Non-Agonist-Binding Subunit Interfaces Confer Distinct Functional Signatures to the Alternate Stoichiometries of the $\alpha 4\beta 2$ Nicotinic Receptor: An $\alpha 4$ – $\alpha 4$ Interface Is Required for Zn^{2+} Potentiation

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The $\alpha 4\beta 2$ subtype is the most abundant nicotinic acetylcholine receptor (nAChR) in the brain and possesses the high-affinity binding site for nicotine. The $\alpha 4$ and $\beta 2$ nAChR subunits assemble into two alternate stoichiometries, $(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4)_3(\beta 2)_2$, which differ in their functional properties and sensitivity to chronic exposure to nicotine. Here, we investigated the sensitivity of both receptor stoichiometries to modulation by Zn^{2+} . We show that Zn^{2+} exerts an inhibitory modulatory effect on $(\alpha 4)_2(\beta 2)_3$ receptors, whereas it potentiates or inhibits, depending on its concentration, the function of $(\alpha 4)_3(\beta 2)_2$ receptors. Furthermore, Zn^{2+} inhibition on $(\alpha 4)_2(\beta 2)_3$ nAChRs is voltage-dependent, whereas it is not on $(\alpha 4)_3(\beta 2)_2$ receptors. We used molecular modeling in conjunction with alanine substitution and functional studies to identify two distinct sets of residues that determine these effects and may coordinate Zn^{2+} . Zn^{2+} inhibition is mediated by a site located on the $\beta 2(+)/\alpha 4(-)$ subunit interfaces on both receptor stoichiometries. $\alpha 4^{\text{H195}}$ and $\beta 2^{\text{D218}}$ are key determinants of this site. Zn^{2+} potentiation on $(\alpha 4)_3(\beta 2)_2$ nAChRs is exerted by a site that resides on the $\alpha 4(+)/\alpha 4(-)$ of this receptor stoichiometry. $\alpha 4^{\text{H195}}$ on the $(-)$ side of the ACh-binding $\alpha 4$ subunit and $\alpha 4^{\text{E224}}$ on the $(+)$ side of the non-ACh-binding $\alpha 4$ subunit critically contribute to this site. We also identified residues within the $\beta 2$ subunit that confer voltage dependency to Zn^{2+} inhibition on $(\alpha 4)_2(\beta 2)_3$, but not on $(\alpha 4)_3(\beta 2)_2$ nAChRs.

Key words: Zn^{2+} modulation; nicotinic receptors; $\alpha 4\beta 2$ receptors; subunit interfaces; acetylcholine; ACh

Introduction

The $\alpha 4\beta 2$ receptor is the most abundant nicotinic acetylcholine receptor (nAChR) in the brain where it forms the high-affinity binding site for nicotine (Picciotto et al., 2001). $\alpha 4\beta 2$ receptors belong to the Cys-loop family of ligand-gated ion channels, which also includes the muscle nAChR and the GABA_A receptor. When expressed in heterologous systems, $\alpha 4$ and $\beta 2$ subunits produce two alternate stoichiometries, $(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4)_3(\beta 2)_2$, which differ in functional pharmacology, desensitization kinetics, unitary conductance (Nelson et al., 2003; Moroni et al., 2006), calcium permeability (Tapia et al., 2007), and sensitivity to chronic exposure to nicotine (Nelson et al., 2003; Kuryatov et al., 2005; Moroni et al., 2006). A mixture of both receptors may be expressed by thalamic neurons (Butt et al., 2002), and

their ratio is altered by an $\alpha 4^{\text{A529T}}$ polymorphism (Kim et al., 2003). By homology to the muscle $\alpha 1\gamma 1\delta\beta 1$ nAChR (Unwin, 2005), it is presumed that the $\alpha 4\beta 2$ nAChR harbors two functional agonist sites and that the order of subunits around the channel is $\alpha 4\beta 2\alpha 4\beta 2(\alpha 4\beta 2)$. On both stoichiometries, the agonist sites reside on $\alpha 4(+)/\beta 2(-)$ interfaces, suggesting similar properties. That the fifth subunit can be either $\alpha 4$ or $\beta 2$ leads to a different composition of non-agonist-binding interfaces. In the $(\alpha 4)_2(\beta 2)_3$ receptor, there will be two $\beta 2(+)/\alpha 4(-)$ interfaces and a $\beta 2(+)/\beta 2(-)$ interface, whereas in the $(\alpha 4)_3(\beta 2)_2$ receptor there will be two $\beta 2(+)/\alpha 4(-)$ interfaces and an $\alpha 4(+)/\alpha 4(-)$ interface. Understanding how receptor stoichiometry influences the properties of $\alpha 4\beta 2$ nAChRs is of general structural interest and has the potential to offer novel therapeutic strategies.

$\alpha 4\beta 2$ nAChRs, like other Cys-loop ligand-gated ion channels, are modulated by Zn^{2+} (Hsiao et al., 2001). Although advances have been made in identifying Zn^{2+} sites on GABA_A (Hosie et al., 2003) and glycine (Miller et al., 2005a,b) receptors, corresponding sites on $\alpha 4\beta 2$ receptors have been relatively unexplored. A potentiating Zn^{2+} -binding site has been proposed on $\alpha 4\beta 4/\beta 2$ nAChRs that involves Zn^{2+} coordination by a histidine residue ($\alpha 4^{\text{H162}}$) and a less direct, probably structural, contribution from $\alpha 4^{\text{E59}}$ (Hsiao et al., 2006). The site is likely to reside on the $\beta 2(+)/$

Received Nov. 19, 2007; revised May 9, 2008; accepted May 12, 2008.

This work was supported by the Oxford Brookes University (M.M., A.C.), the University of Oxford Clarendon Fund (R.V.), the United Kingdom Overseas Research Students Awards Scheme (R.V.), and the Wellcome Trust (P.C.B.). P.C.B. is a Research Councils UK Fellow. We thank J. Connolly and J. Dempster from Strathclyde University, Glasgow, UK and H. Vijverberg from Utrecht University, Utrecht, The Netherlands, for helpful advice on data analysis.

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DOI:10.1523/JNEUROSCI.1228-08.2008

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$\alpha 4(-)$ subunit interfaces that alternate with the ACh-binding $\alpha 4(+)/\beta 2(-)$ subunit interfaces (Hsiao et al., 2006). Given that $\beta 2(+)/\alpha 4(-)$ interfaces are present on both receptor forms, receptor stoichiometry should not influence Zn^{2+} effects on $\alpha 4\beta 2$ receptors (Hsiao et al., 2006). However, conceivably, because of structural homology, residues on non-agonist-binding subunits on $(\alpha 4)_3(\beta 2)_2$ or $(\alpha 4)_2(\beta 2)_3$ receptors could contribute to stoichiometry-specific Zn^{2+} effects.

We show that Zn^{2+} modulates $(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4)_3(\beta 2)_2$ receptors differentially, which was not detected in previous studies (Hsiao et al., 2001, 2006). Whereas Zn^{2+} only inhibits $(\alpha 4)_2(\beta 2)_3$ receptors, the function of $(\alpha 4)_3(\beta 2)_2$ is either potentiated or inhibited, depending on concentration. Furthermore, Zn^{2+} inhibition on $(\alpha 4)_2(\beta 2)_3$ is voltage dependent, whereas it is not on $(\alpha 4)_3(\beta 2)_2$ receptors. By using mutagenesis with receptor modeling and functional studies, we have identified determinants of Zn^{2+} inhibition on both stoichiometries as well as residues implicated in Zn^{2+} potentiation on $(\alpha 4)_3(\beta 2)_2$, providing evidence for a differential role of $\beta 2(+)/\alpha 4(-)$ and $\alpha 4(+)/\alpha 4(-)$ interfaces in Zn^{2+} modulation.

Materials and Methods

Animals. *Xenopus laevis* were purchased from Horst Kaehler. The care and use of *X. laevis* frogs in this study were approved by the Oxford Brookes University Animal Research Committee and meet the guidelines of the Scientific Procedures Act, 1986, of the United Kingdom.

Reagents. ACh, ZnCl_2 , tricine, and diethylpyrocarbonate (DEPC) were purchased from Sigma-Aldrich.

cDNA constructs and mutagenesis. Human nAChR $\alpha 4$ and $\beta 2$ subunit cDNAs were provided by Professor Jon Lindstrom (University of Pennsylvania, Philadelphia, PA) and subcloned into the pCI plasmid vector (Promega). Untranslated regions were eliminated by PCR to minimize differences in the transcription of the two cDNAs that may be brought about by untranslated regions flanking the coding sequence of the cDNAs (Briggs et al., 2006). Site directed mutagenesis was performed using the QuikChange mutagenesis kit (Stratagene). Unless otherwise mentioned, potential Zn^{2+} coordinating residues were replaced with alanine, a residue incapable of coordinating Zn^{2+} . Mutants were tested, individually or in combinations, for their effects on Zn^{2+} modulation of $\alpha 4\beta 2$ nAChRs. The full-length sequence of mutant subunit cDNAs was verified by DNA sequencing (Department of Biochemistry, University of Oxford, Oxford, UK). We present the numbering of the amino acid residues in terms of the full length, including the signal sequence. To obtain the position in the mature form, subtract 30 from the number for $\alpha 4$ and 25 for $\beta 2$.

Injection of cDNA constructs into nucleus of *Xenopus* oocytes. Stage V and VI *Xenopus* oocytes were prepared as described previously (Moroni et al., 2006). Wild-type or mutant human $\alpha 4$ or $\beta 2$ subunit cDNAs were injected into the nuclei of oocytes in a volume of 18.4 nl/oocyte, using a Nanoject Automatic Oocyte Injector (Drummond). To avoid the problem of stoichiometry heterogeneity, we used receptor expression strategies favoring the production of either $(\alpha 4)_2(\beta 2)_3$ or $(\alpha 4)_3(\beta 2)_2$ nAChRs (Moroni et al., 2006). Thus, to express the $(\alpha 4)_2(\beta 2)_3$ nAChRs, $\alpha 4$ and $\beta 2$ subunit cDNAs were combined in a ratio of 1:10, whereas a subunit ratio of 10:1 was used to produce the expression of $(\alpha 4)_3(\beta 2)_2$ nAChRs (Moroni et al., 2006). The total amount of cDNA injected per oocyte was kept constant at 2 ng. After injection, oocytes were incubated at 18°C for 2–5 d in a modified Barth's solution containing 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO_3 , 0.3 mM $\text{Ca}(\text{NO}_3)_2$, 0.41 mM CaCl_2 , 0.82 mM MgSO_4 , 15 mM HEPES, and 50 $\mu\text{g}/\text{ml}$ neomycin, pH 7.6.

Electrophysiology and data analysis. Recordings were performed 2–5 d after injection. Oocytes were placed in a 0.1 ml recording chamber and perfused with modified Ringer solution [containing (in mM) 150 NaCl, 2.8 KCl, 10 HEPES, and 1.8 BaCl_2 , pH 7.2, adjusted with NaOH] at a rate of 15 ml/min. We chose a nominally Ca^{2+} free solution to minimize the contribution to the response of Ca^{2+} -gated chloride channels which are endogenous to the *Xenopus* oocyte and may be activated by Ca^{2+} entry

through the heterologously expressed nAChRs. The possible contribution of contaminant trace levels of Zn^{2+} in the external Ringer solution to the estimated potency of Zn^{2+} was investigated by adding 10 mM tricine to the following concentrations of Zn^{2+} -Ringer solutions (in μM): 0.26, 0.78, 2.6, 7.8, 26, 77.5, and 254. Assuming that the K_D for Zn^{2+} complexation is 10^{-5} M (Paoletti et al., 1997), the concentrations of free Zn^{2+} were estimated to be (in nM) 1, 3, 10, 30, 100, 300, and 1000, respectively. Oocytes were impaled by two agarose-cushioned microelectrodes filled with 3 M KCl ($r = 0.3$ – 1.0 M Ω) and voltage clamped at -60 mV, except for studies of voltage dependency, using a GeneClamp 500B amplifier and pClamp 8 software (Molecular Devices). Typically, traces were filtered at 1 kHz during recording and digitized at 1 kHz using the DigiData 1200 interface (Molecular Devices). All experiments were performed at room temperature.

To construct concentration–response curves for ACh, the peak current (I) was measured in the presence of each concentration of ACh applied at 4 min intervals to prevent desensitization. All peak currents were normalized to the peak amplitude of current induced by 1 mM ACh, the maximum effective concentration of ACh (I_{max}) at $(\alpha 4)_2(\beta 2)_3$ or $(\alpha 4)_3(\beta 2)_2$ nAChRs. Data were fitted with the Hill equation: $I/I_{\text{max}} = [1/(1 + (\text{EC}_{50}/[X])^{n_{\text{Hill}}})]$, where EC_{50} is the concentration of ACh that evokes 50% of the maximal response (I_{max}), I the current response at a given concentration of ACh and n_{Hill} is the Hill coefficient.

The sensitivity to Zn^{2+} was assessed by coapplying a range of Zn^{2+} concentrations with 1 μM ACh at $(\alpha 4)_2(\beta 2)_3$ nAChRs [a concentration close to the ACh EC_{20} at $(\alpha 4)_2(\beta 2)_3$ receptors] (see Fig. 1C), or 10 μM ACh at $(\alpha 4)_3(\beta 2)_2$ receptors [the ACh EC_{10} at $(\alpha 4)_3(\beta 2)_2$ receptors] (see Fig. 1D). For Zn^{2+} to attain equilibrium around impaled oocytes, Zn^{2+} was preapplied for 30 s to the cell before coapplications of ACh and Zn^{2+} . Concentration–response relationships for Zn^{2+} were obtained using this protocol and the peak responses elicited by ACh plus Zn^{2+} were normalized to the peak response of the appropriate ACh alone. Where a single component concentration–response relationship was evident, data were fitted to the Hill equation shown above. When Zn^{2+} produced both a potentiating and inhibiting effect, the data were fitted to the following equation designed to account for the potentiating and inhibitory effects of Zn^{2+} on $\alpha 4\beta 2$ receptors assuming this cation binds to two distinct sites on the receptor: $I = (1 + ((I_{\text{max}} - X)/(1 + 10^{((\log \text{EC}_{50} - X) \times n_{\text{Hill-pot}}))})) / (1 + 10^{((\log \text{IC}_{50} - X) \times n_{\text{Hill-inh}}))})$, where I represents the current responses at a given Zn^{2+} concentration (X), I_{max} represents the maximally potentiated peak, EC_{50} and IC_{50} are the concentrations of Zn^{2+} inducing half-maximal potentiation or inhibition, respectively, and $n_{\text{Hill-pot}}$ and $n_{\text{Hill-inh}}$ are the Hill coefficients for potentiation and inhibition, respectively. F tests were always performed to assess the fitting of the data; the simpler one-component model was preferred unless the extra sum-of-squares F test had a value of $p < 0.05$.

To determine the effects of pH on Zn^{2+} modulation of the ACh responses of $\alpha 4\beta 2$ nAChR, oocytes were superfused with Ringer solutions of pH 5.5 or 8.0. Zn^{2+} and ACh solutions applied onto oocytes were of the same pH as the superfusing Ringer solution. For experiments requiring the use of DEPC, oocytes, before recordings and one at a time, were incubated in Ringer solution containing 1 mM freshly prepared DEPC. Preliminary studies showed that higher concentrations of DEPC caused cell death; therefore, their effects on potentiating or inhibiting Zn^{2+} were not examined further in this study. An incubation time of 10 min was sufficient for 1 mM DEPC to achieve maximal DEPC effects without decreasing cell survival or impairing cell functions. After DEPC incubation the oocyte was placed on a rotating platform and washed for an additional 10 min in DEPC-free Ringer solution. During washing, the solution was exchanged every 2–3 min to allow complete removal of DEPC.

Data analyses were performed using GraphPad Prism 5 software. Data were pooled from at least three different batches of oocytes. Statistical significance was assessed using a two-tailed unpaired t test, or one-way ANOVA followed by the Dunnett's post-test, as appropriate. p values < 0.05 were considered significant.

Molecular modeling. Sequences of the human $\alpha 4$ and $\beta 2$ nAChR subunits were obtained from the ExPASy proteomics server (Gasteiger et al., 2003) with accession numbers P43681 ($\alpha 4$) and P17787 ($\beta 2$). The

homopentameric *Lymnaea stagnalis* acetylcholine-binding protein (AChBP) structure (Protein Data Bank code 1UW6) (Celie et al., 2004) was used to generate models of the extracellular domain of the alternate stoichiometries of $\alpha 4\beta 2$ nAChR, $(\alpha 4)_2(\beta 2)_3$, and $(\alpha 4)_3(\beta 2)_2$ (Nelson et al., 2003; Moroni et al., 2006). Although the sequence identity between AChBP and the subunits of the $\alpha 4\beta 2$ nAChRs is only 18–20%, a similar fold and highly conserved binding-site residues (Brejc et al., 2001) make the AChBP structure suitable for modeling the extracellular domain of nAChRs. Additionally, and to model regions around the transmembrane domain, the 4 Å cryoelectron microscopy structure of the *Torpedo marmorata* nAChR (Protein Data Bank code 2BG9) (Unwin, 2005) was chosen as the template to generate models of the full receptor. In the *Torpedo* nAChR, agonist binding sites exist between $\alpha 1$ - γ and $\alpha 1$ - δ subunits. Hence, the *Torpedo* $\alpha 1$ subunit was used to model the human $\alpha 4$ subunit, γ and δ subunits were used to model human $\beta 2$, and the remaining *Torpedo* $\beta 1$ subunit was used to model the human $\alpha 4/\beta 2$ interface. Multiple sequence alignment was performed using ClustalW (Higgins et al., 1994). One hundred models each of $(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4)_3(\beta 2)_2$ receptors were generated using Modeler 8 (Sali and Blundell, 1993). In the case of the full receptor, the intracellular region, missing in the template, was not modeled. Procheck (Laskowski et al., 1993) was used to evaluate the stereochemical quality of the generated models. The best model for each receptor was energy-minimized *in vacuo*, using the molecular dynamics package Amber 9 (Case et al., 2006), with 500 cycles of the steepest descent method followed by another 500 cycles of the conjugate gradient method. The quality of the energy-minimized structures was checked using Procheck. Images were generated using PyMOL (DeLano, 2002).

Zn^{2+} -binding site analysis. We used information from previous studies (Hsiao et al., 2006) as well as performing a more general search to identify residues involved in Zn^{2+} binding. Because histidines are commonly associated with binding Zn^{2+} (Choi and Lipton, 1999; Low et al., 2000; Castagnetto et al., 2002; Dunne et al., 2002; Hosie et al., 2003; Miller et al., 2005a,b; Nagaya et al., 2005; Hsiao et al., 2006), we searched for histidine residues in our extracellular N-terminal domain models. In this domain, we found six histidines in the $\alpha 4$ subunit (H35, H94, H137, H142, H145, and H195) and four in the $\beta 2$ subunit (H35, H71, H111, and H161). Next, we looked for aspartate and glutamate residues, which are known to form Zn^{2+} -binding sites (Castagnetto et al., 2002), within 13 Å of a histidine residue to include regions that could be brought closer by protein flexibility. Cysteines were not considered because all cysteine residues in the extracellular N-terminal domain are disulfide bridged (Celie et al., 2004; Unwin, 2005) and, hence, not available for coordinating Zn^{2+} . We then narrowed down the list by eliminating locations that were not accessible: intrasubunit or on inflexible regions of the receptor.

$\alpha 4^{H195}$ was selected as a target because the equivalent histidine ($\alpha 4^{H162}$) in the rat $\alpha 4\beta 4$ receptor was identified previously as a site involved in Zn^{2+} potentiation (Hsiao et al., 2006) and also because this residue is located in loop F, which is a flexible loop (Szarecka et al., 2007) that may interact with the adjacent subunit in the non-ACh-binding interface. In the $\alpha 4(+)/\alpha 4(-)$ interface of the $(\alpha 4)_3(\beta 2)_2$ receptor, $\alpha 4(-)^{E92}$ in loop D and $\alpha 4(+)^{D217}$ and $\alpha 4(+)^{D218}$ in loop C, which is a very flexible loop as evident from x-ray crystal structures (Hansen et al., 2005) and molecular dynamics studies (Henchman et al., 2003, 2005), were identified that could form part of the binding site. In the $\beta 2(+)/\alpha 4(-)$ interface of the $(\alpha 4)_2(\beta 2)_3$ receptor, $\beta 2(+)^{E224}$ was selected using this approach.

$\beta 2^{H71}$ was chosen on the basis that it is within a region, near the

Table 1. $(\alpha 4)_2(\beta 2)_3$ nAChR sensitivity to modulation by Zn^{2+}

Receptor type	EC ₅₀ ACh (μ M)	IC ₅₀ Zn^{2+} (μ M)	n_{Hill}	Voltage dependency	n
$(\alpha 4)_2(\beta 2)_3$	4.04 ± 0.3	19 ± 6	0.45 ± 0.014	Y	10
$\alpha 4^{H195A}\beta 2$	3.80 ± 0.2	156 ± 11*	0.41 ± 0.03	Y	5
$\alpha 4^{E92A}\beta 2$	5.20 ± 0.4	51 ± 10*	0.40 ± 0.02	Y	5
$\alpha 4\beta 2^{D218A}$	1.01 ± 0.4	160 ± 14*	0.48 ± 0.01	Y	5
$\alpha 4\beta 2^{D217A}$	4.50 ± 0.3	17 ± 9	0.43 ± 0.03	Y	5
$\alpha 4^{H195A}\beta 2^{D218A}$	7.01 ± 1.5	1144 ± 54*	0.54 ± 0.01	Y	5
$\alpha 4^{H195A}\beta 2^{D217A}$	10.0 ± 1.6	101 ± 23*	0.42 ± 0.09	Y	5
$\alpha 4^{H195A,E92A}\beta 2$	6.70 ± 1.6	156 ± 42*	0.51 ± 0.07	Y	5
$\alpha 4\beta 2^{D218A,D217A}$	4.04 ± 1.2	390 ± 20*	0.48 ± 0.05	Y	4
$\alpha 4^{E92A}\beta 2^{D218A}$	3.50 ± 0.9	600 ± 99*	0.45 ± 0.01	Y	4
$\alpha 4^{E92A}\beta 2^{D217A}$	4.5 ± 1	48 ± 9*	0.44 ± 0.01	Y	4
$\alpha 4^{H195A,E92A}\beta 2^{D218A}$	7.86 ± 0.9	980 ± 27*	0.63 ± 0.02*	Y	5
$\alpha 4\beta 2^{H71A}$	2.30 ± 0.9	19 ± 5	0.52 ± 0.02*	N	4
$\alpha 4\beta 2^{E74A}$	4.50 ± 0.8	19 ± 7	0.65 ± 0.07*	N	4
$\alpha 4\beta 2^{D293A}$	8.30 ± 2.2	21 ± 9	0.63 ± 0.02*	N	3
$\alpha 4\beta 2^{H71A,E74A}$	6.78 ± 0.8	17 ± 2	0.60 ± 0.02*	N	4
$\alpha 4\beta 2^{H71A,E74A,D293A}$	5.01 ± 0.7	12 ± 4	0.60 ± 0.03*	N	4
$\alpha 4^{H195A}\beta 2^{H71A,E74A}$	4.87 ± 0.7	178 ± 23*	0.65 ± 0.04*	N	4
$\alpha 4^{H195A}\beta 2^{H71A,E74A,D293A,D218A}$	4.40 ± 0.9	1085 ± 100*	0.64 ± 0.07*	N	4
$\alpha 4^{E224A}\beta 2$	4.10 ± 0.3	18 ± 6	0.41 ± 0.04	Y	3
$\alpha 4^{E228A}\beta 2$	4.40 ± 0.6	19 ± 5	0.43 ± 0.08	Y	3
$\alpha 4^{E224H}\beta 2$	3.6 ± 0.2	20 ± 7	0.52 ± 0.1	Y	3
$\alpha 4^{H195E}\beta 2$	4.9 ± 0.9	22.04 ± 5	0.47 ± 0.09	Y	3
$\alpha 4^{H195E,E224H}\beta 2$	4.9 ± 0.8	19 ± 11	0.51 ± 0.1	Y	3

The values indicate the ACh EC₅₀ values for activating wild-type and mutant $(\alpha 4)_2(\beta 2)_3$ nAChRs and also the Zn^{2+} IC₅₀ values for inhibiting the EC₂₀ ACh responses. All values are means ± SEM from n cells. ND, Not determined.

* $p < 0.05$ compared with $(\alpha 4)_2(\beta 2)_3$ (see Materials and Methods).

transmembrane domain (from our model of the full-length receptor), with multiple residues ($\beta 2^{E74}$, $\beta 2^{D293}$, and $\beta 2^{E72}$) that could potentially bind Zn^{2+} . $\beta 2^{E72}$ was excluded because it is buried and inaccessible.

Results

A comparison of the sensitivity of the alternate stoichiometries of the $\alpha 4\beta 2$ nAChR to Zn^{2+} modulation was initiated to examine the role of receptor stoichiometry on the effects of Zn^{2+} on $\alpha 4\beta 2$ nAChRs. In a previous study, we showed that *Xenopus* oocytes injected with human $\alpha 4$ and $\beta 2$ nAChR subunit cDNAs at a ratio of 1:10 or 10:1 produce homogeneous populations of $(\alpha 4)_2(\beta 2)_3$ or $(\alpha 4)_3(\beta 2)_2$ nAChRs, respectively (Moroni et al., 2006). $(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4)_3(\beta 2)_2$ nAChRs differ significantly with respect to functional sensitivity to ACh (Tables 1, 2) and other $\alpha 4\beta 2$ -preferring nicotinic ligands, desensitization kinetics, sensitivity to long-term exposure to nicotine (Kuryatov et al., 2005; Moroni et al., 2006; Zwart et al., 2006), and Ca^{2+} permeability (Tapia et al., 2007). We determined the stoichiometries of the $\alpha 4\beta 2$ nAChR using electrophysiological methods in combination with a reporter mutation within the transmembrane domain (TM) 2 of the receptor (Moroni et al., 2006). Human $\alpha 4\beta 2$ nAChR expressed heterologously in human embryonic kidney tsA201 cells have also been shown to assemble as $(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4)_3(\beta 2)_2$ receptors (Nelson et al., 2003).

The ACh current responses of $(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4)_3(\beta 2)_2$ nAChR expressed heterologously in *Xenopus* oocytes were modulated differentially by Zn^{2+} . Zn^{2+} inhibited the ACh responses of $(\alpha 4)_2(\beta 2)_3$ with an IC₅₀ value of $19 \pm 6 \mu$ M ($n = 10$) (Fig. 1A,B). The Zn^{2+} concentration–response curve was best fitted with a single Hill equation ($p = 0.001$, F test; $n = 10$); however, the Hill slope of the curve was 0.45 ± 0.014 , suggesting the likely presence of multiple inhibitory components in the Zn^{2+} concentration–response curve. The steepness of the curve did not change when the buffer tricine was used to remove potential low

Table 2. ($\alpha 4$)₃($\beta 2$)₂ nAChR sensitivity to modulation by Zn²⁺

Receptor type	ACh EC ₅₀ (μ M)	I_{ACh+Zn}/I_{ACh}	Zn ²⁺ EC ₅₀ (μ M)	$n_{Hill-pot}$	Zn ²⁺ IC ₅₀ (μ M)	$n_{Hill-inh}$
($\alpha 4$) ₃ ($\beta 2$) ₂	89 ± 5	1.51 ± 0.3	50 ± 5	1.5 ± 0.5	803 ± 43	1.7 ± 0.3
$\alpha 4^{H195A}\beta 2$	68 ± 5	0.99 ± 0.01	*	*	470 ± 15*	1.3 ± 0.3
$\alpha 4^{E92A}\beta 2$	85 ± 7	1.50 ± 0.1	44 ± 8	1.5 ± 0.1	810 ± 22	1.8 ± 0.2
$\alpha 4\beta 2^{D218A}$	74 ± 7	2.51 ± 0.1*	34 ± 5*	2.0 ± 0.4	762 ± 21	1.7 ± 0.4
$\alpha 4^{H195A,E92A}\beta 2$	76 ± 6	0.99 ± 0.2	*	*	481 ± 17*	1.2 ± 0.06
$\alpha 4^{H195A}\beta 2^{D218A}$	75 ± 12	1.30 ± 0.2	35 ± 13	1.6 ± 0.1	380 ± 25*	1.7 ± 0.1
$\alpha 4^{E92A}\beta 2^{D218A}$	81 ± 7	2.50 ± 0.06	29 ± 5*	1.5 ± 0.08	763 ± 12	1.7 ± 0.07
$\alpha 4^{H195A,E92A}\beta 2^{D218A}$	72 ± 4	1.01 ± 0.03	*	*	457 ± 13*	1.7 ± 0.1
$\alpha 4\beta 2^{D217A}$	71 ± 8	1.60 ± 0.1	42 ± 10	2.0 ± 0.1	796 ± 11	1.6 ± 0.1
$\alpha 4^{H195A}\beta 2^{D217A}$	92 ± 12	0.99 ± 0.03	*	*	480 ± 32*	1.3 ± 0.03
$\alpha 4\beta 2^{D218A,D217A}$	86 ± 10	2.70 ± 0.1*	32 ± 1*	1.7 ± 0.1	798 ± 88	1.4 ± 0.1
$\alpha 4^{E92A}\beta 2^{D217A}$	75 ± 8	1.60 ± 0.1	43 ± 9	1.6 ± 0.1	811 ± 22	1.7 ± 0.3
$\alpha 4^{E224A}\beta 2$	60 ± 18	0.98 ± 0.06	*	*	784 ± 43	0.8 ± 0.01
$\alpha 4^{E228A}\beta 2$	81 ± 9	1.56 ± 0.2	49 ± 5	1.8 ± 0.1	808 ± 99	1.7 ± 0.1
$\alpha 4^{E224A}\beta 2^{D218A}$	78 ± 10	1.01 ± 0.1	*	*	470 ± 43*	1.4 ± 0.1
$\alpha 4^{E224H}\beta 2$	69 ± 10	1.30 ± 0.2	47 ± 8	1.6 ± 0.07	799 ± 85	1.5 ± 0.4
$\alpha 4^{H195E}\beta 2$	71 ± 15	1.10 ± 0.6	ND	ND	700 ± 18	1.4 ± 0.5
$\alpha 4^{H195E,E224H}\beta 2$	80 ± 16	1.30 ± 0.7	47 ± 6	1.8 ± 0.2	766 ± 30	1.7 ± 0.1
$\alpha 4^{H195A,E224A}\beta 2^{D218A}$	83 ± 11	1.00 ± 0.1	*	*	461 ± 58*	1.6 ± 0.1
$\alpha 4\beta 2^{H71A}$	84 ± 15	1.87 ± 0.1	47 ± 5	1.5 ± 0.1	732 ± 70	1.8 ± 0.1
$\alpha 4\beta 2^{E74A}$	69 ± 7	1.82 ± 0.2	43 ± 4	1.6 ± 0.1	753 ± 75	1.8 ± 0.1
$\alpha 4\beta 2^{D293A}$	84 ± 9	1.84 ± 0.1	42 ± 9	1.7 ± 0.1	743 ± 89	1.8 ± 0.9
$\alpha 4\beta 2^{H71A,E74A,D293A}$	91 ± 10	2.91 ± 0.1*	46 ± 8	1.6 ± 0.2	501 ± 53*	1.8 ± 0.1
$\alpha 4\beta 2^{H71A,E74A,D293A,D218A}$	75 ± 10	4.30 ± 0.1*	31 ± 1*	1.7 ± 0.1	499 ± 66*	1.8 ± 0.2
$\alpha 4^{H195A}\beta 2^{H71A,E74A,D293A}$	84 ± 11	1.04 ± 0.1	*	*	507 ± 65*	1.7 ± 0.1
$\alpha 4^{H195A}\beta 2^{H71A,E74A,D293A,D218A}$	90 ± 15	1.18 ± 0.1	29 ± 3*	1.1 ± 0.09	230 ± 43*	1.4 ± 0.1

The values indicate the ACh EC₅₀ values for activating wild-type and mutant ($\alpha 4$)₃($\beta 2$)₂ nAChRs and also the Zn²⁺ EC₅₀ or IC₅₀ values for potentiating or inhibiting, respectively, the EC₁₀ ACh responses. ND, Not determined.

* $p < 0.05$ compared with wild-type controls.

levels of Zn²⁺ contamination in the Ringer solution (data not shown). In contrast, ($\alpha 4$)₃($\beta 2$)₂ nAChR displayed a biphasic response to Zn²⁺ application ($p = 0.003$, F test; $n = 10$). Current responses activated by 10 μ M ACh at ($\alpha 4$)₃($\beta 2$)₂ nAChRs were potentiated by 1–100 μ M Zn²⁺, up to a maximum of 1.51 ± 0.5 at 100 μ M Zn²⁺ relative to controls in the absence of Zn²⁺ (Fig. 1A,B). The EC₅₀ value for potentiation was 50 ± 5 μ M ($n = 10$). Higher concentrations of Zn²⁺ decreased the degree of potentiation until at concentrations >800 μ M Zn²⁺ the amplitudes of the ACh responses elicited in the presence of Zn²⁺ were smaller than those mediated by applications of ACh alone (Fig. 1A,B). The estimated IC₅₀ value for the inhibition of ($\alpha 4$)₃($\beta 2$)₂ nAChR by Zn²⁺ was 803 ± 43 μ M ($n = 10$). Our findings contrast with those of Hsiao et al. (2001, 2006), who reported only Zn²⁺ biphasic effects on rat $\alpha 4\beta 2$ nAChRs expressed heterologously in *Xenopus* oocytes. This is likely because of the use of different expression strategies. Hsiao et al. (2001, 2006) injected oocytes with equal amounts of $\alpha 4$ and $\beta 2$ cRNAs, which is known to produce a mixture of diverse functional types of the $\alpha 4\beta 2$ nAChR, whose predominant component (80–90%) is the low sensitivity form [i.e., ($\alpha 4$)₃($\beta 2$)₂] of the $\alpha 4\beta 2$ nAChR (Zwart and Vijverberg, 1998; Houlihan et al., 2001; Exley et al., 2006; Zwart et al., 2006).

The mechanism of action of Zn²⁺ was subsequently investigated by determining both the effects of Zn²⁺ on the ACh concentration–response curve of the ($\alpha 4$)₂($\beta 2$)₃ and ($\alpha 4$)₃($\beta 2$)₂ nAChRs and the voltage dependency of the Zn²⁺ effects. We chose to investigate Zn²⁺ potentiation on ($\alpha 4$)₃($\beta 2$)₂ nAChR and Zn²⁺ inhibition on the ($\alpha 4$)₂($\beta 2$)₃ nAChR because such distinct effects are likely to be mediated by different Zn²⁺-binding sites. At ($\alpha 4$)₂($\beta 2$)₃ nAChR, 10 μ M Zn²⁺, a concentration slightly lower than the IC₅₀ value for Zn²⁺ inhibition, significantly decreased the amplitude of the current responses

elicited by saturating ACh concentrations ($p < 0.05$; $n = 6$), but had no significant effect on the apparent functional ACh sensitivity of the receptors (EC₅₀ ACh = 4.04 ± 0.3 μ M; EC₅₀ ACh plus Zn²⁺ = 3 ± 0.4 μ M; $n = 6$) (Fig. 1C). These results indicate that Zn²⁺ inhibits the ($\alpha 4$)₂($\beta 2$)₃ nAChR in a noncompetitive manner, which is in keeping with previous findings that residues coordinating Zn²⁺ do not contribute to the agonist binding site in ligand-gated ion channels structurally related to nAChRs (Low et al., 2000; Hosie et al., 2003; Miller et al., 2005; Hsiao et al., 2006). The inhibition of the ACh responses of the ($\alpha 4$)₂($\beta 2$)₃ nAChR by 10 μ M Zn²⁺ was examined at several holding potentials ranging from -120 to -30 mV. The degree of inhibition of the ACh responses of the ($\alpha 4$)₂($\beta 2$)₃ nAChR by Zn²⁺ decreased with hyperpolarization (Fig. 1E). Voltage-dependent effects of Zn²⁺ have been reported previously for $\alpha 2\beta 4$ nAChRs expressed heterologously in *Xenopus* oocytes (García-Colunga et al., 2001) and for native nAChRs in rat parasympathetic ganglia (Nutter and Adams, 1995).

At the ($\alpha 4$)₃($\beta 2$)₂ nAChR, we examined the effects of the maximally potentiating concentration of Zn²⁺ (100 μ M) to minimize possible counteracting effects by inhibitory Zn²⁺, which would reduce the degree of Zn²⁺ potentiation. Zn²⁺ decreased significantly the EC₅₀ value for ACh activation from 89 ± 5 μ M to 34 ± 5 μ M ($n = 8$; $p < 0.05$) (Fig. 1D). However, although Zn²⁺ increased the amplitude of responses to nonmaximal ACh concentrations, it did not increase the amplitude of the response to saturating ACh concentrations. This, as suggested by the concentration–response curve for Zn²⁺ effects on ($\alpha 4$)₃($\beta 2$)₂ nAChRs (Fig. 1B), could reflect masking of Zn²⁺ potentiation by counteracting Zn²⁺ inhibition. The degree of potentiation was similar at all holding potentials tested (Fig. 1E). The degree of inhibition of the ($\alpha 4$)₃($\beta 2$)₂ nAChR by 2 mM Zn²⁺, a concentration that decreased the ACh responses of ($\alpha 4$)₃($\beta 2$)₂ nAChRs by almost

70% the amplitude of the ACh responses of the $(\alpha 4)_3(\beta 2)_2$ nAChR, was also independent of the holding potential from -120 to -30 mV (Fig. 1E).

Histidine residues are involved in the effects of Zn^{2+}

Histidine is the most common residue that participates in Zn^{2+} binding by metalloenzymes (McCall et al., 2000) and by all ligand-gated ion channels examined so far (Choi and Lipton, 1999; Low et al., 2000; Dunne et al., 2002; Hosie et al., 2003; Miller et al., 2005a,b; Nagaya et al., 2005; Hsiao et al., 2006). If histidine residues participate in similar Zn^{2+} site(s) on the alternate forms of the human $\alpha 4\beta 2$ nAChR, then these site(s) should be impaired by DEPC, which reacts with neutral imidazole groups converting the residues to N-carboethoxyhistidyl derivatives. Exposure to 1 mM DEPC significantly reduced the extent of Zn^{2+} inhibition on the current responses activated by ACh at $(\alpha 4)_2(\beta 2)_3$ nAChRs ($I_{\text{ACh}+\text{Zn}^{2+}} = 0.57 \pm 0.009$; after DEPC treatment, $I_{\text{ACh}+\text{Zn}^{2+}} = 0.77 \pm 0.012$, relative to control I_{ACh} ; $p < 0.05$; $n = 5$). In contrast, DEPC treatment abolished both the voltage dependency of Zn^{2+} inhibition on $(\alpha 4)_2(\beta 2)_3$ nAChRs and Zn^{2+} potentiation on $(\alpha 4)_3(\beta 2)_2$ receptors ($I_{\text{ACh}+\text{Zn}^{2+}} = 1.51 \pm 0.05$; after DEPC treatment, $I_{\text{ACh}+\text{Zn}^{2+}} = 0.99 \pm 0.3$, relative to control I_{ACh} ; $p < 0.05$; $n = 5$) (Fig. 2). Because the ability of Zn^{2+} to react with Zn^{2+} -coordinating amino acids is influenced by pH (Miles, 1977), one possible explanation for the failure of DEPC to eliminate completely voltage-independent Zn^{2+} inhibition is that at pH 7.2 DEPC does not react effectively with all the residues implicated in Zn^{2+} inhibition and that partial disruption does not completely abolish the functionality of the site(s). Nevertheless, because DEPC effectively reduces the ability of Zn^{2+} to bind to the imidazole ring of histidines (Dunne et al., 2002; Hosie et al., 2003; Miller et al., 2005b; Nagaya et al., 2005), these findings implicated histidines in the coordination of inhibitory and potentiating Zn^{2+} (Fig. 2).

An additional test for the involvement of histidine residues on the effects of Zn^{2+} on $\alpha 4\beta 2$ receptors is the assessment of the effect of H^+ ions on the responses to ACh in the absence and presence of Zn^{2+} . This test would ascertain whether histidine residues contribute to Zn^{2+} effects because H^+ will compete with Zn^{2+} for binding to the imidazole group on histidine residues. At pH 5.5, the extent of inhibition of the ACh-evoked currents on the $(\alpha 4)_2(\beta 2)_3$ nAChR by $10 \mu\text{M}$ Zn^{2+} was decreased from 0.57 ± 0.03 to 0.84 ± 0.01 , whereas at pH 8.0 inhibition increased to 0.49 ± 0.04 , compared with inhibition at pH 7.2 ($n = 4$). At pH 5.5, the degree of potentiation of the $(\alpha 4)_3(\beta 2)_2$ nAChR by $100 \mu\text{M}$ Zn^{2+} was reduced from 1.48 ± 0.5 ($n = 6$) to 1.1 ± 0.1 ($n = 4$), whereas at pH 8.0 potentiation increased to

Figure 1. Effects of Zn^{2+} on currents activated by ACh in wild-type $(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4)_3(\beta 2)_2$ nAChR expressed heterologously in *Xenopus* oocytes. **A**, Examples of the effects of Zn^{2+} on currents activated by EC_{20} or EC_{10} ACh concentrations on $(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4)_3(\beta 2)_2$ nAChRs, respectively. Zn^{2+} concentrations applied onto both receptor types are indicated in each panel. **B**, Averaged concentration–response for the effects of Zn^{2+} at $(\alpha 4)_2(\beta 2)_3$ (open circles) and $(\alpha 4)_3(\beta 2)_2$ (filled circles) nAChRs. The concentration–response data were fit to the appropriate equations as described in the Materials and Methods section. Averaged parameters of best fits to the concentration–response data are given in Tables 1 and 2. **C**, **D**, ACh concentration–response curves for $(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4)_3(\beta 2)_2$ nAChRs in the absence and presence of Zn^{2+} . For $(\alpha 4)_2(\beta 2)_3$ (C), a concentration of Zn^{2+} ($10 \mu\text{M}$) lower than the IC_{50} value for Zn^{2+} inhibition ($19 \mu\text{M}$) was tested, whereas a maximally potentiating Zn^{2+} concentration ($100 \mu\text{M}$) was used for the studies on $(\alpha 4)_3(\beta 2)_2$ (D) nAChRs. **E**, Voltage dependency of the effects of Zn^{2+} on ACh responses of $\alpha 4\beta 2$ nAChRs. The ACh responses of $(\alpha 4)_2(\beta 2)_3$ were measured in the presence of $10 \mu\text{M}$ Zn^{2+} , whereas those of $(\alpha 4)_3(\beta 2)_2$ nAChRs were studied in the presence of $100 \mu\text{M}$ or 2 mM Zn^{2+} concentrations. The ACh responses were recorded at a range of membrane potentials from -130 to -20 mV. Error bars indicate SEM.

1.65 ± 0.09 ($n = 4$). Because histidine is the only amino acid with a pK_a that would be affected by these pH changes (pK_a 6.1), it clearly contributes to the effects of Zn^{2+} on $\alpha 4\beta 2$ nAChRs.

Structural determinants for Zn^{2+} effects on $(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4)_3(\beta 2)_2$ nAChRs

Previous studies have suggested that $\alpha 4^{\text{H162}}$ within the F loop might be a direct contributor to Zn^{2+} binding forming part of a potentiating site on rat $\alpha 4\beta 4$ or $\alpha 4\beta 2$ nAChRs (Hsiao et al., 2006). We examined this proposal on human $\alpha 4\beta 2$ nAChRs by mutating $\alpha 4^{\text{H195}}$, the human equivalent of rat $\alpha 4^{\text{H162}}$, to alanine. (Note that amino acid numbering is from the initiating methionine of the unprocessed subunit. To obtain the position in the mature form, subtract 30 from the number for $\alpha 4$ and 25 for $\beta 2$.) However, because Zn^{2+} -binding sites require at least three amino acid residues and an activated water molecule for complete tetrahedral coordination (Auld, 2001), we searched for further residues capable of coordinating Zn^{2+} and that were in close

Figure 2. Diethylpyrocarbonate inhibits the voltage-dependent effects of Zn^{2+} on currents activated by ACh at $(\alpha 4)_2(\beta 2)_3$ nAChRs expressed heterologously in *Xenopus* oocytes. Current–voltage relationship of ACh current responses of $(\alpha 4)_2(\beta 2)_3$ nAChRs expressed heterologously in *Xenopus* oocytes. DEPC abolished the voltage dependency of the inhibitory effects of Zn^{2+} on the responses activated by ACh at $(\alpha 4)_2(\beta 2)_3$ nAChRs as shown by the current–voltage relationships for the ACh responses of $(\alpha 4)_2(\beta 2)_3$ in the absence and presence of Zn^{2+} before and after exposure to DEPC. Oocytes were exposed to 1 mM DEPC for 10 min before recordings. Longer periods of exposure did not increase the effect of DEPC. DEPC concentrations >1 mM caused irreversibly damage to oocytes expressing $\alpha 4\beta 2$ nAChRs and were therefore not examined in this study. Error bars indicate SEM.

structural proximity to $\alpha 4^{\text{H195}}$. In our model of the extracellular N-terminal domain of both forms of the human $\alpha 4\beta 2$ nAChR, $\alpha 4^{\text{H195}}$ could interact with $\beta 2^{\text{D218}}$ in loop C to form a Zn^{2+} -binding site at the non-ACh-binding $\beta 2(+)/\alpha 4(-)$ interfaces on both $\alpha 4\beta 2$ nAChR forms (Fig. 3A). An additional residue that could contribute to the proposed Zn^{2+} -binding sites is $\alpha 4^{\text{E92}}$, a residue within loop D (Fig. 3A).

Introducing either $\alpha 4^{\text{H195A}}$ or $\beta 2^{\text{D218A}}$ in the $(\alpha 4)_2(\beta 2)_3$ nAChR decreased sensitivity to Zn^{2+} inhibition significantly by approximately eightfold ($p < 0.05$; $n = 6$) (Fig. 3B, Table 1). By comparison, $\alpha 4^{\text{E92A}}$ reduced the Zn^{2+} IC_{50} value by only threefold (Table 1). Pairing $\alpha 4^{\text{H195A}}$ with $\beta 2^{\text{D218A}}$ further reduced the potency of Zn^{2+} inhibition to $1144 \pm 54 \mu\text{M}$ ($p < 0.05$; $n = 5$) (Fig. 3B, Table 1). However, pairing $\alpha 4^{\text{H195A}}$ with $\alpha 4^{\text{E92A}}$ did not increase the Zn^{2+} IC_{50} value beyond that observed with the single mutations of $\alpha 4^{\text{H195}}$ or $\beta 2^{\text{D218}}$ (Table 1). By comparison, pairing $\beta 2^{\text{D218A}}$ with $\alpha 4^{\text{E92A}}$ significantly increased the Zn^{2+} IC_{50} value relative to that of the single mutants ($p < 0.05$; $n = 5$) (Table 1). Introducing simultaneously $\alpha 4^{\text{H195A}}$, $\alpha 4^{\text{E92A}}$, and $\beta 2^{\text{D218A}}$ did not increase the Zn^{2+} IC_{50} value beyond that observed with the pairing of $\alpha 4^{\text{H195A}}$ and $\beta 2^{\text{D218A}}$ (Table 1). For all mutants, individually or in combinations, the functional sensitivity to ACh was indistinguishable from that of the wild-type $(\alpha 4)_2(\beta 2)_3$ nAChR, suggesting that these mutations selectively affected Zn^{2+} inhibition and did not induce a general perturbation on $(\alpha 4)_2(\beta 2)_3$ function (Table 1). These studies suggested that $\alpha 4^{\text{H195}}$, $\beta 2^{\text{D218}}$, and $\alpha 4^{\text{E92}}$ might contribute to Zn^{2+} binding, forming part of an inhibitory site predicted to reside at the $\beta 2(+)/\alpha 4(-)$ interfaces on both $\alpha 4\beta 2$ nAChR forms.

Although $\alpha 4^{\text{H195A}}$, $\alpha 4^{\text{E92A}}$, or $\beta 2^{\text{D218A}}$ decreased the magnitude of Zn^{2+} inhibition, they did not, singularly or in combinations, abolish it (Table 1), indicating the likely presence of an additional Zn^{2+} site or, conceivably, Zn^{2+} binding was not completely disrupted around the environment of $\alpha 4^{\text{H195}}$, $\alpha 4^{\text{E92}}$, and $\beta 2^{\text{D218}}$. Further examination of our model of the extracellular N-terminal domain of $(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4)_3(\beta 2)_2$ receptors revealed that $\beta 2^{\text{D217}}$ is a residue that could coordinate Zn^{2+} as part of a binding site formed by $\alpha 4^{\text{H195}}$, $\beta 2^{\text{D218}}$, and $\alpha 4^{\text{E92}}$ at $\beta 2(+)/\alpha 4(-)$ interfaces (Fig. 3A). However, as shown in Table 1, introducing $\beta 2^{\text{D217A}}$ decreased sensitivity to Zn^{2+} inhibition only when paired with $\alpha 4^{\text{E92A}}$ or $\beta 2^{\text{D218A}}$, which suggests an indirect role on Zn^{2+} inhibition for this residue. Although these findings

did not answer the question of whether alanine substitutions of $\alpha 4^{\text{H195}}$, $\beta 2^{\text{D218}}$, or $\alpha 4^{\text{E92}}$ fully disrupted the inhibiting Zn^{2+} locus predicted to reside at $\beta 2(+)/\alpha 4(-)$ interfaces, the observation that none of these mutations, singularly or combined, affected the voltage dependency of Zn^{2+} inhibition suggested additional inhibitory Zn^{2+} sites.

We have shown that $\alpha 4^{\text{H195}}$, $\alpha 4^{\text{E92}}$, and $\beta 2^{\text{D218}}$ residues are implicated in non-voltage-dependent Zn^{2+} inhibition of $(\alpha 4)_2(\beta 2)_3$ receptors. Given that these residues are predicted to reside on the non-ACh-binding $\beta 2(+)/\alpha 4(-)$ interfaces (Fig. 3A), which are common to both $\alpha 4\beta 2$ stoichiometries, we expected these residues to contribute to Zn^{2+} inhibition on the $(\alpha 4)_3(\beta 2)_2$ stoichiometry as well. This implied that replacement of any of these residues with alanine could result in an increase in sensitivity to Zn^{2+} potentiation and maximal potentiation, if, as suggested by the concentration–response curve for Zn^{2+} inhibition on $(\alpha 4)_2(\beta 2)_3$, Zn^{2+} potentiation and inhibition had overlapping concentration ranges. In accord with our prediction, $\beta 2^{\text{D218A}}$ increased maximal Zn^{2+} potentiation on $(\alpha 4)_3(\beta 2)_2$ and significantly increased the sensitivity of $(\alpha 4)_3(\beta 2)_2$ to Zn^{2+} potentiation from 50 to 31 μM ($p < 0.05$; $n = 6$) (Fig. 3C, Table 2). Moreover, $\beta 2^{\text{D218A}}$ caused potentiation of maximal ACh currents by Zn^{2+} (1.34 ± 0.09) (see Fig. 6B), which did not occur on wild-type $(\alpha 4)_3(\beta 2)_2$ (Fig. 1D). However, unexpectedly, $\alpha 4^{\text{H195A}}$ abolished Zn^{2+} potentiation on its own and increased sensitivity to Zn^{2+} inhibition, and when paired with $\beta 2^{\text{D218A}}$, it reduced maximal potentiation from 1.51 to 1.18 (Fig. 3C, Table 2). $\alpha 4^{\text{E92A}}$ reduced potentiation, albeit not significantly, and did not increase or reduce the individual or combined effects of $\alpha 4^{\text{H195A}}$ or $\beta 2^{\text{D218A}}$ (Table 2), suggesting that it might not form part of the Zn^{2+} potentiating site. As for Zn^{2+} inhibition on $(\alpha 4)_2(\beta 2)_3$ nAChRs, $\beta 2^{\text{D217A}}$ did not impact on the potentiating effects of Zn^{2+} on $(\alpha 4)_3(\beta 2)_2$ nAChRs (Table 2).

The $\alpha 4(+)/\alpha 4(-)$ subunit interface bears a potentiating Zn^{2+} site in $(\alpha 4)_3(\beta 2)_2$ nAChRs

The discovery that Zn^{2+} potentiation on $(\alpha 4)_3(\beta 2)_2$ nAChRs is abolished by $\alpha 4^{\text{H195A}}$ but enhanced by $\beta 2^{\text{D218A}}$ suggested that the signal transduction pathway associated with the Zn^{2+} inhibiting site located at the $\beta 2(+)/\alpha 4(-)$ subunit interfaces of the $\alpha 4\beta 2$ nAChR may differ in the $(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4)_3(\beta 2)_2$ receptors. An alternative explanation, given the structural homology of the subunits of the nAChR family, is that $\alpha 4^{\text{H195}}$ contributes to two independent Zn^{2+} -binding sites on the $(\alpha 4)_3(\beta 2)_2$ nAChR. One site is inhibitory and resides on the $\beta 2(+)/\alpha 4(-)$ subunit interfaces of both receptors; $\alpha 4^{\text{H195}}$, $\beta 2^{\text{D218}}$, and $\alpha 4^{\text{E92}}$ contribute to this site that is represented twice in both receptor stoichiometries (Fig. 4A). The other site is potentiating and resides on the $\alpha 4(+)/\alpha 4(-)$ interface of the $(\alpha 4)_3(\beta 2)_2$ nAChR (Fig. 4A). Conceivably, H195 on the $(-)$ side of the ACh-binding $\alpha 4$ subunit could contribute to the proposed Zn^{2+} potentiating site together with residues homologous to $\beta 2^{\text{D218}}$ on the $(+)$ side of the non-ACh-binding $\alpha 4$ subunit. Because D218 on the $\beta 2$ subunit is within loop C, this hypothesis was examined by introducing $\alpha 4(+)^{\text{E224A}}$ or $\alpha 4(-)^{\text{E228A}}$ in $(\alpha 4)_3(\beta 2)_2$ (Fig. 4B). Because of their location within loop C, $\alpha 4(+)^{\text{E224A}}$ or $\alpha 4(+)^{\text{E228A}}$ could contribute to a Zn^{2+} -binding site together with $\alpha 4^{\text{H195}}$ on the $(-)$ side of the adjacent ACh-binding $\alpha 4$ subunit. Other residues in loop C such as C225 and C226 were not considered as likely contributors to a Zn^{2+} -binding site because these residues are disulfide bridged (Celie et al., 2004; Unwin, 2005) and, therefore, unable to coordinate Zn^{2+} . Figure 4C shows that introducing mutant $\alpha 4^{\text{E224A}}$ abolished Zn^{2+} potentiation. Furthermore, pairing $\alpha 4^{\text{E224A}}$ with

$\beta 2^{\text{D218A}}$ (Table 2) or with $\alpha 4^{\text{H195A}}$ and $\beta 2^{\text{D218A}}$ (Fig. 4C) also eliminated Zn^{2+} potentiation. In contrast, introducing $\alpha 4^{\text{E228A}}$ did not affect Zn^{2+} potentiation (Table 2), supporting a role for $\alpha 4^{\text{E224}}$, but not for $\alpha 4^{\text{E228}}$, in potentiating Zn^{2+} . Abolition of Zn^{2+} potentiation by $\alpha 4^{\text{E224A}}$ could reflect a perturbation of the general properties of the receptor or involvement in downstream signal transduction pathways. As shown in Table 2, $\alpha 4^{\text{E224A}}$, individually or in combination with other residues, did not change the sensitivity to activation by ACh, indicating that the effect of $\alpha 4^{\text{E224A}}$ was specific on Zn^{2+} potentiation.

To test whether $\alpha 4^{\text{E224}}$ might be a direct contributor to Zn^{2+} binding, we substituted E224 with histidine and then examined whether DEPC treatment abolished Zn^{2+} potentiation on receptors containing this mutation. The more common strategy of cysteine substitution in combination with exposure to the cysteine-modifying reagent methyl thiosulfonate ethylammonium (MTSEA) was not considered appropriate for these studies because of the presence of neighboring C225 and C226. For these studies, we also mutated $\alpha 4^{\text{H195}}$ to glutamate to eliminate possible ambiguities that could have arisen from modification of $\alpha 4^{\text{H195}}$ by DEPC. Although introducing $\alpha 4^{\text{H195E}}$ abolished potentiation (Table 2), pairing of $\alpha 4^{\text{E224H}}$ with $\alpha 4^{\text{H195E}}$ produced levels of Zn^{2+} potentiation comparable with those obtained on wild receptor (Fig. 4D, Table 2), indicating that the mutations introduced maintained the functionality of the Zn^{2+} potentiating site. $\alpha 4^{\text{E224H}}$ on its own did not eliminate Zn^{2+} potentiation, although it reduced its potency (Table 2). Exposure to 1 mM DEPC completely abolished Zn^{2+} potentiation on $\alpha 4^{\text{H195E,E224H}}$ $\beta 2$ nAChRs (Fig. 4D). Furthermore, introducing $\alpha 4^{\text{E224H}}$ and $\alpha 4^{\text{H195E}}$ into $(\alpha 4)_3(\beta 2)_2$ nAChRs did not influence the sensitivity of $(\alpha 4)_3(\beta 2)_2$ nAChRs for activation by ACh (Table 2), or indeed that of $(\alpha 4)_2(\beta 2)_3$ nAChRs (Table 1). These data therefore are consistent with our view that $\alpha 4^{\text{E224}}$ and $\alpha 4^{\text{H195}}$ directly contribute to Zn^{2+} binding, forming part of a potentiating site at the $\alpha 4(+)/\alpha 4(-)$ subunit interface on the $(\alpha 4)_3(\beta 2)_2$ nAChR. Of importance, Zn^{2+} inhibition on $(\alpha 4)_2(\beta 2)_3$ nAChRs was not influenced by $\alpha 4^{\text{E224H}}$ or $\alpha 4^{\text{E224A}}$, further indicating that this residue specifically influences Zn^{2+} effects on $(\alpha 4)_3(\beta 2)_2$ nAChRs (Table 1).

Interestingly, the presence of a Zn^{2+} potentiating site on the $\alpha 4(+)/\alpha 4(-)$ interface reduced the apparent sensitivity of $(\alpha 4)_3(\beta 2)_2$ nAChRs to Zn^{2+} inhibition by 42-fold compared with the sensitivity of the $(\alpha 4)_2(\beta 2)_3$ stoichiometry to Zn^{2+} inhibition (Table 2). This may reflect allosteric interactions between the Zn^{2+} potentiating and Zn^{2+} sites present on $(\alpha 4)_3(\beta 2)_2$ nAChRs; however, we cannot discount the possibility

Figure 3. $\alpha 4$ and $\beta 2$ subunit residues that influence non-voltage-dependent Zn^{2+} inhibition on $\alpha 4\beta 2$ nAChRs. **A**, Left, Diagram showing the position of Zn^{2+} inhibitory sites on $\beta 2(+)/\alpha 4(-)$ interfaces on $(\alpha 4)_2(\beta 2)_3$ nAChR. ACh-binding sites on $\alpha 4(+)/\beta 2(-)$ interfaces are also shown. On the right is a side-on view of the $\beta 2(+)/\alpha 4(-)$ subunit interface region on the left showing the location of $\alpha 4^{\text{E92}}$ (in β sheet $\beta 2$, colored yellow), $\alpha 4^{\text{H195}}$ (in loop F, colored violet), and $\beta 2^{\text{D217}}$ and $\beta 2^{\text{D218}}$ (in loop C, colored orange). **B**, Zn^{2+} concentration–response curves constructed for mutant $(\alpha 4^{\text{H195A}})_2(\beta 2)_3$, $(\alpha 4)_2(\beta 2^{\text{D218A}})_3$, and $(\alpha 4^{\text{H195A}})_2(\beta 2^{\text{D218A}})_3$ receptors. Averaged parameters of best fits to concentration–response data are given in Tables 1. **C**, Zn^{2+} concentration–response curves constructed for mutant $(\alpha 4^{\text{H195A}})_3(\beta 2)_2$, $(\alpha 4)_3(\beta 2^{\text{D218A}})_2$, and $(\alpha 4^{\text{H195A}})_3(\beta 2^{\text{D218A}})_2$ receptors. The concentration–response data were fit to monophasic or the biphasic equations described in the Materials and Methods section. Averaged parameters of best fits to concentration–response data are given in Table 2. Error bars indicate SEM.

of subtly different properties between the Zn^{2+} inhibiting sites on both stoichiometries. At least one of these is flanked by different subunits in both receptor stoichiometries (Fig. 4A).

Voltage-dependent inhibition of $(\alpha 4)_2(\beta 2)_3$ by Zn^{2+}

The discovery that the voltage dependency of Zn^{2+} inhibition was abolished by DEPC treatment but not by substitution of $\alpha 4^{\text{H195}}$, $\alpha 4^{\text{E92}}$, or $\beta 2^{\text{D218}}$ by alanine suggested an additional inhibitory Zn^{2+} site. As the suggested site should be close to the ion channel to exert voltage-dependent effects, we searched our model of the $(\alpha 4)_2(\beta 2)_3$ receptor for histidine residues close to the ion channel and that could be accessible to external Zn^{2+} ions. According to our model, $\beta 2^{\text{H71}}$ and $\beta 2^{\text{E74}}$ both within the $\beta 1$ – $\beta 2$ loop and $\beta 2^{\text{D293}}$ within the TM2–TM3 extracellular

Figure 4. nAChR $\alpha 4$ subunit residues that influence Zn^{2+} potentiation on $(\alpha 4)_3(\beta 2)_2$ nAChRs. **A**, The left panel schematically illustrates the proposed low-affinity arrangement of the subunits and the location of the Zn^{2+} potentiation site at the $\alpha 4(+)/\alpha 4(-)$ interface. The right panel shows a side-on view of the model and the location of $\alpha 4^{\text{E224}}$ (in loop C, colored orange) and $\alpha 4^{\text{H195}}$ (in loop F, colored violet). It is clear that some additional movement probably from the C loop would be required to make the binding site competent. **B**, Alignment of the C loop of the $\alpha 4$ and $\beta 2$ nAChR subunits. **C**, Zn^{2+} concentration–response curves constructed for mutant $(\alpha 4^{\text{E224A}})_3(\beta 2)_2$ and $(\alpha 4^{\text{H195A,E224A}})_3(\beta 2^{\text{D218A}})_2$ receptors. The concentration–response data were fit to the appropriate equations as described in the Materials and Methods section. Averaged parameters of best fits to concentration–response data are given in Table 2. **D**, Zn^{2+} concentration–response curve constructed for mutant $(\alpha 4^{\text{H195E,E224H}})_3(\beta 2)_2$ receptor before and after DEPC treatment. Oocytes were exposed to 1 mM DEPC for 10 min as described in Materials and Methods. The concentration–response data were fit to the appropriate equations as described in Materials and Methods. Averaged parameters of best fits to concentration–response data are given in Table 2. Error bars indicate SEM.

linker could form a Zn^{2+} -binding site located near the ion channel region (Fig. 5A).

Introducing $\beta 2^{\text{H71A}}$, $\beta 2^{\text{E74A}}$, or $\beta 2^{\text{D293A}}$ individually or in combinations abolished the ability of Zn^{2+} to inhibit the ACh responses of $(\alpha 4)_2(\beta 2)_3$ nAChRs in a voltage-dependent manner without affecting the functional sensitivity of $(\alpha 4)_2(\beta 2)_3$ nAChRs to activation by ACh (Fig. 5B, Table 1). $\beta 2^{\text{H71A}}$, $\beta 2^{\text{E74A}}$, or $\beta 2^{\text{D293A}}$, individually or in combinations, had no effects on the estimated IC_{50} value for Zn^{2+} inhibition on wild-type $(\alpha 4)_2(\beta 2)_3$ nAChRs, but significantly increased the n_{Hill} coefficient (Fig. 5C, Table 1). Furthermore, when $\beta 2^{\text{H71A}}$, $\beta 2^{\text{E74A}}$, and $\beta 2^{\text{D293A}}$ were introduced with $\alpha 4^{\text{H195A}}$ or $\alpha 4^{\text{H195A}}$ and $\beta 2^{\text{D218A}}$, the n_{Hill} coefficient was further increased, whereas the sensitivity to Zn^{2+} inhibition, compared with that obtained with $\alpha 4^{\text{H195A}}$ alone or in combination with $\beta 2^{\text{D218A}}$, remained unchanged (Table 1).

Introduction of $\beta 2^{\text{H71A}}$, $\beta 2^{\text{E74A}}$, or $\beta 2^{\text{D293A}}$ had little effect on the ACh sensitivity of $(\alpha 4)_3(\beta 2)_2$ nAChRs (Table 2), but doubled

maximal potentiation by Zn^{2+} compared with wild-type $(\alpha 4)_3(\beta 2)_2$ nAChRs (Fig. 6A, Table 2). Furthermore, the stepwise introduction of these mutants into $(\alpha 4)_3(\beta 2)_2$ nAChRs progressively and significantly increased the degree of Zn^{2+} potentiation to levels greater than those produced by the individual mutations (Fig. 6A, Table 2). These results suggested that the site within the $\beta 2$ subunit producing voltage-dependent Zn^{2+} inhibition on the $(\alpha 4)_2(\beta 2)_3$ receptor is also present on $(\alpha 4)_3(\beta 2)_2$ nAChRs. Further evidence for the involvement of $\beta 2^{\text{H71A}}$, $\beta 2^{\text{E74A}}$, and $\beta 2^{\text{D293A}}$ in the inhibitory effects of Zn^{2+} on $(\alpha 4)_3(\beta 2)_2$ nAChRs was obtained by determining the effects of these mutations on the concentration–response curve for ACh at this receptor type. As shown in Figure 6B, Zn^{2+} potentiated the maximal ACh responses of $(\alpha 4)_3(\beta 2^{\text{H71A,E74A,D293A}})_2$ receptors. Furthermore, when $\beta 2^{\text{D218A}}$ was introduced against the background of $\beta 2^{\text{H71A,E74A,D293A}}$, potentiation of maximal ACh responses increased additively from 1.17 ± 0.2 ($\alpha 4)_3(\beta 2^{\text{H71A,E74A,D293A}})_2$ and 1.34 ± 0.03 at $(\alpha 4)_3(\beta 2^{\text{D218A}})_2$ to 1.52 ± 0.02 at $(\alpha 4)_3(\beta 2^{\text{H71A,E74A,D293A,D218A}})_2$ (Fig. 6B).

Discussion

The most important conclusions from this study are (1) that the effects of Zn^{2+} on $\alpha 4\beta 2$ nAChRs are heavily influenced by the receptor stoichiometry, (2) that Zn^{2+} potentiation on the $(\alpha 4)_3(\beta 2)_2$ stoichiometry is determined by a site located at the $\alpha 4(+)/\alpha 4(-)$ subunit interface, and (3) that the $\beta 2(+)/\alpha 4(-)$ interfaces at both receptor types house a Zn^{2+} inhibitory site. These findings have important structural and functional implications about the role of subunit composition on the structural-functional properties of the $\alpha 4\beta 2$ nAChR and the manner by which Zn^{2+} influences $\alpha 4\beta 2$ nAChR function.

Indeed, this study shows for the first time that non-ACh-binding interfaces confer distinct functional signatures to the alternate stoichiometries of the $\alpha 4\beta 2$ nAChR.

Effects of Zn^{2+} on the $\alpha 4\beta 2$ nAChR are determined by subunit composition

Zn^{2+} exerts an inhibitory effect on $(\alpha 4)_2(\beta 2)_3$ receptors, whereas it potentiates or inhibits, depending on its concentration, the function of $(\alpha 4)_3(\beta 2)_2$ receptors. The finding that subunit composition influences the effects of Zn^{2+} on heteromeric receptors is not limited to the $\alpha 4\beta 2$ nAChR. Glycine $\alpha 1$ and $\alpha 2$ receptors, which are homologous to the $\alpha 4\beta 2$ nAChR, differ markedly in sensitivity to both Zn^{2+} inhibition (Miller et al., 2005a) and Zn^{2+} potentiation (Miller et al., 2005b), the differences reflecting divergence in key residues (Miller et al., 2005a,b). Subunits adjacent to subunits harboring Zn^{2+} sites may also alter the sensitiv-

ity of ligand-gated ion channels to Zn^{2+} effects. For example, incorporation of the $\gamma 2$ subunit to $\alpha \beta$ GABA_A receptors markedly decreases sensitivity to Zn^{2+} inhibition by disrupting the high-affinity Zn^{2+} -binding sites that reside at the α - β subunit interface (Hosie et al., 2003). Regarding the $\alpha 4\beta 2$ nAChR, subunit composition not only affects the sensitivity to Zn^{2+} modulation, but also the manner by which Zn^{2+} modulates the function of the receptors.

Residues on $\beta 2(+)/\alpha 4(-)$ interfaces contribute to Zn^{2+} inhibition on $(\alpha 4)_2(\beta 2)_3$ and $(\alpha 4)_3(\beta 2)_2$ receptors

From our model of the $\alpha 4\beta 2$ nAChRs and the findings of our alanine substitution studies, we conclude that $\alpha 4^{\text{H195}}$ and $\beta 2^{\text{D218}}$ critically contribute to an inhibiting Zn^{2+} -binding site predicted to reside at the $\beta 2(+)/\alpha 4(-)$ interfaces on both $\alpha 4\beta 2$ nAChR forms. Despite the lack of structural information on the $\alpha 4\beta 2$ nAChR, the findings are compatible with $\alpha 4^{\text{H195}}$ and $\beta 2^{\text{D218}}$ binding inhibiting Zn^{2+} . First, the identified residues are all capable of coordinating Zn^{2+} and when they were replaced with alanine, the sensitivity to Zn^{2+} decreased markedly. Second, mutation of either residue specifically disrupted Zn^{2+} inhibition without significant changes in the sensitivity of the receptor to activation by ACh. Third, $\beta 2^{\text{D218}}$ is within loop C, which, as we highlight above, can undergo large conformational changes. Such motions would allow $\beta 2^{\text{D218}}$ to come into close enough proximity to $\alpha 4^{\text{H195}}$ to form part of a discrete Zn^{2+} -binding site with other suitably located Zn^{2+} coordinating residues. Although $\alpha 4^{\text{E92}}$ is close to $\alpha 4^{\text{H195}}$ and $\beta 2^{\text{D218}}$, the effects of alanine substitutions at this position have considerably less impact than what is observed with $\alpha 4^{\text{H195}}$ or $\beta 2^{\text{D218}}$, suggesting that $\alpha 4^{\text{E92}}$ might not contribute directly to Zn^{2+} binding. A similar view is held by Hsiao et al. (2006), who showed that mutagenesis or MTSEA treatment are less effective on the rat equivalent of $\alpha 4^{\text{E92}}$ than on $\alpha 4^{\text{H162}}$ (the rat equivalent of $\alpha 4^{\text{H195}}$).

The $\alpha 4(+)/\alpha 4(-)$ subunit interface houses a Zn^{2+} potentiating site

Here, we provide evidence that is consistent with $\alpha 4^{\text{H195}}$, a residue located on the $(-)$ side of ACh-binding $\alpha 4$ subunit, playing a determinant role on Zn^{2+} potentiation on $(\alpha 4)_3(\beta 2)_2$ nAChRs. This finding is in agreement with those of Hsiao et al. (2006), who suggested $\alpha 4^{\text{H162}}$, the rat equivalent of human $\alpha 4^{\text{H195}}$, as a key determinant of Zn^{2+} potentiation on $\alpha 4\beta 4\beta 2$ nAChRs. However, our study also demonstrated that $\alpha 4^{\text{H195}}$ is critically involved in Zn^{2+} inhibition on both $\alpha 4\beta 2$ nAChR stoichiometries. Given these conflicting data, we investigated whether the $\alpha 4(-)/\alpha 4(+)$ subunit interface of the $(\alpha 4)_3(\beta 2)_2$ nAChRs could harbor a Zn^{2+} potentiating site partly formed by $\alpha 4^{\text{H195}}$ on the $(-)$ face of an ACh-binding $\alpha 4$ subunit and $\alpha 4^{\text{E224}}$, on the $(+)$ side of the nonbinding $\alpha 4$ subunit. Our findings are in accord with this possibility. When these residues were substituted by alanine, the sensitivity to Zn^{2+} potentiation was abolished, without perturbations of the general functional properties of the receptor. Furthermore, alanine sub-

Figure 5. nAChR $\beta 2$ subunit residues that affect voltage-dependent Zn^{2+} inhibition. **A**, The left panel shows the overall location of the residues involved in voltage-dependent Zn^{2+} inhibition in the $\beta 2$ subunit of the model of the full-length receptor. The right panel is a magnification of that region, from within the vestibule, and illustrates that the residues are in close proximity. **B**, Current–voltage relationship for wild-type $(\alpha 4)_2(\beta 2)_3$ and mutant $(\alpha 4)_2(\beta 2^{\text{H71A}})_3$, $(\alpha 4)_2(\beta 2^{\text{E74A}})_3$, and $(\alpha 4)_2(\beta 2^{\text{D293A}})_3$ receptors. **C**, Concentration–response curves for the effects of Zn^{2+} on wild-type $(\alpha 4)_2(\beta 2)_3$ and mutant $(\alpha 4)_2(\beta 2^{\text{H71A}})_3$, $(\alpha 4)_2(\beta 2^{\text{E74A}})_3$, $(\alpha 4)_2(\beta 2^{\text{D293A}})_3$, and $(\alpha 4^{\text{H195A}})_2(\beta 2^{\text{H71A,E74A,D293A}})_3$ receptors. The concentration–response data were fit to the appropriate equations as described in Materials and Methods. Averaged parameters of best fits to the concentration–response data are given in Table 1. Error bars indicate SEM.

stitution of $\alpha 4^{\text{E224}}$ did not affect Zn^{2+} modulation on $(\alpha 4)_2(\beta 2)_3$ or the overall function of this receptor type, confirming that $\alpha 4^{\text{E224}}$ specifically confers a Zn^{2+} potentiating site on the $(\alpha 4)_3(\beta 2)_2$ stoichiometry. The fact that the Zn^{2+} inhibiting and Zn^{2+} potentiating sites are located at homologous positions arises because of subunit homology, and such a phenomenon has been observed previously in other pseudosymmetric proteins. For example, it has been shown that some of the residues contributing to the benzodiazepine site of GABA_A receptors are homologous to residues implicated in agonist binding (Sigel and Buhr, 1997) and recent work on the ACh-binding protein suggests that aromatic residues found in the binding pocket are conserved at non- α interfaces, where they contribute to noncompetitive galanthamine-binding sites (Hansen and Taylor, 2007).

Figure 6. nAChR $\beta 2$ subunit residues that influence Zn^{2+} effects on $(\alpha 4)_3(\beta 2)_2$ nAChRs. **A**, Concentration-response curve for Zn^{2+} effects on mutant $(\alpha 4)_3(\beta 2)^{H71A}_2$, $(\alpha 4)_3(\beta 2)^{E74A}_2$, $(\alpha 4)_3(\beta 2)^{D293A}_2$, and $(\alpha 4)_3(\beta 2)^{H71A,E74A,D293A}_2$ nAChRs. **B**, ACh concentration-response curve in the presence of a maximally potentiating Zn^{2+} concentration (100 μM) constructed for mutant $(\alpha 4)_3(\beta 2)^{D218A}_2$, $(\alpha 4)_3(\beta 2)^{H71A,E74A,D293A}_2$, and $(\alpha 4)_3(\beta 2)^{H71A,E74A,D218A,D293A}_2$ nAChRs. For comparison, the ACh concentration-response curve in the presence of 100 μM Zn^{2+} constructed for wild-type $(\alpha 4)_3(\beta 2)_2$ has been included. Error bars indicate SEM.

Do $\alpha 4^{H195}$ and $\alpha 4^{E224}$ contribute to a Zn^{2+} -binding site?

Our findings make a strong case for a role in binding. Both $\alpha 4^{H195}$ and $\alpha 4^{E224}$ are chemically able to coordinate Zn^{2+} and when substituted by alanine or covalently modified by DEPC, only the sensitivity to Zn^{2+} potentiation was altered. Furthermore, $\alpha 4^{H195}$ is sensitive to MTSEA (Hsiao et al., 2006), which indicates that it is accessible to Zn^{2+} . In the $\alpha 4(-)/\alpha 4(+)$ interface, and in terms of a potential Zn^{2+} -binding site, $\alpha 4^{E224}$ is situated on what would normally be considered the principal (+) interface. The distance between these residues in the model is somewhat larger (~ 12 Å) than would be expected for a normal Zn^{2+} -binding site (typical values between coordinating groups is of the order of 6–7 Å) (Auld, 2001). Thus, to form a competent binding site, it seems likely that some movement at the interface is required. There is a precedent for this in that loop C has been shown (in AChBP at least) to adopt a variety of different conformations (Hansen et al., 2005). Furthermore, molecular dynamics simulations (Henchman et al., 2003, 2005) have suggested that this loop is highly flexible. Thus, the formation of a Zn^{2+} -binding site by these residues is a distinct possibility. In addition, analysis of the metalloprotein database (Castagnetto et al., 2002) suggests that water molecules are often involved in the coordination of Zn^{2+} ions. Water molecules are not explicitly considered in our modeling here, but could help to stabilize potential Zn^{2+} coordination. However, we cannot at this stage rule out alternative modes of action.

Voltage-dependent Zn^{2+} inhibition is mediated by $\beta 2$ subunit residues

The relative positioning of the $\beta 2^{H71}$, $\beta 2^{E74}$, and $\beta 2^{D293}$ in the predicted quaternary structure of $\alpha 4\beta 2$ nAChR is in accord with these residues contributing to a site that may exert voltage-dependent Zn^{2+} inhibition on $(\alpha 4)_2(\beta 2)_3$ nAChRs. Clearly, this

presumed site is independent of the inhibiting site located at $\beta 2(+)/\alpha 4(-)$ interfaces; $\beta 2^{H71}$ and $\beta 2^{E74}$ are in a loop tilting toward the channel, whereas $\beta 2^{D293}$ is on the pore lining the M2 helix (Fig. 6A). Although mutating $\beta 2^{H71}$, $\beta 2^{E74}$, or $\beta 2^{D293}$ eliminated voltage-dependent inhibition on $(\alpha 4)_2(\beta 2)_3$ nAChRs, it did not significantly alter the IC_{50} value for Zn^{2+} inhibition. Moreover, although alanine substitutions increased maximal Zn^{2+} potentiation on $(\alpha 4)_3(\beta 2)_2$ nAChRs, the estimated EC_{50} value for Zn^{2+} potentiation was not significantly altered. These findings suggest that $\beta 2^{H71}$, $\beta 2^{E74}$, and $\beta 2^{D293}$ may coordinate Zn^{2+} as part of a site that may influence the downstream pathways associated with inhibiting and/or potentiating Zn^{2+} .

It should be noted that although $\beta 2^{H71A}$, $\beta 2^{E74A}$, or $\beta 2^{D293A}$ increased the degree of Zn^{2+} potentiation on $(\alpha 4)_3(\beta 2)_2$ receptors, no evidence was found of voltage-dependent Zn^{2+} effects on $(\alpha 4)_3(\beta 2)_2$ receptors lacking the Zn^{2+} potentiating site (Table 2). However, because the functional properties of the alternate forms of the $\alpha 4\beta 2$ nAChR differ substantially, a Zn^{2+} -binding site that modulates Zn^{2+} signal pathways would be expected to influence receptor function in a stoichiometry-specific manner.

Overall, our findings show that the effects of Zn^{2+} on $\alpha 4\beta 2$ nAChRs are heavily influenced by subunit stoichiometry. Among the nAChR assembled from two different subunits, this is the first time that it has been shown that the non-ACh-binding subunit drastically changes the responses to modulators and that subunit homology and stoichiometry might result in one residue contributing to two independent modulatory sites. Although more work, including structural data, will be needed to ultimately understand the effects of Zn^{2+} on $\alpha 4\beta 2$ receptors and how modulatory sites may be formed, our studies have highlighted a crucial role played by non-ACh-binding interfaces on receptor modulation.

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