Zinc Potentiates Neuronal Nicotinic Receptors by Increasing Burst Duration

Bernard Hsiao\textsuperscript{1}, Karla B. Mihalak\textsuperscript{1}, Karl L. Magleby\textsuperscript{2,3} and Charles W. Luetje\textsuperscript{1,3}

Departments of \textsuperscript{1}Molecular and Cellular Pharmacology, \textsuperscript{2}Physiology and Biophysics and the \textsuperscript{3}Neuroscience Program, University of Miami Miller School of Medicine, Miami, Florida 33101.

**Running Head:** Zinc potentiation of neuronal nicotinic receptors

**Corresponding Author:**
Dr. Charles W. Luetje  
Department of Molecular and Cellular Pharmacology (R-189)  
University of Miami Miller School of Medicine, P.O. Box 016189  
Miami, Florida 33101

Tel: (305) 243-4458  
Fax: (305) 243-4555  
E-mail: cluetje@med.miami.edu
Abstract

Micromolar zinc potentiates neuronal nicotinic acetylcholine receptors (nAChRs) in a subtype dependent manner. Zinc potentiates receptor function even at saturating agonist concentrations, without altering receptor desensitization rate. Potentiation could occur through an increase in the number of available receptors, an increase in single channel current amplitude, or an increase in single channel open probability. To distinguish among these possibilities, we examined rat neuronal nAChRs expressed in Xenopus oocytes. Blockade of a large fraction of ACh activated α4β4 or α4β2 receptors by the open channel blocker hexamethonium failed to change the extent of potentiation by zinc, suggesting that zinc does not change the number of available receptors. The single channel amplitudes of ACh (1 µM) activated α4β4 receptors in outside-out patches were similar in the absence and presence of 100 µM zinc (3.0 ± 0.1 pA and 2.9 ± 0.1 pA, respectively). To determine the effect of zinc on single channel open probability, we examined α4β4 receptors in cell-attached patches. The open probability at 100 nM ACh (0.011 ± 0.002) was increased 4.5 fold by 100 µM zinc (0.050 ± 0.008), accounting for most of the potentiation observed at the whole cell level. The increase in open probability was due to an increase in burst duration, which increased from 207 ± 38 msec in the absence of zinc, to 830 ± 189 msec in the presence of zinc. Our results suggest that potentiation of neuronal nAChRs by zinc is due to a stabilization of the bursting states of the receptor.
Ionic zinc is found in neurons throughout the brain, with highest concentrations in the cerebral cortex and limbic areas (Frederickson et al., 2000). Zn\(^{2+}\) is packaged in synaptic vesicles and released during neuronal activity (Assaf and Chung 1984; Howell et al. 1984). Zinc serves as an endogenous modulator of nervous system function (Li et al. 2003; Smart et al. 2004). For example, synaptically released Zn\(^{2+}\) serves as a modulator of long term potentiation in the hippocampus and the amygdala (Vogt et al. 2000; Ueno et al. 2002; Izumi et al. 2006; Kodirov et al. 2006). The effects of Zn\(^{2+}\) are thought to occur through the modulation of a variety of ligand-gated ion channels including glutamate and GABA receptors, as well as glycine and ATP receptors (Huang 1997; Smart et al. 2004).

Neuronal nicotinic acetylcholine receptors (nAChRs) are also modulated by Zn\(^{2+}\) (Palma et al. 1998; Garcia-Colunga 2001; Hsiao et al. 2001). Micromolar concentrations of Zn\(^{2+}\) inhibit homomeric α7 receptors (Palma et al. 1998) and heteromeric α3β2 receptors (Hsiao et al. 2001). In contrast, the effect of Zn\(^{2+}\) on many heteromeric neuronal nAChRs is biphasic. These receptors are potentiated by micromolar Zn\(^{2+}\) and inhibited by millimolar Zn\(^{2+}\) (Hsiao et al. 2001). The extent of potentiation varies depending on subunit composition, with α4 containing receptors displaying the most dramatic potentiation. At low ACh concentrations, the α4β2 and α4β4 receptors are potentiated by Zn\(^{2+}\), with EC\textsubscript{50}s of 16 µM and 22 µM (respectively) and with maximum potentiation of approximately 2.5- and 5-fold (respectively) reached at 100 µM Zn\(^{2+}\). Inhibition of both receptors occurs at [Zn\(^{2+}\)] > 100µM (Hsiao et al., 2001). Estimates of extracellular synaptic [Zn\(^{2+}\)] during neuronal activity vary widely, from 7-8 µM to perhaps as much as 100-300 µM (Assaf and Chung, 1984; Xie et al., 1994; Vogt et al., 2000; Li et al., 2001; Ueno et al. 2002; but see Kay 2003). Zn\(^{2+}\) modulation of neuronal nAChRs occurs throughout this concentration range (Hsiao et al. 2001).
There are a variety of possible mechanisms through which Zn$^{2+}$ might potentiate the function of neuronal nAChRs. Our earlier demonstration that Zn$^{2+}$ potentiates neuronal nAChRs even at saturating ACh concentrations (Hsiao et al. 2001) suggests that potentiation cannot be explained solely by a lateral shift in the macroscopic dose-response relationship. Our recent work has identified the sites at which Zn$^{2+}$ binds and potentiates the receptors as the subunit-subunit interfaces that alternate with interfaces that bind ACh (Hsiao et al. 2006). In the context of the recent “C-loop closure” model of receptor activation (Hansen et al. 2005), Zn$^{2+}$ might act by stabilizing an open or bursting conformation of the receptor. Here we examine the effect of Zn$^{2+}$ on the parameters that contribute to the total current ($I$) through a population of receptors (as described by the equation $I = n \cdot i \cdot P_o$): $n$, the number of active receptors; $i$, the single channel current amplitude; and $P_o$, the single channel open probability. We rule out an effect of Zn$^{2+}$ on $n$ and $i$, finding that an increase in $P_o$, due to an increase in burst duration accounts for the potentiation of neuronal nAChRs by Zn$^{2+}$. 
Materials and Methods

Materials. *Xenopus laevis* frogs were purchased from Nasco (Fort Atkinson, WI). The care and use of *X. laevis* were approved by the University of Miami Animal Research Committee and meet 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). Zinc containing solutions were prepared prior to each experiment from a 1 M stock solution of zinc acetate. We have previously shown that zinc acetate solutions yield results that are identical to those obtained with zinc chloride solutions (Hsiao et al., 2001).

Neuronal nAChR expression in *X. laevis* oocytes. m$^7$G(5’)-ppp(5’)-G capped cRNA transcripts encoding nAChR subunits were prepared by *in vitro* transcription from linearized template DNA encoding the α4, β2, and β4 subunits. Mature *X. laevis* frogs were anesthetized by submersion in 0.1% 3-aminobenzoic acid ethyl ester and oocytes were surgically removed. Follicle cells were removed by treatment with Collagenase B for 2 hours at room temperature. Stage V oocytes were injected with 1-30 ng of each cRNA in 15-50 nl of water and incubated at 18°C in modified Barth’s saline (88 mM NaCl, 1mM KCl, 2.4 mM NaHCO₃, 0.3 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 0.82 mM MgSO₄, 100 µg/ml gentamicin, 15 mM HEPES, pH 7.6) for 1-7 days.

Whole-cell recording. Current responses were measured under two-electrode voltage clamp. Oocytes were perfused at room temperature (20°-25°C) with perfusion solution (115 mM NaCl, 1.8 mM CaCl₂, 2.5 mM KCl, 0.1 µM atropine, 10 mM HEPES, pH 7.2) at a rate of approximately 4 ml/min. Acetylcholine, hexamethonium and zinc were diluted in the perfusion
solution and applied under control of solenoid valves. Micropipettes were filled with 3 M KCl and had resistances of 0.3-2.0 MΩ. Oocytes were held at −70 mV (Figure 1) or −85 mV (Figure 2). Experiments were performed using a TEV-200 voltage clamp unit (Dagan, Minneapolis, MN). Current responses were filtered (4-pole, Bessel low pass) at 20 Hz (-3db) and sampled at 100 Hz. Data were acquired, and analyzed on a Macintosh Power PC 7100 computer using AxoData 1.2.2 and AxoGraph 4.6 software (Molecular Devices, Union City, CA) or on a Pentium III PC running pClamp 8 software (Molecular Devices).

**Patch clamp.** Current flowing through single α4β4 nAChRs was recorded from patches of oocyte surface membrane in both the outside-out and cell-attached configurations. α4β4 nAChRs were used in these experiments because this subunit combination shows robust expression in oocytes and displays much less desensitization than β2 containing receptors. Vitelline membranes were manually removed from the oocytes prior to patch formation. Recording pipets were pulled from borosilicate glass capillary tubing (Warner Instrument Corp.) to a resistance of 7-15 megaohms. During outside-out patch recordings, the recording chamber solution consisted of 150 mM NaCl, 2.8 mM KCl, 1 mM CaCl₂, and 10 mM Hepes, pH adjusted to 7.2 with NaOH. The recording pipet solution consisted of 80 mM CsCl, 60 mM CsF, 10 mM EGTA, 1 mM CaCl₂, 10 mM Hepes pH adjusted to 7.2 with KOH. Recording chamber solution containing 1 μM ACh or 1 μM ACh with 100 μM Zn²⁺ was applied to the patches using a Warner Instruments SF-77B “Perfusion Fast-Step” stepper with a three barreled, square glass tube. Based on preliminary experiments involving steps between solutions of differing concentrations of KCl, solution changes were achieved in 20-30 msec. In cell-attached patch experiments, the recording pipet solution was identical to the recording chamber solution (see
above) with the addition of either 100 nM ACh alone or 100 nM ACh with 100 μM Zn\(^{2+}\).

Patches were voltage clamped using an Axopatch 200A integrating patch clamp (Molecular Devices) and held at –100 mV. A +60mV holding command voltage was applied in order to hold the patch at approximately –120 mV (assuming a resting potential of –60 mV). Current responses were filtered (8-pole, Bessel low-pass) at 4 kHz (-3db) and sampled at 15.4 kHz using a Digidata 1322A (Molecular Devices) and a PC running Clampex 8.2 (Molecular Devices).

**Data analysis.** Prism software (GraphPad, San Diego, CA) was used to assess statistical significance using a two-tailed unpaired t-test or one-way ANOVA with a Dunnett’s post test, as appropriate. The magnitude of Zn\(^{2+}\) potentiation in Figures 1 and 2 was measured as previously described (Hsiao et al. 2001, 2006). Briefly, when no desensitization was apparent (all α4β4 currents), control current in response to ACh was determined from a 1 sec average beginning 29 sec after initiation of agonist application and compared to a 1 sec average of baseline current immediately prior to ACh application. Current levels during Zn\(^{2+}\) co-application were determined from a 1 sec average beginning 29 sec after initiation of Zn\(^{2+}\) application and compared to the control current. When desensitization was apparent (all α4β2 currents), the initial 30 sec ACh response in the absence of Zn\(^{2+}\) was fit to a single exponential decay function. This fit was projected over the next 30 sec during which both ACh and Zn\(^{2+}\) were co-applied. The degree of modulation was measured by taking a 1 sec average 29 sec after initiation of Zn\(^{2+}\) application and comparing it to a 1 sec average of the projected response to ACh alone during the same time period. Single channel data were analyzed using Clampfit 8.2 software (Molecular Devices), custom software developed by K.L.M., and the QUB software suite, v. 1.1.0.2538 developed by Qin, Auerbach, and Sachs at the State University of New York at
Buffalo (Qin et al. 1996). Data were pre-conditioned for analysis using Clampfit by low-pass four-pole Bessel filtering at 1.5 kHz, adjusting for baseline variation, and eliminating artifactual events.

Single channel amplitude was measured in outside-out patches. Single channel activity was observed following inactivation of the multiple-channel response. The average amplitude of all openings at the single channel level was measured from each patch. Outside-out patches were not used to measure open probability because channel activity ceased in the outside-out configuration within 1 minute of continual agonist application. Even after extended periods of wash in control solution and the addition of ATP in the pipet solution, channel activity was never restored. This phenomenon, termed “run-down”, has been reported by others (Ballivet, 1988; Buisson 1996). For this reason, cell-attached patches were used to obtain data to evaluate open probability.

Open probability of single channels in cell-attached patches was assessed using both 50% threshold analysis with custom software and SKM in the QUB software suite. Only patches lasting for more than 10 minutes were analyzed. Binomial analysis has shown that when P_o is low, it is not possible to estimate the maximum number of channels in a patch from overlap of channel opening (Horn 1991). For this reason, we will represent our measurement of open probability as nP_o and this should be regarded as an upper estimate of P_o. Patches containing stretch activated channels were excluded from analysis (Silberberg and Magleby 1997).

Stable data, as evaluated with stability plots (Weiss and Magleby 1990), were used for the burst analysis, which is based on six patches with ACh alone and five patches with ACh and Zn^{2+}. Burst analysis was conducted using custom software by first defining the critical time (t_crit), the length of time between bursts as described by Magleby and Pallotta (1983). The t_crit
was determined by fitting the distributions of closed-interval durations with the sum of several exponential components. The intervals in the exponentials with the longest time constants were then used to define the gaps between bursts. Because there was typically a difference of about three orders of magnitude in the mean durations of the closed intervals generating gaps between bursts from the much briefer duration closed intervals generating gaps within bursts, there was no ambiguity in assigning exponential components to gaps between burst or within bursts. The $t_{\text{crit}}$ was defined such that the number of closed intervals between bursts misclassified as closed intervals within bursts was equal to the number of closed intervals within bursts misclassified as closed intervals between bursts. Thus, errors resulting from misclassification of intervals between and within bursts would tend to cancel out. Analysis of the intraburst subconductance architecture was accomplished by using the $t_{\text{crit}}$ to isolate individual bursts in QUB, assigning each of the four states (closed, two subconductance levels, and fully open – see Figure 4) to a corresponding state in a model, and idealizing the data utilizing the SKM method in QUB.

As will be shown in the results, Zn$^{2+}$ increased the ACh induced peak currents recorded from oocytes at the whole cell level by about four to five fold, while having much less of an effect on the peak macroscopic currents recorded from outside patches. To determine whether this difference might be associated with the rapid rundown/desensitization observed with outside out patches, simulations with a model for desensitization were carried out. Since little is known about desensitization of neuronal ACh receptors, a simple model without cyclic recovery was assumed: D-C-O-D, where D, C, and O are desensitized, closed, and open states respectively. Similar results were obtained with a more complex model: D-C-C-O-D. Rate constants were selected to obtain a response like that in Fig. 3 for ACh and then the rate constants were changed to make the open probability four fold greater and the response recalculated. This was not meant
to be a study of desensitization, but to answer the question of whether desensitization could
differentially alter the peak responses, depending on the open probability.
Results

Zn\(^{2+}\) potentiates neuronal nicotinic receptors.

The \(\alpha4\beta2\) and \(\alpha4\beta4\) neuronal nAChRs are potentiated by Zn\(^{2+}\) (Figure 1). We previously found that maximal potentiation of these receptors occurred at 100 µM Zn\(^{2+}\) and that potentiation was most pronounced at low ACh concentrations (Hsiao et al. 2001). For this reason, we used 100 µM Zn\(^{2+}\) and low ACh concentrations (\(\leq EC_{10}\)) throughout the present study. Coapplication of 100 µM Zn\(^{2+}\) potentiates the response of \(\alpha4\beta2\) receptors to 10 µM ACh by 215 ± 26% (n = 4), while the response of \(\alpha4\beta4\) receptors to 1 µM ACh is potentiated by 435 ± 30% (n = 7). These values are similar to our previous work (Hsiao et al. 2001, 2006).

Zn\(^{2+}\) does not increase the number of available receptors.

One possible mechanism for Zn\(^{2+}\) potentiation of the macroscopic current response is through an increase in \(n\), the number of available receptors. In this mechanism, Zn\(^{2+}\) might reveal a population of neuronal nAChRs that were previously inactive despite the presence of agonist. We used the antagonist hexamethonium (HEX) to test this possibility. Experiments with both parasympathetic ganglion neurons and \(\alpha4\beta2\) expressing *Xenopus* oocytes have shown that in the presence of agonist and under hyperpolarizing conditions, HEX can become trapped within the ion pore of the receptor (Ascher et al. 1979; Gurney and Rang 1984; Bertrand et al. 1990). Depolarization in the presence of agonist is required to eject HEX from the pore and allow normal function of the channel to resume (Gurney and Rang 1984; Bertrand et al. 1990). We took advantage of these properties of HEX to determine whether a fixed population of receptors is passing additional current in the presence of Zn\(^{2+}\) (Fig. 2A, bottom left), or whether the number of available receptors is increased in the presence of Zn\(^{2+}\) (Fig. 2A, bottom right). If
a portion of the active receptors is blocked by HEX, the extent of potentiation of the remaining unblocked receptors should allow us to distinguish between these two possibilities. If, after partial HEX blockade, the remaining unblocked receptors pass additional current (Fig. 2B, bottom left), then the extent of potentiation should be similar to that seen prior to HEX blockade. If there is an increase in the number of available receptors (Fig. 2B, bottom right), then the extent of potentiation should be greater than that seen prior to HEX blockade.

A representative trace is provided in Figure 2C to illustrate the HEX experiment. Before application of HEX, the response of α4β2 receptors to 10 µM ACh is potentiated upon coapplication of 100 µM Zn²⁺ by approximately two-fold (left portion of trace). Following a 20 sec application of 30 µM HEX, a substantial fraction of the ACh response is blocked (approximately 85%, when desensitization is taken into account). The initial blockade of the receptor activity by HEX is a combination of both competitive block and open channel block (discussed above). After a brief wash period (~1 min) to remove the HEX, only the open channel blockade remains. Potentiation of the remaining ACh response by the second application of 100 µM Zn²⁺ was also approximately two-fold (middle portion of trace). Results with multiple α4β2 expressing oocytes are provided in Figure 2D. Potentiation of α4β4 receptors was also unaffected by HEX blockade. Potentiation before and after HEX (10 µM) application had blocked approximately half of the active receptors was 437 ± 28% and 488 ± 44%, respectively (Figure 2E). For both α4β2 and α4β4, potentiation after relief of HEX block (by depolarization in the presence of ACh) did not differ from the extent of potentiation prior to HEX application. For both receptors, the similar extent of potentiation before and after HEX blockade indicates that Zn²⁺ does not reveal a new population of receptors. Instead, Zn²⁺ must act by altering the properties of a fixed population of receptors.
**Zn$^{2+}$ does not increase the single channel current amplitude.**

A second possible mechanism for Zn$^{2+}$ potentiation is through an increase in the current through individual channels. If a change in single channel current amplitude were to account for the potentiation of α4β4 receptors by Zn$^{2+}$, then single channel current amplitude would have to increase 4-5 fold in the presence of Zn$^{2+}$. To examine this possibility, channel activity of α4β4 receptors in response to 1 µM ACh was recorded in patches in the outside-out configuration in the absence and presence of 100 µM Zn$^{2+}$ (Figure 3). The current recordings in Figure 3 were selected to be of similar peak amplitude for ease of comparison. After an initial multi-channel response, the current decayed to a level where individual channels could be observed. Channel amplitude was assessed from these single channel events (here we are measuring the largest and most prevalent channel amplitude, two subconductance levels are discussed below). The mean current amplitude for 33 open intervals from 3 separate patches in the presence of ACh alone and 34 open intervals from 3 patches in the presence of ACh and Zn$^{2+}$ were measured. When activated by 1 µM ACh, the single channel amplitude was 3.0 ± 0.1 pA. When activated by 1 µM ACh in the presence of 100 µM Zn$^{2+}$, the single channel amplitude was 2.9 ± 0.1 pA. Thus, the single channel current amplitude of these receptors is not altered by the presence of Zn$^{2+}$ (p = 0.24, two-tailed, unpaired t-test).

The current recordings in Figure 3 were selected to be of similar peak amplitude for ease of comparison. Results from 4 patches exposed to ACh yielded a peak amplitude of 44 ± 12 pA, while 4 patches exposed to ACh + Zn$^{2+}$ yielded a peak amplitude of 88 ± 29 pA (mean ± SEM). These values were not significantly different (p = 0.21, two-tailed, unpaired t-test). The large variability presumably reflects differing numbers of channels in the patches. The question that
arises is why aren’t the peak amplitudes in the presence of zinc 4-fold greater that the peak amplitudes in the absence of zinc? In contrast to Figure 1, these responses are obtained in excised outside-out patches that display rapid rundown/desensitization. To explore whether rapid rundown/desensitization might be involved in determining peak amplitudes, simulations of gating with simple models for rundown/desensitization (see Materials and Methods) indicated that peak macro current amplitudes resulting from a step application of ACh in the presence of Zn\(^{2+}\) could be increased only 30-100% over that in ACh alone, even though mean open time before desensitization was increased 400%. These simulations (not shown) suggest that the pronounced rundown/desensitization associated with outside out patches could lead to less of a difference between peak currents in ACh and ACh plus Zn\(^{2+}\) in the presence of rundown/desensitization than in its absence. In any case, these experiments in the outside-out patch configuration, which allows precise control of voltage, are sufficient to show that single channel amplitude is unchanged by zinc. Because of the rapid rundown/desensitization, single channel data for the remainder of our study were collected in the cell-attached configuration.

**Zn\(^{2+}\) increases the single channel open probability.**

A third potential mechanism for Zn\(^{2+}\) potentiation is an increase in the open probability of individual receptors. To examine this possibility, single channel openings were recorded in the cell-attached patch configuration on α4β4 expressing oocytes. In initial experiments we applied 1 µM ACh, but this concentration resulted in a loss of channel activity in approximately 1 minute, presumably due to desensitization. To obtain suitably long recordings, we tried lower ACh concentrations and found that use of 100 nM ACh would yield channel activity in individual patches that could be recorded for the duration of the patch (10 - 20 minutes). At the
whole cell level, potentiation of the response to this low concentration of ACh by 100 µM Zn$^{2+}$ was 548 ± 39% (n = 7, Figure 1C).

In the cell attached patch configuration the receptors are in constant contact with the agonist in the patch pipette, so that exposure of receptors in an individual patch to solutions with and without agonist was not done. Thus, it was important to establish that the channel activity we observe is due to receptor activation. First, channel activity was not observed when ACh was not included in the pipette solution. Second, the channel activity occurred in bursts lasting for hundreds of milliseconds, interspersed with longer periods of inactivity (Figure 4A). This bursting activity is typical of β4 subunit containing receptors (Papke and Heinemann 1991). Our use of a very low concentration of ACh (100 nM) suggests that each burst represents a single receptor activation; the channel activity during a single episode of agonist binding (Gibb and Colquhoun 1991). Lastly, two distinct subconductance levels (at approximately 34% and 69% of the amplitude of the fully open state) could be observed within the bursts (Figure 4C). Bursting activity and the presence of two distinct subconductance levels were also observed in patches recorded in the outside-out configuration where channel activity can be compared in the presence and absence of ACh (Figure 3).

Analysis of the cell attached patch recordings revealed that Zn$^{2+}$ exerts a large effect on the $nP_o$ of α4β4 receptors (Figure 5, Table 1). Using 50% threshold analysis (see Materials and Methods), an $nP_o$ of 0.0111 ± 0.0016 was observed in the presence of 100 nM ACh. Co-application of 100 µM Zn$^{2+}$ with 100 nM ACh increased $nP_o$ to 0.0495 ± 0.0084. Results were similar when using the SKM algorithm in QUB ($nP_o = 0.0118 ± 0.0018$ in the absence of Zn$^{2+}$, and $nP_o = 0.0541 ± 0.0083$ in the presence of Zn$^{2+}$). Thus, $nP_o$ was increased by approximately 4.5-fold in the presence of Zn$^{2+}$. This accounts for most of the 5.5-fold increase in current seen
at the whole cell level when 100 µM Zn²⁺ is co-applied with 100 nM ACh. SKM also allowed analysis of the effect of Zn²⁺ on the individual subconductance levels. As we observed for the largest conductance level (see above), the amplitudes of the two subconductance levels were unaffected by Zn²⁺. While Zn²⁺ did not significantly increase the open probability of the rare (accounting for ~2% of the time spent in bursts) lower subconductance level (p = 0.089, two-tailed unpaired t-test), the open probabilities of the more common upper subconductance state and the fully open conductance state were increased by 3.1 fold and 5.7 fold respectively (p < 0.0001 and p < 0.01, respectively, two-tailed unpaired t-test).

As expected from the stochastic nature of single channel gating (Colquhoun and Hawkes 1982), a wide range of burst durations was observed in both the absence and presence of Zn²⁺, as shown by the representative traces in Figure 5 where it can be seen that the presence of Zn²⁺ greatly increases the mean duration of the bursts. Analysis of the burst durations (Figure 6, Table 1) reveals a 4-fold increase in burst duration due to the coapplication of Zn²⁺, which accounts for the increase in \(nP_o\).

Discussion

Zinc dramatically potentiates the function of \(\alpha 4\) containing neuronal nAChRs (Hsiao et al. 2001, 2006). We investigated the mechanism of Zn²⁺ potentiation by examining the effect of Zn²⁺ on the factors that contribute to the total amount of current passed by a population of these receptors. One possible mechanism is an increase in the total number of available receptors, either by activating a previously unresponsive population or by slowing the rate at which receptors enter a closed desensitized state. However, we found no evidence for the activation of a cryptic population of receptors by Zn²⁺. We have also ruled out an effect of Zn²⁺ on the time
course of desensitization onset in our previous work (Hsiao et al. 2006) and in the current study, where in Fig. 3 and additional experiments, the time constant for desensitization in the absence and presence of Zn$^{2+}$ was $\tau = 3.2 \pm 0.8$ sec and $3.0 \pm 0.7$ sec, respectively ($n = 4$ for each condition, $p = 0.836$, two-tailed unpaired t-test). Another possible mechanism for the potentiation by Zn$^{2+}$ is an increase in the single channel current amplitude. However, we found the amplitude of single channel events to be unchanged in the presence and absence of Zn$^{2+}$. Instead, we found Zn$^{2+}$ potentiation is due to an increase in channel open probability.

Burst analysis of the recordings from cell-attached patches revealed that the Zn$^{2+}$ mediated increase in open probability was associated with a 4-fold lengthening of the mean burst duration, with little effect on the gap between bursts (Table 1). Thus, once a channel enters a bursting mode, Zn$^{2+}$ appears to stabilize it within the bursting states before it returns to the long lasting closed states. If Zn$^{2+}$ acted instead by facilitating the entrance of the channels into bursts by facilitating the binding of ACh, then the gap between bursts might be expected to decrease about four fold. Our observation that the gap between bursts is unchanged by zinc most likely rules out the possibility that Zn$^{2+}$ acts by facilitating the binding of ACh; i.e., Zn$^{2+}$ does not act by inducing an allosteric conformational change that increases the access of ACh to its binding site or increases the probability that a collision of ACh with its binding site results in a successful binding. It should be noted, however, that a conclusion of a lack of effect of zinc on interburst gap duration requires that the average number of channels in patches examined with and without zinc be the same. We have no reason to suspect that the number of channels in each patch would be different, in part because some patches exposed to ACh or ACh plus zinc were obtained from the same oocyte. Thus the only way that zinc might increase the number of channels is by a mechanism of activating “silent” channels, a mechanism that we ruled out in Figure 2.
Therefore, it is likely that zinc has little effect on the interburst gap duration, and consequently, is not facilitating the effective binding rate of ACh to its receptor as a mechanism of receptor potentiation. Our use of a very low agonist concentration (100 nM ACh) suggests that the termination of a burst represents a dissociation of agonist from the receptor, rather than the entry of the receptor into a desensitized state (Gibb and Colquhoun, 1991). Further support for burst termination from agonist dissociation rather than desensitization with 100 nM ACh are the observations of no apparent desensitization with 100 nM or 1 µM agonist application over many tens of seconds (Figure 1B, C). Desensitization only becomes apparent when the agonist concentration is increased to 10 µM (data not shown), 100-fold greater than what we used for the bursting analysis. Taken together, these observations suggest that Zn\(^{2+}\) acts by stabilizing the agonist bound set of open and closed states that generate bursts, mainly by decreasing the probability of leaving the bursting states. The dramatic increase in burst duration with Zn\(^{2+}\) in our experiments suggests that Zn\(^{2+}\), at an appropriate concentration, might increase the durations of excitatory post-synaptic potentials generated by neuronal nicotinic receptors.

Our observations and other studies (Lewis et al. 1997; Sivilotti et al. 1997) suggest that the gating of neuronal ACh receptors is complex, with multiple conductance levels. We find that the α4β4 channel enters at least two subconductance states during gating: one with a subconductance level of 69% of the fully open level that is a consistent feature of gating, and one with a subconductance level 34% of the fully open level that is entered only about 2% of the time. Zn\(^{2+}\) does not affect the amplitude of either of the subconductance states or the fully open state. However, Zn\(^{2+}\) does increase the open probability of both the upper subconductance state and the fully open state. Zn\(^{2+}\) does not appear to affect the open probability of the lower subconductance state.
Zn\textsuperscript{2+} also potentiates glycine receptors (Bloomenthal et al. 1994; Laube et al. 1995). While the Zn\textsuperscript{2+} potentiation sites on glycine receptors and neuronal nAChRs are structurally distinct (Harvey et al. 1999; Laube et al. 2002; Hsiao et al. 2006), the mechanism of Zn\textsuperscript{2+} potentiation of these two receptor classes shows some similarity. Much like our findings with neuronal nAChRs, potentiating concentrations of Zn\textsuperscript{2+} increase the burst duration without altering the single channel amplitude of glycine receptors (Laube et al. 2000). Zn\textsuperscript{2+} also modulates the function of GABA\textsubscript{A} receptors, but this modulation is functionally distinct (GABA\textsubscript{A} receptors are inhibited, not potentiated) and structurally distinct from neuronal nAChR modulation (Hosie et al. 2003; Smart et al. 2004; Hsiao et al. 2006).

The mechanism of Zn\textsuperscript{2+} potentiation of neuronal nAChRs shows some similarity to the mechanism of benzodiazepine potentiation of GABA receptors; both modulators increase the $P_o$. However, benzodiazepine potentiation of GABA receptors appears to be achieved by an increase in channel opening frequency, without an effect on burst duration (Vicini et al. 1987; Rogers et al. 1994). More importantly, the location of the Zn\textsuperscript{2+} potentiation sites of neuronal nAChRs, at the subunit-subunit interfaces that alternate with the interfaces that bind ACh (Hsiao et al. 2006), is analogous to the location of the high affinity benzodiazepine-binding site of GABA receptors (Amin et al. 1997; Boileau et al. 1998; Kucken et al. 2000; Teissere and Czajkowski 2001). In particular, the $\alpha$4E59 residue that we have localized to the zinc potentiation site of neuronal nAChRs (Hsiao et al., 2006) is in a location identical to that of the benzodiazepine-binding site residue $\gamma$2T81 of GABA\textsubscript{A} receptors (Teissere and Czajkowski 2001; Kucken et al. 2003).

The potentiating action of benzodiazepines is thought to occur through an allosteric mechanism in which the relative stability of the closed and open states is altered, shifting the equilibrium toward the open state (Downing et al. 2005; Campo-Soria et al. 2006). The increase
in burst duration that we observe suggests that Zn\textsuperscript{2+} potentiates neuronal nAChRs by stabilizing the bursting states of the receptor. The recent examination of atomic structures of the soluble acetylcholine binding protein complexed with a variety of nicotinic ligands (Hansen et al. 2005) provides a structural framework for understanding this mechanism of Zn\textsuperscript{2+} potentiation. In the heteromeric neuronal nAChRs we are studying, agonist-binding sites are located at the \(\alpha/\beta\) interface. When the agonist-binding sites are occupied by agonist, the C-loop of the \(\alpha\) subunit accommodates the structure of the agonist by moving closer to the vertical axis of the receptor. This movement, termed “C-loop closure”, may be part of a conformational change associated with channel opening (Hansen et al. 2005). We previously identified residues on the \(\alpha4\) subunit that mediate zinc potentiation and proposed that these sites are located at the \(\beta/\alpha\) interfaces (Hsiao et al., 2006). It has also recently been proposed that these residues can participate in forming a zinc potentiation site at the \(\alpha-\alpha\) interface in an \((\alpha4)_3(\beta2)_2\) receptor (Moroni et al., 2007). If the bursting episodes that we observe represent gating of agonist bound receptors (see above), then the increase in the duration of these episodes in the presence of Zn\textsuperscript{2+} suggests that Zn\textsuperscript{2+} may be acting at the \(\beta-\alpha\) interfaces (or possibly an \(\alpha-\alpha\) interface) to stabilize C-loop closure, thus prolonging the burst and potentiating the macroscopic response. The structural similarity with the high affinity benzodiazepine sites of GABA receptors, together with the proposed C-loop closure model of subunit-subunit interface function, make the Zn\textsuperscript{2+} potentiation sites of neuronal nAChRs an attractive target for future drug development.
Acknowledgements

We thank Dr. David Weiss for helpful discussions regarding the mechanism of benzodiazepine action and Drs. Richard Bookman and Arthur Bassett for generous loans of equipment. We thank Floyd Maddox and Ana Mederos for excellent technical assistance.

Support

This work was supported by NIH MH66038 (CWL), NIH DA08102 (CWL) and NIH AR032805 (KLM). B.H. and K.B.M. were supported in part by T32HL07188. B.H. was supported in part by a PhRMA Foundation Medical Student Research Fellowship and was a Lois Pope LIFE Fellow.
References


Silberberg SD, Magleby KL. Voltage-induced slow activation and de-activation of mechanosensitive channels in Xenopus oocytes. *J Physiol (Lond)* 505: 551-569, 1997.


Ueno S, Tsukamoto M, Hirano T, Kikuchi K, Yamada MK, Nishiyama N, NAgano T, Matsuki N, Ikekaya Y. Mossy fiber Zn\textsuperscript{2+} spillover modulates heterosynaptic N-methyl-


Figure Legends

Figure 1. Zinc potentiates neuronal nicotinic receptors.

A, Current response of an α4β2 expressing oocyte to 10 µM ACh before, during and after coapplication of 100 µM Zn²⁺. B, Current response of an α4β4 expressing oocyte to 1 µM ACh before, during and after coapplication of 100 µM Zn²⁺. C, Current response of an α4β4 expressing oocyte to 100 nM ACh before, during and after coapplication of 100 µM Zn²⁺. Panels B and C show responses from different oocytes. The wide variation in expression level from oocyte to oocyte results in these responses to two different ACh concentrations having similar amplitudes.

Figure 2. Zinc does not increase n, the number of available receptors.

A, Potentiation of nAChRs by Zn²⁺ may be due to an increase in the activity of individual receptors (bottom left) or an increase in the number of receptors due to activation of a previously inactive population (bottom right). B, After open channel blockade of a portion of the active receptors by hexamethonium (HEX), the extent of Zn²⁺ potentiation should be similar if the properties of individual receptors change (bottom left) or should be greater if a previously inactive population of receptors becomes active (bottom right). C, Potentiation of the ACh (10 µM) response of an α4β2-expressing oocyte by 100 µM Zn²⁺ is shown before and after application of HEX (30 µM) and following reversal of blockade by depolarization to +85 mV. The dashed line indicates the baseline current level. D, Open channel blockade does not alter the extent of potentiation of α4β2 receptors. The response of α4β2-expressing oocytes to coapplication of 10 µM ACh and 100 µM Zn²⁺ is plotted as a percentage of the response to ACh alone (mean ± SEM, n = 3). There were no significant differences in potentiation by Zn²⁺ before
and after application of 30 µM HEX or following reversal of HEX blockade (p = 0.128, one-way ANOVA). E, Open channel blockade does not alter the extent of potentiation of α4β4 receptors. The response of α4β4-expressing oocytes to coapplication of 1 µM ACh and 100 µM Zn$^{2+}$ is plotted as a percentage of the response to ACh alone (mean ± SEM, n = 5). There were no significant differences in potentiation by Zn$^{2+}$ before and after application of 10 µM HEX or following reversal of HEX blockade (p = 0.073, one-way ANOVA).

**Figure 3. Zinc does not increase $i$, the single channel current amplitude.**

Recordings were obtained from outside-out patches (holding potential: -100 mV) containing multiple α4β4 receptors in response to application (arrows) of 1 µM ACh alone (A), or 1 µM ACh and 100 µM Zn$^{2+}$ (B). The multi-channel response rapidly inactivated during maintained application of agonist until single channel events could be discerned. At the bottom of each panel, the indicated sections of each recording are expanded to show the amplitude of a single channel in the absence (A) and presence (B) of Zn$^{2+}$.

**Figure 4.** Single channel recording from a cell attached patch of an α4β4-expressing oocyte. The recording pipet was filled with 0.1 µM ACh and 100 µM Zn$^{2+}$. A, Three successive bursts of activity are shown. B, An expanded view of the circled burst from panel A reveals multiple subconductance levels. C, Further expansion of the third burst illustrates two distinct subconductance levels (s1 and s2) at 34% and 69% of the fully open state. The lower subconductance level (s1) was rare, accounting for ~2% of the time spent in bursts. This record was selected to illustrate this infrequent subconductance level.
Figure 5. A, A continuous trace from a single cell-attached patch on an α4β4-expressing oocyte in the presence of 0.1 μM ACh. Open events are in an upward direction and are approximately 3 pA when fully open. B, A continuous trace from a single cell-attached patch on an α4β4-expressing oocyte in the presence of both 0.1 μM ACh and 100 μM Zn²⁺. C, Representative bursts from cell-attached patches from α4β4-expressing oocytes are shown when only 0.1 μM ACh was in the recording pipet. D, Representative bursts from patches when the recording pipet contained 0.1 μM ACh and 100 μM Zn²⁺.

Figure 6. Zinc increases burst duration.
Cumulative histograms of burst durations are shown. Each bar represents the number of bursts of that duration or greater. A, Burst durations in the presence of 100 nM ACh alone. 351 bursts from 6 patches are analyzed. The mean burst duration is 207 ± 38 msec. The numbers of observations are multiplied by 415/351 to allow direct comparison with panel B. B, Burst durations in the presence of 100 nM ACh and 100 μM Zn²⁺. 415 bursts from 5 patches are analyzed. The mean burst duration is 830 ± 189 msec.
Table 1. Single channel and burst analysis of α4β4 nAChRs.

<table>
<thead>
<tr>
<th></th>
<th>No Zn^{2+}</th>
<th>With Zn^{2+}</th>
<th>With Zn^{2+}/No Zn^{2+}</th>
</tr>
</thead>
<tbody>
<tr>
<td>nP_o (half-threshold method)</td>
<td>0.0111 ± 0.0016</td>
<td>0.0495 ± 0.0084***</td>
<td>4.46</td>
</tr>
<tr>
<td>nP_o (SKM method)</td>
<td>0.0118 ± 0.0018</td>
<td>0.0541 ± 0.0083***</td>
<td>4.58</td>
</tr>
<tr>
<td>Critical time</td>
<td>494 ± 142 msec</td>
<td>682 ± 190 msec</td>
<td>1.38</td>
</tr>
<tr>
<td>Mean open time</td>
<td>15 ± 4 msec</td>
<td>22 ± 5 msec</td>
<td>1.47</td>
</tr>
<tr>
<td>Mean shut time</td>
<td>1466 ± 513 msec</td>
<td>667 ± 210 msec</td>
<td>0.45</td>
</tr>
<tr>
<td>Mean No. bursts per patch</td>
<td>71 ± 19</td>
<td>81 ± 27</td>
<td>1.14</td>
</tr>
<tr>
<td>No. intraburst openings</td>
<td>9 ± 1</td>
<td>30 ± 8*</td>
<td>3.33</td>
</tr>
<tr>
<td>No. intraburst closings</td>
<td>8 ± 1</td>
<td>29 ± 8*</td>
<td>3.62</td>
</tr>
<tr>
<td>Duration intraburst open intervals</td>
<td>18 ± 3 msec</td>
<td>24 ± 4 msec</td>
<td>1.33</td>
</tr>
<tr>
<td>Duration intraburst shut intervals</td>
<td>8 ± 2 msec</td>
<td>8 ± 3 msec</td>
<td>1.00</td>
</tr>
<tr>
<td>Burst duration</td>
<td>207 ± 38 msec</td>
<td>830 ± 189 msec**</td>
<td>4.01</td>
</tr>
<tr>
<td>Interburst gap duration</td>
<td>15710 ± 5300 msec</td>
<td>14370 ± 4720 msec</td>
<td>0.91</td>
</tr>
<tr>
<td>Single channel amplitude</td>
<td>3.0 ± 0.1 pA</td>
<td>2.9 ± 0.1 pA</td>
<td>0.97</td>
</tr>
</tbody>
</table>

Values that are significantly different in the presence of Zn^{2+} are denoted with asterisks (* p<0.05, ** p<0.01, *** p<0.001). Results are reported as mean ± SEM and are based on 6 patches without Zn^{2+} and 5 patches in the presence of Zn^{2+}, with an average of 560 open intervals per patch without Zn^{2+} and 1860 per patch in the presence of Zn^{2+}. 
Figure 1

A  \( \alpha 4 \beta 2 \)
10 ACh  \( \frac{100 \text{ Zn}^{2+}}{} \)

B  \( \alpha 4 \beta 4 \)
1 ACh  \( \frac{100 \text{ Zn}^{2+}}{} \)

C  \( \alpha 4 \beta 4 \)
0.1 ACh  \( \frac{100 \text{ Zn}^{2+}}{} \)
Figure 2

A

Zn^{2+}

B

HEX

Zn^{2+}

C

\(\alpha 4\beta 2\)

ACh

\(\text{Zn}^{2+}\)

HEX

\(\text{Zn}^{2+}\)

\(\text{Zn}^{2+}\)

+ 85 mV

- 85 mV

D

\(\alpha 4\beta 2\)

Per cent of ACh Alone

pre HEX post HEX depol

E

\(\alpha 4\beta 4\)

Percent of ACh alone

pre HEX post HEX depol
Figure 5

A. _1 sec_  ACh  

B. _1 sec_  ACh + zinc  

C. _0.1 sec_  ACh  

D. _0.1 sec_  ACh + zinc
Figure 6

A

B

# bursts with durations ≥ to bin time

Burst duration (s)

ACh

ACh + Zn²⁺