Thus, based on our results and the results of Palma et al., (1998), many of the nAChRs expressed by these neurons would be expected to be inhibited by Zn$^{2+}$.

CNS synaptic terminals have been shown to be capable of taking up, storing, and releasing Zn$^{2+}$ (Huang, 1997; Frederickson et al., 2000). Extracellular Zn$^{2+}$ is estimated to reach concentrations as high as several hundred micromolar during neuronal activity (Frederickson et al., 1983; Assaf and Chung, 1984). Neuronal nAChRs are located both presynaptically and postsynaptically. Although the neuronal nAChR subunit expression in these neurons is heterogeneous, the predominant subunits are α3, α7, and a varying ratio of β2 and β4 (Poht et al., 1996, 1997). Thus, based on our results and the results of Palma et al., (1998), many of the nAChRs expressed by these neurons would be expected to be inhibited by Zn$^{2+}$.

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