0026-895X/07/7103-769–776$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 71:769–776, 2007

Vol. 71, No. 3
30445/3177148
Printed in U.S.A.

Ca^{2+} Permeability of the $(\alpha4)_3(\beta2)_2$ Stoichiometry Greatly Exceeds That of $(\alpha4)_2(\beta2)_3$ Human Acetylcholine Receptors

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Received September 5, 2006; accepted November 28, 2006

ABSTRACT

Human $\alpha4\beta2$ nicotinic acetylcholine receptors (AChRs) expressed in *Xenopus laevis* oocytes or transfected cell lines are present as a mixture of two stoichiometries, $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$, which differ depending on whether a $\beta2$ or $\alpha4$ subunit occupies the accessory subunit position corresponding to $\beta1$ subunits of muscle AChRs. Pure populations of each stoichiometry can be expressed in oocytes by combining a linked pair of $\alpha4$ and $\beta2$ with free $\beta2$ to produce the $(\alpha4)_2(\beta2)_3$ stoichiometry or with free $\alpha4$ to produce the $(\alpha4)_3(\beta2)_2$ stoichiometry. We show that the $(\alpha4)_3(\beta2)_2$ stoichiometry and the $(\alpha4)_2(\beta2)_2\beta3$ and $(\alpha4)_2(\beta2)_2\alpha5$ subtypes in which $\beta3$ or $\alpha5$ occupy the accessory positions have much higher permeability to Ca^{2+} than does $(\alpha4)_2(\beta2)_3$ and suggest that this could be physiologically significant in triggering signaling cascades if this stoichiometry or these subtypes were found in vivo. We show that Ca^{2+} permeability is determined by charged amino acids at the extracellular end of the M2 transmembrane domain, which could form a ring of amino acids at the outer end of the cation channel. $\alpha4$, $\alpha5$, and $\beta3$ subunits all have a homologous glutamate in M2 that contributes to high Ca^{2+} permeability, whereas $\beta2$ has a lysine at this position. Subunit combinations or single amino acids changes at this ring that have all negative charges or a mixture of positive and negative charged amino acids are permeable to Ca^{2+}. All positive charges in the ring prevent Ca^{2+} permeability. Increasing the proportion of negative charges is associated with increasing permeability to Ca^{2+}.

Nicotinic acetylcholine receptors (AChRs) belong to the cysteine loop superfamily of ligand-gated ion channels that includes muscle and neuronal AChRs, GABA types A and C receptors, 5-hydroxytryptamine type 3 receptor, and glycine receptors (Sine and Engel, 2006). These receptors are formed by five homologous subunits arranged around a central ion pore. Neuronal AChRs are formed from a variety of pentameric combinations of subunits $\alpha2$–$\alpha10$ and $\beta2$–$\beta4$ (Lindstrom, 2000; Gotti et al., 2006; Sine and Engel, 2006).

$\alpha4\beta2$* AChRs are the predominant subtypes with high affinity for nicotine in the mammalian brain (Flores et al., 1992). The asterisk indicates that additional subunits such as $\alpha5$ are present in some of these AChRs (Gerzanich et al., 1998; Gotti et al., 2006). Most $\alpha4\beta2$* AChRs are believed to be located presynaptically and modulate the release of several neurotransmitters (Dani, 2001; Dajas-Bailador and Wonnacott, 2004). $\alpha4\beta2$* AChRs are sufficient for nicotine reward, tolerance, and sensitization (Tapper et al., 2004; Maskos et al., 2005). Mutations in either the $\alpha4$ or $\beta2$ subunits can cause autosomal dominant nocturnal frontal lobe epilepsy (Combi et al., 2004). In addition, the $\alpha4\beta2$ AChR subtype is believed to be involved in neurodegenerative diseases such as Alzheimer's and Parkinson's diseases and Lewy body dementia because in postmortem brain tissues from patients of these diseases, $\alpha4\beta2$* AChRs are significantly reduced with respect to age-matched normal subjects (Zanardi et al., 2002).

The stoichiometry of chicken $\alpha4\beta2$ AChRs heterologously expressed in oocytes using a 1:1 (α/β) ratio of cRNAs was initially shown to be $(\alpha4)_2(\beta2)_3$ by two different methods (Anand et al., 1991; Cooper et al., 1991). However, later studies with human AChRs showed that the stoichiometry $(\alpha4)_3(\beta2)_2$ could also be formed (Zwart and Vijverberg, 1998; Nelson et al., 2003; Zhou et al., 2003; Moroni et al., 2006). The $(\alpha4)_3(\beta2)_2$ stoichiometry has much lower sensitivity to activation and up-regulation by agonists and desensitizes more rapidly. It is unknown whether the $(\alpha4)_3(\beta2)_2$ stoichiometry is expressed in brain. Its properties seem most appropriate for a postsynaptic AChR (Nelson et al., 2003). Long-term exposure to nicotine increased the proportion of the more nicotine-sensitive $(\alpha4)_2(\beta2)_3$ stoichiometry in human embryonic kidney cells permanently transfected with human $\alpha4\beta2$ AChRs (Nelson et al., 2003; Kuryatov et al.,

This work was supported by National Institutes of Health grant NS11323 (to J.L.) and the Phillip Morris External Research Program.
L.T. and A.K. contributed equally to this work.
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.106.030445.

ABBREVIATIONS: AChR, acetylcholine receptor; ACh, acetylcholine; TC-2559, (*E*)-*N*-methyl-4-[3-(5-ethoxypyridin)y1]-3-buten-1-amine.

2005). The proportion of the $(\alpha4)_2(\beta2)_3$ stoichiometry can be increased by increasing expression of $\beta2$ (Nelson et al., 2003). The proportion of the $(\alpha4)_3(\beta2)_2$ stoichiometry can be increased by increasing the amount of $\alpha4$ through protein kinase A or the 14-3-3 chaperone (Exley et al., 2006). In addition, the presence of untranslated regions in $\alpha4$ and $\beta2$ subunits can influence the proportion of stoichiometries expressed (Briggs et al., 2006), presumably by influencing the efficiency of translation. In *X. laevis* oocytes, greatly increasing the proportion of $\beta2$ or $\alpha4$ mRNA injected to 1:10 or 10:1 forces the expression of one stoichiometry or the other, but the lopsided subunit ratio reduces total AChR expression (Moroni et al., 2006), presumably by producing nonproductive assembly intermediates. $\alpha3\beta2$ AChRs, at least, are also subject to expression in two similar stoichiometries (Briggs et al., 2006).

Drugs that are at least somewhat selective for each $\alpha4\beta2$ AChR stoichiometry have been identified recently (Briggs et al., 2006; Moroni et al., 2006; Zwart et al., 2006). Pharmacological evidence suggests that the $(\alpha4)_2(\beta2)_3$ stoichiometry is expressed in the brain but does not eliminate the possibility that the $(\alpha4)_3(\beta2)_2$ stoichiometry is also expressed. The observation that the $(\alpha4)_3(\beta2)_2$ stoichiometry is more sensitive to the channel-blocking drugs mecamylamine and chlorisondamine (Briggs et al., 2006) indicates that its channel differs from that of the $(\alpha4)_2(\beta2)_3$ stoichiometry.

We take advantage of the use of $\alpha4\beta2$ AChRs formed from linked subunits to obtain exclusively either of the stoichiometries in *X. laevis* oocytes (Zhou et al., 2003). Here, we show that the $(\alpha4)_3(\beta2)_2$ stoichiometry is much more permeable to Ca^{2+} than is the $(\alpha4)_2(\beta2)_3$ stoichiometry. If the $(\alpha4)_3$ stoichiometry were expressed in brain, this would allow it to be much more potent in triggering signaling cascades triggered by the influx of Ca^{2+}.

Materials and Methods

cDNAs and cRNAs. Synthesis of cDNAs for human $\alpha4$ (Kuryatov et al., 1997), human $\beta2$ (Anand and Lindstrom, 1990), and tandem constructs of human $\alpha4$ and $\beta2$ subunits (Zhou et al., 2003) were described previously. The cDNA for rat $\beta3$ was a gift from Steve Heinemann and Jim Boulter. The cDNA for human $\alpha5$ was kindly provided by Dr. Francesco Clementi (University of Milan, Milan, Italy). It was subcloned in the pSP64poly(A) vector (Kuryatov et al., 1997). cRNAs from linearized cDNA templates were synthesized in vitro using the SP6 or T7 mMessage mMachine kit (Ambion, Austin, TX). The tandem construct used in this study was the β-6-α type (according to nomenclature in Zhou et al., 2003), in which the C terminus of the $\beta2$ subunit is linked via an AGS sequence repeated six times to the N terminus of the $\alpha4$ subunit. Preparation of cDNAs and cRNAs for human $\alpha1$ and δ muscle AChR subunits and human $\alpha7$ subunit was described previously (Luther et al., 1989; Peng et al., 1994a). $\beta1$ and ε human cDNAs subunits were kindly provided by Dr. A. G. Engel. Mutations E261K into human $\alpha4$ subunit and K258E into human $\beta2$ subunit were introduced using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and sequenced to verify that only the desired mutation was present.

Oocyte Removal and Injection. Oocytes were removed surgically from *X. laevis* and placed in an OR-2 solution containing 82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, and pH 7.5. They were defolliculated in this buffer containing 2 mg/ml collagenase type IA (Sigma, St. Louis, MO) for 1.5 h. After defolliculation, oocytes were incubated at 18°C in semisterile L-15 medium (Invitrogen, Carlsbad, CA) diluted by half in 10 mM HEPES buffer with 10 U/ml penicillin and 10 μg/ml streptomycin, pH 7.5. Oocytes were injected cytosolically with combinations of $\alpha7$, $\alpha4+\beta2$, β-6-$\alpha+\alpha4$, β-6-$\alpha+\beta2$, β-6-$\alpha+\alpha5$, or β-6-$\alpha+\beta3$ subunit cRNAs subunits (50 ng of $\alpha7$; 5 ng of either $\alpha4$, $\alpha4^{EK}$, $\beta2$, $\beta2^{KE}$, $\beta3$, or $\alpha5$ subunit; and 10 ng of the concatamer β-6-α in a total volume of 46 nl). To express muscle AChR, 10 ng of $\alpha1$ and 5 ng of each of the other subunit cRNAs ($\beta1$, δ, and ε) were injected in a total volume of 46 nl. Five nanograms of the mutant K258E $\beta2$ subunit ($\beta2^{KE}$) cRNA were injected with 5 ng of either the wild-type $\alpha4$ subunit or the mutant E261K $\alpha4$ ($\alpha4^{EK}$) subunit cRNAs. Twenty nanograms of each cRNA were injected for the $\alpha4^{EK}$ and wild-type $\beta2$ combination.

Electrophysiological Recordings and Data Analysis. Three to six days after injection, whole-cell membrane currents evoked by ACh (I_{ACh}) were recorded in oocytes at room temperature with a standard two-electrode voltage-clamp amplifier (Oocyte Clamp OC-725; Warner Instrument, Hamden, CT). Recordings were performed at a holding potential of −50 mV unless otherwise stated. All perfusion solutions contained 0.5 μM atropine to block responses of endogenous muscarinic AChRs that might be present in oocytes. Agonists were applied by means of a set of 2-mm glass tubes directed to the animal pole of the oocytes. Application was achieved by manual unclamping/clamping of a flexible tube connected to the glass tubes and to reservoirs with the test solutions. The recording chamber was perfused at a flow rate of 15 to 20 ml/min.

In a set of experiments, the perfusion solution was ND-96 with either normal or high Ca^{2+}. These solutions consisted of 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 (normal) or 18 mM (high) $CaCl_2$ and 5 mM HEPES, pH 7.5. Intracellular electrodes were filled with 3 M KCl. In another set of experiments, to prevent activation of endogenous Ca^{2+}-dependent Cl^- channels of oocytes, Cl^--free solutions were used instead for oocyte preincubation (6–16 h) and during recordings (Francis and Papke, 1996). In this case, normal or high Ca^{2+} solutions included 90 mM NaOH, 2.5 mM KOH, 1.8 or 18 mM $Ca(OH)_2$, and 10 mM HEPES, buffered with methanesulfonic acid to pH 7.3. Intracellular electrodes were filled in this case with 2.5 M potassium aspartate. In addition, 40 mM dextrose was supplemented to the normal Ca^{2+} solutions (with or without chloride) to maintain the same osmolarity as the high Ca^{2+} solutions. In a third set of experiments, to determine I_{ACh} carried only by Ca^{2+} ions, a perfusion solution containing only 1.8 mM $Ca(OH)_2$ buffered to pH 7.5 with HEPES was used. Dextrose (178 mM) was added to this solution to preserve normal osmolarity. Resistance of the voltage and current electrodes were 2.5 to 6 and 0.5 to 2 MΩ, respectively, in all cases.

Reversal potentials of I_{ACh} in normal and high Ca^{2+} solutions were determined by applying 2-s ramps from −70 to +50 mV during agonist application after the current had reached a steady-state value. Currents obtained in response to the voltage ramp in the absence of agonist application (reflecting passive membrane currents) were subtracted from the ramp currents during AChR activation (Kuryatov et al., 1997). In all experiments, oocytes were superfused for at least 5 min with every new test solution to ensure complete exchange of the bath solutions. A 1% agarose + 3 M KCl bridge was used between the bath solution and the ground electrode bath to minimize the differences in junction potentials along the recording circuit.

ACh concentration-response curves were obtained by normalizing the responses to different ACh concentrations to the response to 300 μM ACh in each oocyte, except for the $\alpha4^{EK}\beta2$ combination, in which the responses were normalized to 1 mM ACh, and 3 mM ACh was used for the β-6-$\alpha+\alpha4$ and β-6-$\alpha+\beta3$ combinations. The concentration-response curves were fitted using a nonlinear least-squares error curve-fit method (KaleidaGraph, Abelbeck Software; Synergy Software, Reading, PA) to the double independent Hill equation: $I(x) = I_{max}1x^n/(x^n + EC_{50}1^n) + I_{max}2x^m/(x^m + EC_{50}2^m)$, where $I(x)$ is the maximal current measured at the agonist concentration x, $I_{max}1$ and $I_{max}2$ are the maximal current responses at the saturating agonist concentration, $EC_{50}1$ and $EC_{50}2$ are the agonist concentra-

tions required for the half-maximal response, and n and m are the Hill coefficients for each component of the fitted curve.

Stimulation and data acquisition were digitized at 100 Hz with a MacLab 2e interface and Scope 3.4.3 software (AD Instruments, Castle Hill, Australia). Current and voltage traces were analyzed using the Scope software. Representative traces were constructed opening data files in Scope and exporting data segments to Origin 5.0 (OriginLab Corp., Northampton, MA). Plots were also made with the Origin software.

Statistics. Data sets are expressed as means ± S.E. Statistical significance between data were determined by Student's t test. Differences were considered significant at the level of $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

Results

Ca^{2+} Permeability of $(\alpha4)_3(\beta2)_2$ and $(\alpha4)_2(\beta2)_3$ Stoichiometries Assayed by Effects on Reversal Potential. *X. laevis* oocytes were injected with three different combinations of $\alpha4$ and $\beta2$ AChR subunits: free $\alpha4$ plus free $\beta2$ subunits ($\alpha4\beta2$) in a 1:1 ratio to obtain the wild-type $\alpha4\beta2$ AChR, expected to predominantly have the $(\alpha4)_2(\beta2)_3$ stoichiometry (Anand et al., 1991; Cooper et al., 1991), or a mixed population of both $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$ stoichiometries (Zwart and Vijverberg, 1998; Nelson et al., 2003); the β-6-α concatamer plus free $\alpha4$ subunits (β-6-α+$\alpha4$), which forms only $(\alpha4)_3(\beta2)_2$ AChRs (Zhou et al., 2003); and β-6-α concatamer plus free $\beta2$ subunits (β-6-α+$\beta2$), which forms only $(\alpha4)_2(\beta2)_3$ AChRs (Zhou et al., 2003). It is reasonable to predict that a change in subunit composition in $\alpha4\beta2$ AChR would change the residues lining the lumen of the channel and, therefore, many of its functional properties, including ionic selectivity (Bertrand et al., 1993).

Ca^{2+} permeability was investigated by measuring the shift in the reversal potential of I_{ACh} when changing the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) from 1.8 to 18 mM [10-fold increase in $[Ca^{2+}]_o$, based on the Goldman-Hodgkin-Katz equation (Fucile, 2004)]. The reversal potentials of I_{ACh} were estimated using ramp protocols (see *Materials and Methods*) applied during the stable phase of response to applications of 30 μM ACh (Fig. 1). To prevent activation of endogenous Ca^{2+}-dependent Cl^- channels, ND-96 solutions were substituted with Cl^--free media during a 6- to 16-h period of preincubation of oocytes and during the recordings. A 10-fold increase in $[Ca^{2+}]_o$ caused the reversal potential of I_{ACh} to shift in the positive direction, as expected if Ca^{2+} were permeating through the channel, by 17.9 ± 3.0 mV in $\alpha4\beta2$ ($n = 18$), 26.9 ± 1.3 mV in β-6-α+$\alpha4$ ($n = 32$), and 7.3 ± 2.2 mV in β-6-α+$\beta2$ ($n = 12$) (Fig. 2).

The shift in reversal potentials shows significant differences in calcium permeabilities between the three combinations tested. The β-6-α+$\alpha4$ combination, which produces only the $(\alpha4)_3(\beta2)_2$ stoichiometry, was the most permeable to calcium. The $\alpha4\beta2$ combination, which produces a mixture of the two stoichiometries, had lower calcium permeability. The β-6-α+$\beta2$ combination, which produces only the $(\alpha4)_2(\beta2)_3$ stoichiometry, was least permeable to calcium.

For comparison, human $\alpha7$ AChR (the AChR subtype with greatest Ca^{2+} permeability) and adult muscle AChR [$(\alpha1)_2\beta1\gamma\delta$] (significantly less permeable to Ca^{2+} than other AChR subtypes) (Fucile, 2004) were also tested under the same experimental conditions. They gave shifts in their reversal potentials of 28.6 ± 2.4 mV ($n = 9$) for $\alpha7$ AChR and −0.4 ± 3.5 mV ($n = 7$) for muscle AChR. In this assay, the Ca^{2+} permeability of the $(\alpha4)_3(\beta2)_2$ stoichiometry is statistically indistinguishable from that of $\alpha7$ AChRs (Fig. 2).

Currents when Calcium Was the Only Extracellular Ion Available to $(\alpha4)_3(\beta2)_2$ and $(\alpha4)_2(\beta2)_3$ AChR Stoichiometries in Solutions without Cl^-. Differences in Ca^{2+} permeability between $\alpha4\beta2$ AChR stoichiometries were also tested in solutions without chloride by an alternative method. When all cations but Ca^{2+} in the extracellular buffer were replaced by an equiosmotic concentration of dextrose, all combinations tested still conducted detectable inward currents (Fig. 3), indicating that they were all permeable to Ca^{2+}. The amplitude of the currents, however, normalized to the currents induced by the same concentration of agonist in the normal extracellular buffer, varied significantly between

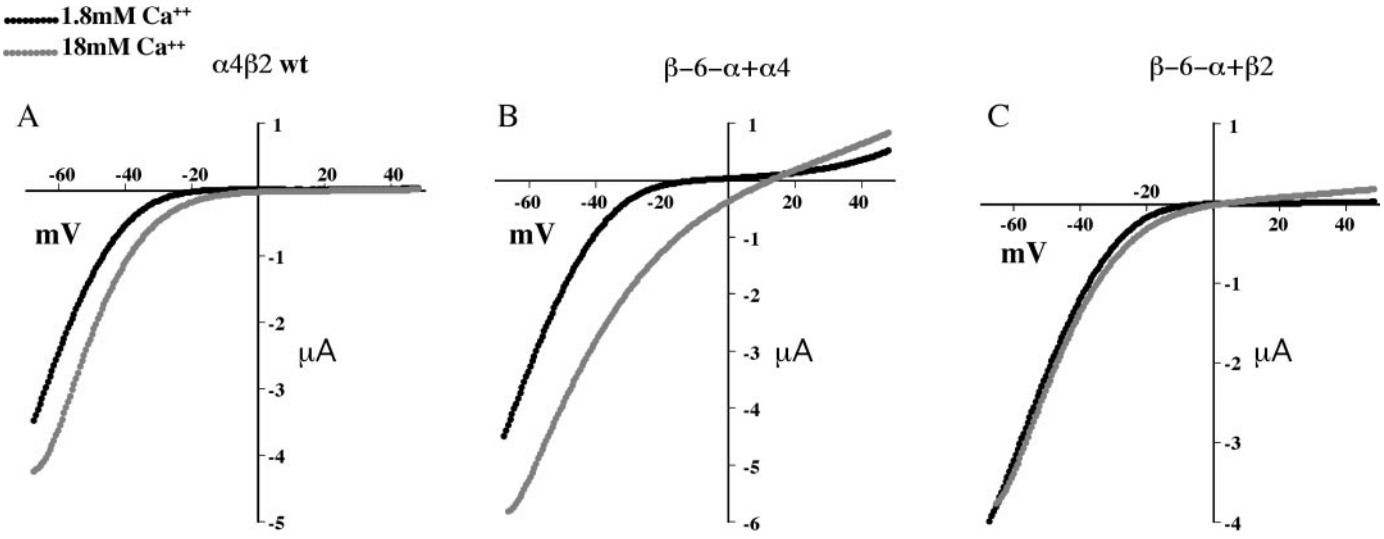

Fig. 1. Illustrative currents from the ramp protocols used to determine the reversal potential of I_{ACh}. Oocytes injected with the cRNA combinations shown ($\alpha4\beta2$, β-6-α+$\alpha4$, or β-6-α+$\beta2$) were held at −50 mV and stimulated with applications of 30 μM ACh. When I_{ACh} had reached a steady value, a 2-s voltage ramp from −70 to +50 mV was applied. This ramp protocol was applied for each oocyte in normal $[Ca^{2+}]_o$ solutions (boldface traces) and in solutions with 10 times the $[Ca^{2+}]_o$ (gray traces) and in the absence of ACh to get the passive membrane currents, which were subtracted from the values of the ramps in the presence of ACh. Cl^--free medium was used to avoid activating Ca^{2+}-dependent Cl^- channels.

the stoichiometries: 20.2 ± 1.0% for β-6-α+α4 (n = 9), 8.4 ± 1.3% for β-6-α+β2 (n = 12), and 6.4 ± 1.6% for α4β2 (n = 10). This value for human α7 AChR (used as a reference value for an AChR with high Ca^{2+} permeability) under the same experimental conditions was 33.8 ± 5.5% (n = 12), 49.8 ± 9.2% for β-6-α+α5 (n = 9), and 22.7 ± 8.4% for β-6-α+β3 (n = 9) (Fig. 4). In this assay, the Ca^{2+} permeability of the $(\alpha4)_3(\beta2)_2$ stoichiometry is significantly lower than that of α7 ($p < 0.001$) or β-6-α+α5 ($p < 0.01$) AChRs. These results further confirm greater calcium permeability for the $(\alpha4)_3(\beta2)_2$ stoichiometry compared with the $(\alpha4)_2(\beta2)_3$ stoichiometry.

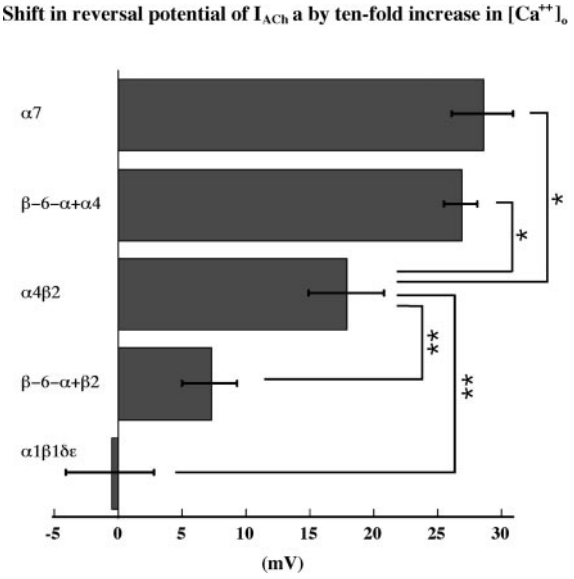

Fig. 2. Shift in the reversal potential of I_{ACh} when increasing $[Ca^{2+}]_o$ 10 times in solutions without chloride. The reversal potential of I_{ACh} in oocytes injected with the cRNAs indicated was determined by applying a ramp protocol during the stable response to 30 μM ACh as described in Fig. 1. The shift in reversal potential as a result of increasing $[Ca^{2+}]_o$ from 1.8 to 18 mM is plotted. The values statistically different are shown with *, $p < 0.05$, and **, $p < 0.01$. Values are means ± S.E. of the shift (in millivolts) in 11 to 20 oocytes per cRNA combination from at least two different batches of oocytes.

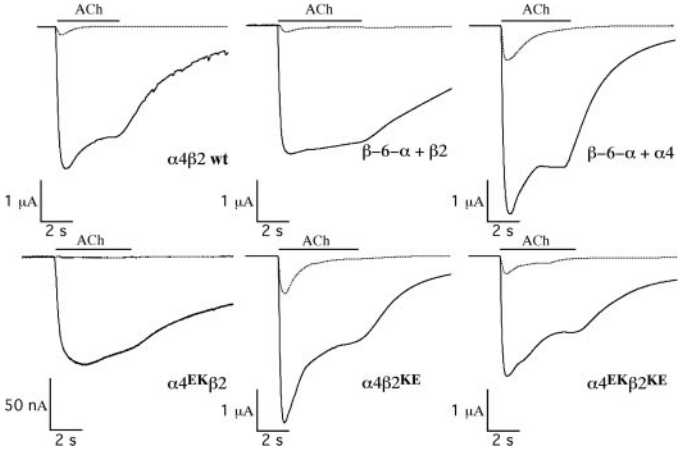

Fig. 3. Illustrative recordings of I_{ACh} carried only by Ca^{2+} ions. Oocytes expressing each of the subunit combinations shown were stimulated with ACh applications (horizontal bars; 300 μM ACh for $\alpha4^{EK}\beta2$, 30 μM ACh for the rest). In each oocyte, I_{ACh} was recorded first in solutions without Cl^- but with the rest of the standard extracellular ions (Na^+, K^+) (boldface traces) and next in solutions without Cl^-, which contained Ca^{2+} as the only extracellular ion (broken traces).

Effect of a Ring of Charged Residues at the Mouth of the Channel on Ca^{2+} Permeability of α4β2 AChR. The charged residues at the outer edge of the M2 transmembrane domain are known to be determinants for the cationic selectivity of the nicotinic channel (Imoto et al., 1986; Bertrand et al., 1993). Thus, the anionic residue glutamate Glu261 in the α4 subunit was changed to cationic lysine (referred as $\alpha4^{EK}$) and the homologous cationic residue Lys258 in β2 was changed to the anionic E ($\beta2^{KE}$) (Fig. 5). Table 1 and Fig. 4 show the effects on the ring of M2 amino acids indicated in Fig. 5 of the various subunit combinations used. Their contributions to Ca^{2+} permeability in α4β2 AChRs were investigated in the same conditions as before for concatamers (Fig. 4). Oocytes were injected with equimolar amounts of cRNAs for wild-type α4+β2, $\alpha4^{EK}$+β2, α4+$\beta2^{KE}$, $\alpha4^{EK}$+$\beta2^{KE}$, β-6-α+$\alpha4^{EK}$, or β-6-α+$\beta2^{KE}$.

Wild-type subunits produced nearly equal amounts of both stoichiometries, each of which contained either a 3:2 or 2:3 ratio of − to + charged amino acids in the putative ring of amino acids formed at the extracellular end of M2, resulting in a net average charge of approximately +0.11 averaged over the two stoichiometries (Table 1). This nearly equal mix of + and − charged residues was associated with Ca^{2+} permeability as indicated by a current when only Ca^{2+} was present equal to 6.4% of the current under control conditions. β-6-α+α4 produced only the $(\alpha4)_3(\beta2)_2$ stoichiometry and a larger net negative charge of −1, resulting in higher Ca^{2+} permeability (20% of control current). Using β-6-α+β2 to produce only the $(\alpha4)_2(\beta2)_3$ stoichiometry and a net charge of + 1 reduced the Ca^{2+} permeability to 8.7% of control. To further test the importance of changes in the putative M2 ring, we reversed the charges in α4 and β2 subunits at this position and tested β-6-α concatamers with $\alpha4^{EK}$ and $\beta2^{KE}$ subunits. The Ca^{2+} permeabilities in these AChRs were virtually opposite to wild-type combinations: 12.2% for β-6-α+$\alpha4^{EK}$ and 19.9% for β-6-α+$\beta2^{KE}$ (Table 1). The extreme case of $\alpha4^{EK}\beta2$ resulted in a much larger net positive charge of +5, independent of stoichiometry, and no Ca^{2+} permeability. The opposite extreme case of $\alpha4\beta2^{KE}$ resulted in a net charge of −5 independent of stoichiometry and the Ca^{2+} permeability of 20%. The double mutant $\alpha4^{EK}\beta2^{KE}$, in which the charges on both free subunits were reversed, resulted in a net negative charge of −0.42 and a Ca^{2+} permeability of 16.3% of control. Overall, all negative charges or a mix of charges in the ring permitted some Ca^{2+} permeability, with Ca^{2+} permeability increasing as the net charge became more negative, or decreasing to 0 if all five residues were positively charged.

Because previous studies showed that mutations in the channel domain might alter not only ion selectivity but also sensitivity to agonists or antagonists and desensitization properties of the channel (Bertrand et al., 1993), ACh concentration-response curves were obtained for these mutants (Fig. 6, A and B) for comparison with the α4β2 wild type. Fitting the wild-type dose-response curve for the properties of each stoichiometry indicates that a 1:1 mixture of subunit mRNAs produces a 5:4 ratio of the $(\alpha4)_2$ to $(\alpha4)_3$ stoichiometries. All concentration-response curves of the mutants can be similarly resolved into two components. The proportions of the stoichiometries presumably reflect the relative affinities of the β2 or α4 subunits for assembly in the β1 position. The Ca^{2+} permeability of each stoichiometry reflects the number

of positively charged lysine or negatively charged glutamate residues in the ring at the extracellular end of M2. The $\alpha4\beta2^{KE}$ subunit combination presumed $(\alpha4)_2(\beta2^{KE})_3$ component was most sensitive to ACh. With this subunit combination, in all stoichiometries the M2 ring was occupied only by glutamate. This ring of negative charge would be expected to not only select for Ca^{2+} permeability but also to increase cation concentration near the channel entrance and increase currents. Note that currents through $\alpha4\beta2^{KE}$ with five negative charges are 47-fold greater than currents through $\alpha4^{EK}\beta2$ with five plus charges (Fig. 3). The $\alpha4^{EK}\beta2$ subunit combination presumed $(\alpha4^{EK})_2(\beta2)_3$ component was least sensitive to ACh.

Concentration-response curves using linked subunits to fix the stoichiometries (Fig. 6C) revealed the expected high ACh sensitivity of $(\alpha4)_2(\beta2)_3$ stoichiometry with the β-6-α+$\beta2$ combination and the expected much lower sensitivity of $(\alpha4)_3(\beta2)_2$ with the β-6-α+$\alpha4$ combination. The β-6-α+$\alpha4$ combination showed a trace (6%) of high-sensitivity AChRs. These probably resulted because at the 1:1 M ratio of 5 ng of $\alpha4$ mRNA to 10 ng of β-6-α mRNA used, there was not sufficient excess of free $\alpha4$ subunit to completely prevent formation of the linked dimers one of each stoichiometry, which are formed when β-6-α is expressed alone (Zhou et al., 2003).

Both $\alpha5$ and $\beta3$ subunits, like $\alpha4$, have glutamate residues at the M2 ring position that was shown to be critical for regulating Ca^{2+} permeability; therefore, it would be expected that $(\alpha4)_2(\beta2)_2\alpha5$ and $(\alpha4)_2(\beta2)_2\beta3$ AChRs would exhibit high

AChR Subunits	M2 segment
	K
α4	ITLCISVLLSLTVFLLLITEI
β2	MTLCISVLLALTVFLLLISKI
	E

Fig. 5. Alignment of the M2 transmembrane domains in human $\alpha4$ and $\beta2$ AChR subunits showing the residues mutated for this study shaded. The mutated residues are part of a ring at the extracellular end of M2, which is believed to influence the ion-selectivity of the channel.

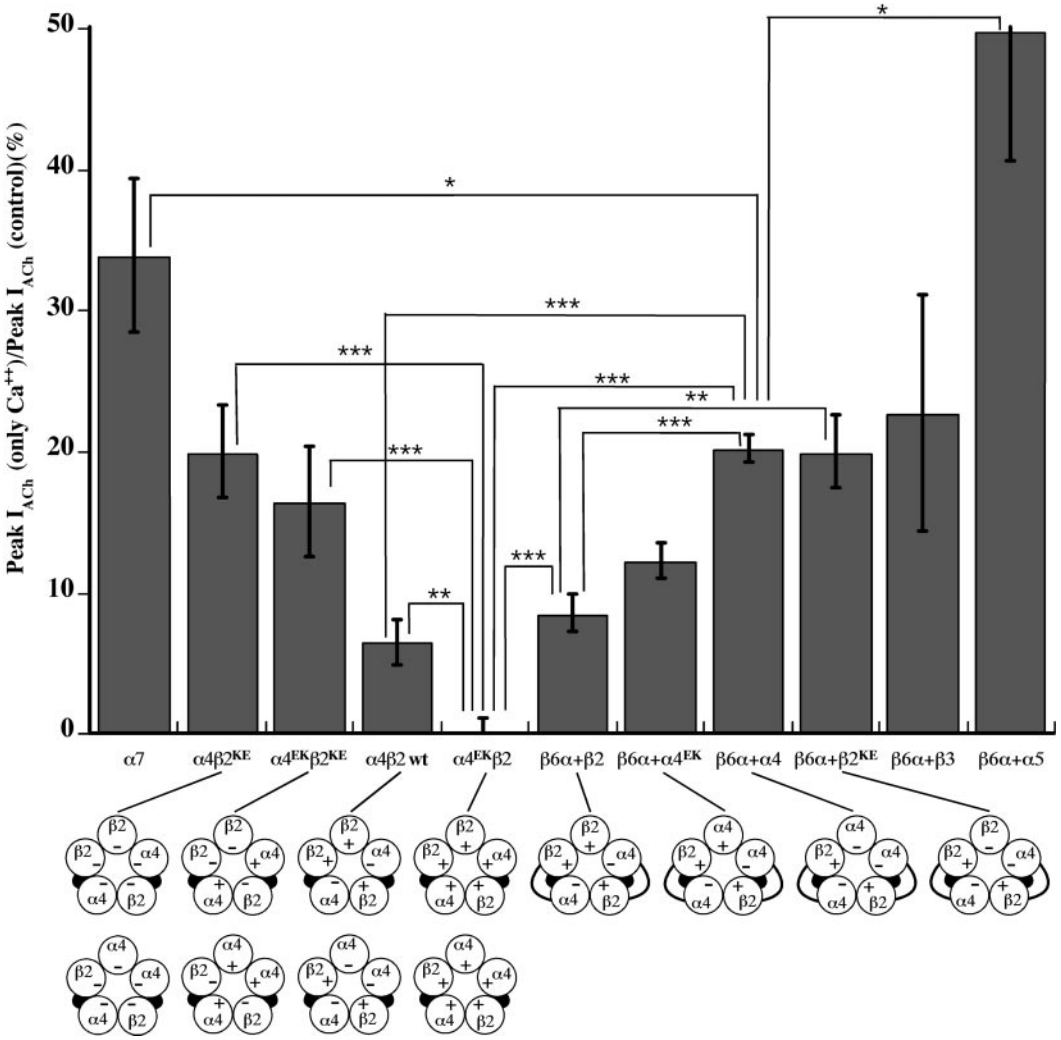

Fig. 4. I_{ACh} peaks in solutions with Ca^{2+} as the only extracellular ion normalized to I_{ACh} peaks in solutions with standard extracellular ions. For each oocyte, the peak of I_{ACh} to 30 μM ACh (or 300 μM for $\alpha4^{EK}\beta2$, β-6-α+$\alpha4$, β-6-α+$\alpha4^{EK}$, β-6-α+$\alpha5$, and β-6-α+$\beta3$) in the "only Ca^{2+}" solution was normalized to the peak of I_{ACh} in a solution with standard extracellular ions. Data are means ± S.E. from 9 to 15 oocytes per cRNA combination tested from at least two different batches. The values statistically different are shown with *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$. The graph also includes this value obtained for human $\alpha7$ AChR (12 oocytes, 2 different batches) as a reference value for a highly Ca^{2+}-permeable AChR. The inserts below show the charge of the residues at positions mutated at the outer edge of the M2 domain for each combination tested. Where the stoichiometry is not defined by the use of linked subunits, both stoichiometries present are shown. Table 1 summarizes these results and accounts for the proportions of each stoichiometry in the mixtures. The Ca^{2+} permeability of β-6-α+$\alpha5$ is greater than wild-type $\alpha4\beta2$ ($p < 0.01$).

Ca^{2+} permeability similar to $(\alpha4)_3(\beta2)_2$ AChRs. These stoichiometries were achieved by expressing free $\alpha5$ and $\beta3$ with linked β-6-α subunits. As expected, both $\alpha5$ and $\beta3$ conferred much higher permeability to Ca^{2+} than did $\beta2$ (Fig. 4).

The β-6-α+$\alpha5$ combination exhibited the same high sensitivity to activation by ACh as the β-6-α+$\beta2$ combination (Fig. 6C). This indicates that the $(\alpha4)_2(\beta2)_3$ stoichiometry and $(\alpha4)_2(\beta2)_2\alpha5$ subtype are equally highly sensitive to ACh but are dramatically different in Ca^{2+} permeability, with the $(\alpha4)_2(\beta2)_2\alpha5$ subtype having 6-fold greater permeability to Ca^{2+} (Fig. 4) than the $(\alpha4)_2(\beta2)_3$ stoichiometry. This will probably to be an important component of the biological significance of $\alpha4\beta2\alpha5$ AChRs.

The β-6-α+$\beta3$ combination exhibited even lower sensitivity to ACh than the β-6-α+$\alpha4$ combination (Fig. 6C) and showed a similarly high permeability to Ca^{2+}. The similar high Ca^{2+} permeabilities in the presence of either $\beta3$ or $\alpha5$ accessory subunits further confirms the vital importance of the ring of charge in determining Ca^{2+} permeability, which was critically demonstrated using single amino acid mutations of $\alpha4$ and $\beta2$ subunits. The greatly differing ACh sensitivities in the presence of $\beta3$ or $\alpha5$ demonstrate that, although accessory subunits do not participate in forming ACh binding sites, they can greatly influence the sensitivity to the global conformation changes in AChRs that are required to open their cation channels.

Discussion

Expression of an equal amount of $\alpha4$ and $\beta2$ subunits in *X. laevis* oocytes results in nearly equal amounts of AChRs with an $(\alpha4)_2(\beta2)_3$ or $(\alpha4)_3(\beta2)_2$ stoichiometry. By expressing a β-6-α concatamer with free $\beta2$ subunits, a pure population of the $(\alpha4)_2(\beta2)_3$ was produced, and by expressing this concatamer with free $\alpha4$ subunits, only the $(\alpha4)_3(\beta2)_2$ stoichiometry was produced. We assayed Ca^{2+} permeability of these recombinant human $\alpha4\beta2$ AChRs by two methods: 1) the shift in the reversal potential of I_{ACh} toward more positive values when native Ca^{2+}-activated Cl^- channel effects were avoided by eliminating Cl^-, and 2) measurement of inward currents when Ca^{2+} was the only extracellular permeant ion. Both methods showed that the $(\alpha4)_3(\beta2)_2$ stoichiometry had greater Ca^{2+} permeability than the $(\alpha4)_2(\beta2)_3$ stoichiometry. Furthermore, the differences in permeability were shown to result from charged residues at the outer side of the M2 transmembrane domain, which form a pentameric ring at the extracellular end of the cation channel. $\alpha4$, $\alpha5$, and $\beta3$ subunits all have a homologous glutamate in M2, which contributes to high Ca^{2+} permeability, whereas $\beta2$ has a lysine at this position that reduces Ca^{2+} permeability. All positive charges in the ring prevented Ca^{2+} permeability, and Ca^{2+} permeability increased with the increasing numbers of negative charges in this ring. The presence of a ring of negative charges near the mouth of the channel would also be expected to increase the cation concentration near the channel entrance and increase currents.

Physiological Implications of the Results. There is clear evidence that coexpression of $\alpha4$ and $\beta2$ subunits can result in a mixture of AChRs in $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$ stoichiometries (Nelson et al., 2003; Zhou et al., 2003; Briggs et al., 2006; Moroni et al., 2006; Zwart et al., 2006). It has not been demonstrated that both stoichiometries are expressed in brain neurons, but there is evidence for heterogeneity of apparent $\alpha4\beta2$ AChR properties in neurons that could be accounted for either by a mixture of these stoichiometries and/or subunit combinations such as $\alpha4\beta2\alpha5$, $\alpha4\beta2\beta3$, or $\alpha4\alpha6\beta2\beta3$ (Marks et al., 1999; Shafaee et al., 1999). The $(\alpha4)_3(\beta2)_2$ stoichiometry has been shown to differ from the $(\alpha4)_2(\beta2)_3$ stoichiometry in that it is less sensitive to nicotine-induced up-regulation caused by increased assembly as a result of pharmacological chaperone effects of nicotine (Nelson et al., 2003; Kuryatov et al., 2005; Moroni et al., 2006). The $(\alpha4)_3(\beta2)_2$ stoichiometry desensitizes more rapidly (Nelson et al., 2003). The $(\alpha4)_3(\beta2)_2$ stoichiometry is less sensitive to activation or competitive inhibition by many ligands (Kuryatov et al., 2005; Moroni et al., 2006; Zwart et al., 2006). The efficacy of some agonists differs greatly between stoichiometries. For example, cytisine fails to activate the $(\alpha4)_2(\beta2)_3$ stoichiometry but gives 22% of the maximum effect of ACh on the $(\alpha4)_3(\beta2)_2$ stoichiometry, whereas TC-2559 has 260% of the potency of ACh on the $(\alpha4)_2(\beta2)_3$ stoichiometry but only 22% efficacy on the $(\alpha4)_3(\beta2)_2$ stoichiometry (Moroni et al., 2006; Zwart et al., 2006). The newly approved drug for smoking cessation, varenicline, is believed to act like cytisine as a

TABLE 1

Ca^{2+} permeabilities of wild-type and mutant AChRs in both $(\alpha4)_2$ and $(\alpha4)_3$ stoichiometries

The glutamic acid (E) or lysine (K) residues at the extracellular end of the M2 transmembrane domain as shown in Fig. 5 were presumed to form a ring of charge lining the cation channel which regulated its permeability to Ca^{2+}. The proportion of more ACh-sensitive $(\alpha4)_2(\beta2)_3$ and less ACh-sensitive $(\alpha4)_3(\beta2)_2$ stoichiometries of each subunit combination were determined as shown in Fig. 6B. The net ratio of − charged glutamic acid to + charged lysine residues in the pentameric M2 ring weighted for the proportion of each stoichiometry was determined by multiplying the charge composition of each stoichiometry by its proportion of the total and summing charges. When linked subunits were used, the stoichiometry was all $(\alpha4)_2(\beta2)_3$ if the free subunit was $\beta2$ or $(\alpha4)_3(\beta2)_2$ if the free subunit was $\alpha4$.

AChR	Amino Acid Composition of Cation Selectivity Ring in M2 in Each Stoichiometry		Fraction of Each Stoichiometry $(\alpha4)_2/(\alpha4)_3$	Net Charge[a]	Observed Ca^{2+} Permeability[b]
	$(\alpha4)_2$	$(\alpha4)_3$			
					%
Wild type	$(E)_2(K)_3$	$(E)_3(K)_2$	0.555/0.445	+0.11	6.4 ± 1.6 (n=10)
β-6-α+$\alpha4$	$(E)_2(K)_3$	$(E)_3(K)_2$	0.06/0.94	−1.0	20.2 ± 1.0 (n= 9)
β-6-α+$\alpha4^{EK}$		$(E)_2(K)_3$	0/1	+1.0	12.2 ± 1.2 (n=10)
β-6-α+$\beta2$	$(E)_2(K)_3$		1/0	+1.0	8.4 ± 1.3 (n=12)
β-6-α+$\beta2^{KE}$	$(E)_3(K)_2$		1/0	−1.0	19.9 ± 2.6 (n= 9)
$\alpha4^{EK}\beta2$	$(K)_5$	$(K)_5$	0.498/0.502	+5.0	−0.79 ± 1.8 (n=14)
$\alpha4\beta2^{KE}$	$(E)_5$	$(E)_5$	0.627/0.373	−5.0	19.9 ± 3.3 (n=15)
$\alpha4^{EK}\beta2^{KE}$	$(K)_2(E)_3$	$(K)_3(E)_2$	0.714/0.286	−0.43	16.3 ± 3.9 (n=15)

[a] Of the five amino acids in the ring formed by the extracellular ends of M2 domains.
[b] For mixture of stoichiometries (control currents observed with Ca^{2+} as the only cation).

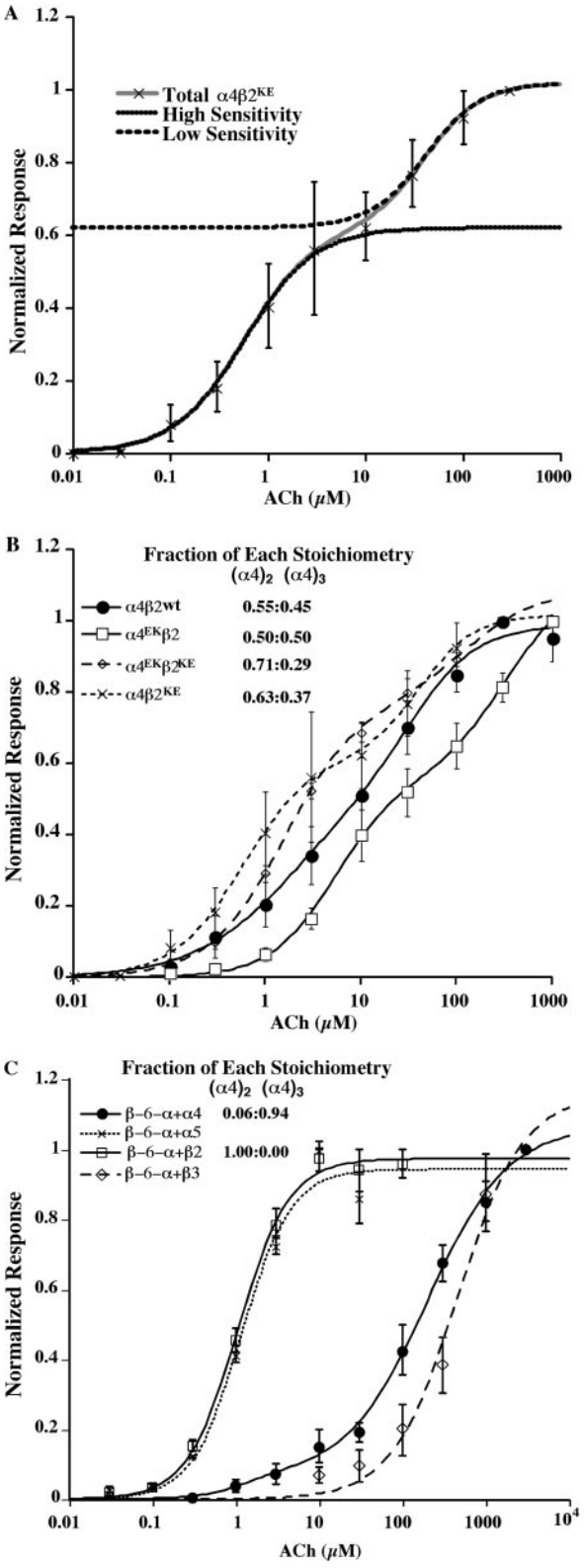

Fig. 6. ACh concentration-response curves of mutants and wt $\alpha4\beta2$ AChRs. Sensitivity to activation by ACh reveals the fraction of each stoichiometry in various subunit mixtures. A, the proportion of high-sensitivity and low-sensitivity components in each dose-response curve were determined as shown here for $\alpha4\beta2^{KE}$. B, the two component concentration-response curves are shown for equal mixtures of wild-type $\alpha4$ and $\beta2$ subunits and for the various mutant subunit combinations studied in the previous figures. C, the concentration-response curves are shown for combinations of linked $\alpha4\beta2$ subunit pairs with free subunits to provide subtypes of defined stoichiometry. The $(\alpha4)_2(\beta2)_3$ stoichiometry has the expected high sensitivity to ACh. $(\alpha4)_3(\beta2)_2$ stoichiometry has the expected low sensitivity but shows a trace of high-affinity component because insufficient excess of free $\alpha4$ was added to prevent all formation of the dimers, which β-6-α forms when expressed alone. These dimers contain linked AChRs, one of each stoichiometry (Zhou et al., 2003). The effects of trace amounts of $(\alpha4)_2(\beta2)_3$ AChRs are seen.

partial agonist on $\alpha4\beta2$ AChRs in oocytes and on dopaminergic neurons (Coe et al., 2005; Jorenby et al., 2006). If it behaved like cytisine, it would only be a partial agonist if $(\alpha4)_3(\beta2)_2$ AChRs existed in neurons and would be an antagonist on the $(\alpha4)_2(\beta2)_3$ stoichiometry. Nicotine is only a 28% partial agonist on the $(\alpha4)_2(\beta2)_3$ stoichiometry but a 62% partial agonist on the $(\alpha4)_3(\beta2)_2$ stoichiometry (Moroni et al., 2006). The channel-blocking antagonists mecamylamine and chlorisondamine more potently block the $(\alpha4)_3(\beta2)_2$ stoichiometry (Briggs et al., 2006), which is consistent with our observation that the channel properties of the two stoichiometries differ.

Here, we show that the $(\alpha4)_3(\beta2)_2$ stoichiometry has much greater Ca^{2+} permeability than does the $(\alpha4)_2(\beta2)_3$ stoichiometry. The increased Ca^{2+} flux through the $(\alpha4)_3(\beta2)_2$ stoichiometry would increase transmitter release in response to $\alpha4\beta2$ AChRs located presynaptically and increase Ca^{2+}-activated signaling cascades in response to activation of postsynaptic $\alpha4\beta2$ AChRs. We proposed (Nelson et al., 2003) that the $(\alpha4)_2(\beta2)_3$ stoichiometry might be localized presynaptically where tonic volume transmission depending on ACh released at a distance would depend on its high sensitivity and slow desensitization, whereas the $(\alpha4)_3(\beta2)_2$ stoichiometry might be most effective as a postsynaptic AChR where it would be exposed transiently to high concentrations of ACh in the course of short-term rapid synaptic transmission that would be sufficient to activate it. Further investigations are required to localize $\alpha4\beta2$ AChR stoichiometries in neurons and to determine their physiological and pharmacological significance. This would be aided by identifying drugs that are highly selective for each stoichiometry. Note also that the observation that $\alpha3\beta2$ AChRs can be expressed in two stoichiometries (Briggs et al., 2006) suggests that this mode of regulation may apply to the expression of many heteromeric AChRs.

The presence of $\alpha5$ or $\beta3$ in the $\beta1$-like accessory position would determine the $(\alpha4)_2$ stoichiometry of the $\alpha4\beta2^*$ AChR. Depending on the extent of incorporation of $\alpha5$ or $\beta3$, the presence of $(\alpha4)_3$ AChRs would be reduced or eliminated. However, because both $\alpha5$ and $\beta3$ have glutamate in the M2 ring at the same position as does $\alpha4$, one can expect that the $(\alpha4)_3(\beta2)_2$, $(\alpha4)_2(\beta2)_2\alpha5$, and $(\alpha4)_2(\beta2)_2\beta3$ AChRs would all have similarly high permeability to Ca^{2+}. Kuryatov et al. (1997) showed that, as expected, coexpressing $\alpha5$ with $\alpha4$ and $\beta2$ increased Ca^{2+} permeability. Here we show that $(\alpha4)_2(\beta2)_2\alpha5$ has the highest Ca^{2+} permeability of any combination tested and that $(\alpha4)_2(\beta2)_2\beta3$ has Ca^{2+} permeability similar to that of $(\alpha4)_3(\beta2)_2$ stoichiometry. The combination in $(\alpha4)_2(\beta2)_2\alpha5$ of the high ACh sensitivity of the $(\alpha4)_2(\beta2)_3$ stoichiometry and even higher Ca^{2+} permeability than the $(\alpha4)_3(\beta2)_2$ stoichiometry would make this subtype exceptionally potent at functional roles in which Ca^{2+} influx was important, such as presynaptic promotion of transmitter release or postsynaptic triggering of signaling cascades.

The $(\alpha4)_2(\beta2)_2\beta3$ subtype was found to have even lower

ACh sensitivity than the $(\alpha4)_3(\beta2)_2$ stoichiometry and a similarly high Ca^{2+} permeability. Both $\beta3$ and $\alpha5$ can assemble only in the accessory position, comparable with that of $\beta1$ in the muscle $(\alpha1)_2\beta1\gamma\delta$ AChRs, in which they do not contribute to the formation of ACh binding sites. As shown here and elsewhere, accessory subunits can influence both sensitivity to the conformation changes involved in channel opening and channel conductance. For example, $\alpha5$ increases Ca^{2+} permeability and desensitization of both $\alpha3\beta2$ and $\alpha3\beta4$ AChRs and greatly increases ACh sensitivity of $\alpha3\beta2$ but not $\alpha3\beta4$ AChRs (Gerzanich et al., 1998). $\beta3$ greatly increases assembly and sensitivity to nicotine-induced up-regulation of $\alpha6\beta2$ and $\alpha6\beta4$ AChRs (Tumkosit et al., 2006). It has recently been reported that $\beta3$ led to nearly complete loss of function of $\alpha4\beta2$, $\alpha4\beta4$, and $\alpha3\beta2$ AChRs and proposed that $\beta3$ served a universal role as a dominant-negative regulator (Broadbent et al., 2006). However, here we show that $\beta3$ formed functional but much lower affinity AChRs with $\alpha4\beta2$. Thus, $\beta3$ does not behave as a universal dominant-negative regulator. The greatly reduced AChR function that Broadbent et al. (2006) observed in the presence of $\beta3$ resulted from using a cRNA ratio in oocytes of $\alpha/\beta/\beta3$ of 1:1:20, which probably provokes the assembly of nonproductive intermediates, thereby reducing the total amount of AChR expressed, and the low sensitivity of $\alpha4\beta2\beta3$ AChRs to activation would further reduce detectable function.

Acknowledgments

We thank Barbara Campling for her comments on the manuscript.

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