0026-895X/05/6802-487–501$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2005 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 68:487–501, 2005

Vol. 68, No. 2
11155/3044683
Printed in U.S.A.

Mutations Linked to Autosomal Dominant Nocturnal Frontal Lobe Epilepsy Affect Allosteric Ca^{2+} Activation of the $\alpha4\beta2$ Nicotinic Acetylcholine Receptor[S]

Nivalda O. Rodrigues-Pinguet, Thierry J. Pinguet, Antonio Figl, Henry A. Lester, and Bruce N. Cohen

Division of Biology, California Institute of Technology, Pasadena, California (N.O.R.-P., H.A.L., B.N.C.); Luxtera, Inc., Carlsbad, California (T.J.P.); and Molecular Devices Corp., Union City, California (A.F.)

Received January 18, 2005; accepted May 17, 2005

ABSTRACT

Extracellular Ca^{2+} robustly potentiates the acetylcholine response of $\alpha4\beta2$ nicotinic receptors. Rat orthologs of five mutations linked to autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE)—$\alpha4$(S252F), $\alpha4$(S256L), $\alpha4$(+L264), $\beta2$(V262L), and $\beta2$(V262M)—reduced 2 mM Ca^{2+} potentiation of the $\alpha4\beta2$ 1 mM acetylcholine response by 55 to 74%. To determine whether altered allosteric Ca^{2+} activation or enhanced Ca^{2+} block caused this reduction, we coexpressed the rat ADNFLE mutations with an $\alpha4$ N-terminal mutation, $\alpha4$(E180Q), that abolished $\alpha4\beta2$ allosteric Ca^{2+} activation. In each case, Ca^{2+} inhibition of the double mutants was less than that expected from a Ca^{2+} blocking mechanism. In fact, the effects of Ca^{2+} on the ADNFLE mutations near the intracellular end of the M2 region—$\alpha4$(S252F) and $\alpha4$(S256L)—were consistent with a straightforward allosteric mechanism. In contrast, the effects of Ca^{2+} on the ADNFLE mutations near the extracellular end of the M2 region—$\alpha4$(+L264)$\beta2$, $\beta2$(V262L), and $\beta2$(V262M)—were consistent with a mixed mechanism involving both altered allosteric activation and enhanced block. However, the effects of 2 mM Ca^{2+} on the $\alpha4\beta2$, $\alpha4$(+L264)$\beta2$, and $\alpha4\beta2$(V262L) single-channel conductances, the effects of membrane potential on the $\beta2$(V262L)-mediated reduction in Ca^{2+} potentiation, and the effects of eliminating the negative charges in the extracellular ring on this reduction failed to provide any direct evidence of mutant-enhanced Ca^{2+} block. Moreover, analyses of the $\alpha4\beta2$, $\alpha4$(S256L), and $\alpha4$(+L264) Ca^{2+} concentration-potentiation relations suggested that the ADNFLE mutations reduce Ca^{2+} potentiation of the $\alpha4\beta2$ acetylcholine response by altering allosteric activation rather than by enhancing block.

ADNFLE is a monogenic partial epilepsy linked to four $\alpha4$ and two $\beta2$ nicotinic subunit mutations (Steinlein et al., 1995, 1997; Hirose et al., 1999; De Fusco et al., 2000; Phillips et al., 2001; Leniger et al., 2003). ADNFLE seizures occur primarily during slow-wave sleep and seem to originate in the frontal lobe (Scheffer et al., 1995). However, the physiological mechanism that generates these seizures has not been established.

At physiological concentrations (1.5–2 mM), Ca^{2+} potentiates the neuronal nicotinic agonist response by binding to an N-terminal site in the receptor protein (Galzi et al., 1996; Le Novere et al., 2002; Rodrigues-Pinguet et al., 2003). Allosteric Ca^{2+} activation of the neuronal nicotinic receptors is conserved across species (rat, human, chicken) and across nicotinic receptor subtypes (Mulle et al., 1992; Vernino et al., 1992; Galzi et al., 1996; Steinlein et al., 1997; Liu and Berg, 1999). Adding 2 to 2.5 mM Ca^{2+} to the extracellular saline increases the wild-type $\alpha4\beta2$ acetylcholine response by 300 to 500% (Steinlein et al., 1997; Figl et al., 1998; Rodrigues-Pinguet et al., 2003). A common feature of the ADNFLE mutations is that they reduce 2 to 2.5 mM Ca^{2+} potentiation of the $\alpha4\beta2$ acetylcholine response by 50 to 72%, at acetylcholine concentrations $\geq$30 μM (Steinlein et al., 1997; Figl et al., 1998; Rodrigues-Pinguet et al., 2003). Because the extracellular Ca^{2+} concentration in the mammalian brain is normally 1.5 to 2 mM (Egelman and Montague, 1999), a decrease in Ca^{2+} potentiation of the $\alpha4\beta2$ acetylcholine response could contribute to ADNFLE seizures either by (1) reducing $\alpha4\beta2$-

This work was supported by grants from the National Institute of Health (NS0438000 and NS011756, to B.N.C. and H.A.L., respectively and a minority supplement (NS0438000-04S1 to N.O.R.).
Portions of this work were published previously by UMI publishers in a doctoral dissertation by N.O.R.
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.105.011155.
[S] The online version of this article (available at http://molpharm.aspetjournals.org) contains supplemental material.

ABBREVIATIONS: ADNFLE, autosomal dominant nocturnal frontal lobe epilepsy; $[Ca^{2+}]_o$, added extracellular Ca^{2+} concentration; i_s, single-channel current; g_s, single-channel conductance; *SSD*, percentage of steady-state desensitization; τ_D, time constant of desensitization.

mediated inhibitory transmitter release in the cortex (McNamara, 1999) or (2) shifting the balance between $\alpha4\beta2$-mediated excitatory and inhibitory transmitter release during bouts of high-frequency cortical synaptic activity, in favor of excitatory transmitter release (Rodrigues-Pinguet et al., 2003).

At concentrations outside the physiological range (≥20 mM), Ca^{2+} also reduces the single-channel conductance of the $\alpha4\beta2$ receptor (Buisson et al., 1996). The ability of Ca^{2+} to both block and potentiate $\alpha4\beta2$ nicotinic receptors means that at least two distinct molecular mechanisms could account for the effects of the ADNFLE mutations on Ca^{2+} potentiation. The mutations could 1) interfere with allosteric Ca^{2+} activation or 2) enhance the potency of uncompetitive or noncompetitive Ca^{2+} block. There are several classes of possible Ca^{2+} blocking mechanisms. We refer to them simply as "noncompetitive."

The $\alpha4$ N-terminal mutation $\alpha4$(E180Q) eliminates allosteric Ca^{2+} activation of the $\alpha4\beta2$ receptor (Rodrigues-Pinguet et al., 2003). To decide whether the ADNFLE mutations reduce Ca^{2+} potentiation by interfering with allosteric Ca^{2+} activation or by enhancing Ca^{2+} block, we constructed double-mutant receptors containing this mutation and one of five rat orthologs—$\alpha4$(S252F), $\alpha4$(S256L), $\alpha4$(+L264), $\beta2$(V262L), and $\beta2$(V262M)—of the human ADNFLE mutations—$\alpha4$(S248F), $\alpha4$(S252L), $\alpha4$(776ins3), $\beta2$(V287L), and $\beta2$(V287M). We refer to these receptors as $\alpha4$(E180Q):ADNFLE double mutants. The rat $\alpha4$(+L264) mutation is a 3-base pair insertion that adds a leucine at position 264 to the $\alpha4$ amino acid sequence.

If the Ca^{2+} blocking mechanism is correct, then the percentages by which Ca^{2+} blocks the acetylcholine response of $\alpha4$(E180Q):ADNFLE double-mutant receptors and by which the corresponding single ADNFLE mutations reduce Ca^{2+} potentiation of the wild-type acetylcholine response should be the same. On the other hand, if the mutations reduce Ca^{2+} potentiation of the acetylcholine response by altering allosteric Ca^{2+} activation, then the $\alpha4$(E180Q) mutation should nullify the effects of the ADNFLE mutations, and Ca^{2+} should not block the $\alpha4$(E180Q):ADNFLE double-mutant acetylcholine response any more than it blocks $\alpha4$(E180Q)$\beta2$ acetylcholine response. We also measured the effects of the $\alpha4$(S256L) and $\alpha4$(+L264) mutations on the Ca^{2+} concentration-potentiation relation for the acetylcholine response, the effects of 2 mM Ca^{2+} on the wild-type, $\alpha4$(+L264)$\beta2$, and $\alpha4\beta2$(V262L) single-channel conductance, and the voltage dependence of the $\beta2$(V262L)-mediated reduction in Ca^{2+} potentiation. Finally, we determined whether eliminating the fixed negative charge in the extracellular ring at the extracellular pore entrance could reverse the effects of the ADNFLE mutations on Ca^{2+} potentiation. The results show that the ADNFLE mutations reduce Ca^{2+} potentiation of the $\alpha4\beta2$ acetylcholine response by disrupting allosteric Ca^{2+} activation of the receptor rather than by enhancing Ca^{2+} block.

Materials and Methods

Oocyte Expression. Stage V to VI *Xenopus laevis* oocytes were surgically isolated as described previously (Quick and Lester, 1994). Female *X. laevis* frogs were anesthetized by immersion in 0.2% tricaine methanesulfonate (Sigma, St. Louis, MO), pH 7.4, for 45 to 60 min, and the ovarian lobes were extracted through a small abdominal incision. The oocyte follicular layer was removed using Type A collagenase (1–2 h in a 2 mg/ml collagenase solution; Sigma). To increase nicotinic receptor expression, the rat $\alpha4$–1 (Goldman et al., 1987) and $\beta2$ inserts (Deneris et al., 1988) were subcloned into a vector containing a 5′-untranslated region from the alfalfa mosaic virus that enhanced protein translation and a long 3′ poly A tail (Figl et al., 1998). We used the QuikChange single and multiple site-directed mutagenesis kit (Stratagene, La Jolla, CA) to construct the rat $\alpha4$ and $\beta2$ mutations and verified them by DNA sequencing. Capped cRNA was synthesized in vitro using the mMessage mMachine RNA transcription kit (Ambion, Austin, TX). After a 24-h incubation in a modified Barth's solution containing 96 mM NaCl , 5 mM HEPES, 2.5 mM sodium pyruvate, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.6 mM theophylline (Sigma) with 2.5 μg/ml Gentamicin (Sigma) and 5% horse serum, pH 7.4 (Irvine Scientific, Santa Ana, CA), the isolated oocytes were injected with rat $\alpha4$ and $\beta2$ cRNA.

Whole-Oocyte Electrophysiology. Injected oocytes were incubated for ≥24 h in the modified Barth's solution at 15–19°C before electrophysiological recordings were attempted. We voltage-clamped the oocytes with two 3-MΩ, KCl-filled microelectodes (1.5- to 4-MΩ resistance) using a GeneClamp 500 voltage clamp (Axon Instruments, Union City, CA). During the voltage-clamp recordings, the oocytes were continually superfused with a nominally Ca^{2+}-free saline (ND98) containing 98 mM NaCl, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.4, at 20–23°C, unless otherwise stated. We added 0.1 to 2 mM $CaCl_2$ to the ND98 solution to measure Ca^{2+}-induced changes in the acetylcholine response. Acetylcholine was applied to the oocytes using a U-tube microperfusion system (Cohen et al., 1995). The time constant for solution exchange was ~0.5 s. The voltage-clamp currents were digitized using a personal computer equipped with a DigiData 1322A analog-to-digital interface and pCLAMP version 8 software (Axon Instruments). To avoid aliasing, the voltage-clamp currents were filtered at one fourth to one fifth of the sampling frequency (typically 20 Hz) with an 8-pole, low-pass Bessel filter. We measured the current-voltage relation of the acetylcholine-induced current by ramping the membrane potential between −120 and +50 mV over a 0.5-s interval, in the presence and absence of 1 μM acetylcholine, and digitally subtracting the ramp current in the presence of acetylcholine from that in the absence of acetylcholine. We used Dunnett's test (SigmaStat ver. 1; SPSS Inc., Chicago, IL) to determine whether the ADNFLE mutations significantly affected Ca^{2+} potentiation of the peak acetylcholine response. The errors reported in the text are S.E.M. or S.E. (for fitted parameters). We used a previous approximation for the variance of the ratio of two random variables (Mood et al., 1974) to calculate the S.E.M. for the mutant-induced fractional reductions in Ca^{2+} potentiation of the acetylcholine response.

Desensitization Analysis. To compare desensitization of the wild-type and mutant responses, we fit their desensitizing phases to the sum of an exponential component and a constant term using the curve-fitting routine in pCLAMP version 8.2. From these fits, we obtained an apparent time constant of desensitization (τ_D) and the percentage of steady-state desensitization of the response (*SSD*), defined as

$$SSD = \left(1 - \frac{I_{exp}}{I_{exp} + I_C}\right) \times 100$$

where I_{exp} and I_C are the fitted amplitudes of the exponential component and constant, respectively. We analyzed the effects of the mutations and Ca^{2+} on these two parameters using a two-way analysis of variance with receptor type and Ca^{2+} concentration as factors. Post hoc comparisons were carried out on the ranked data using the Student-Newman-Keuls test.

BAPTA Injections. To prevent Ca^{2+} influx through the wild-type and mutant nicotinic receptors from activating endogenous Ca^{2+}-activated Cl^- currents, we injected the oocytes with 50 nl of a 100 mM K_4BAPTA solution buffered to pH 7.4 with 10 mM HEPES, 5 to

10 min before recording from them (Haghighi and Cooper, 2000; Rodrigues-Pinguet et al., 2003). Assuming an oocyte volume of 500 nl, these injections produced a final intracellular BAPTA concentration of ~10 mM.

Single-Channel Recordings. We recorded single wild-type and mutant channels in cell-attached patches at 20–23°C using the patch-clamp option of the GeneClamp 500 voltage clamp. Patch electrodes were pulled from borosilicate capillary tubing (1.6-mm o.d., 0.80 mm

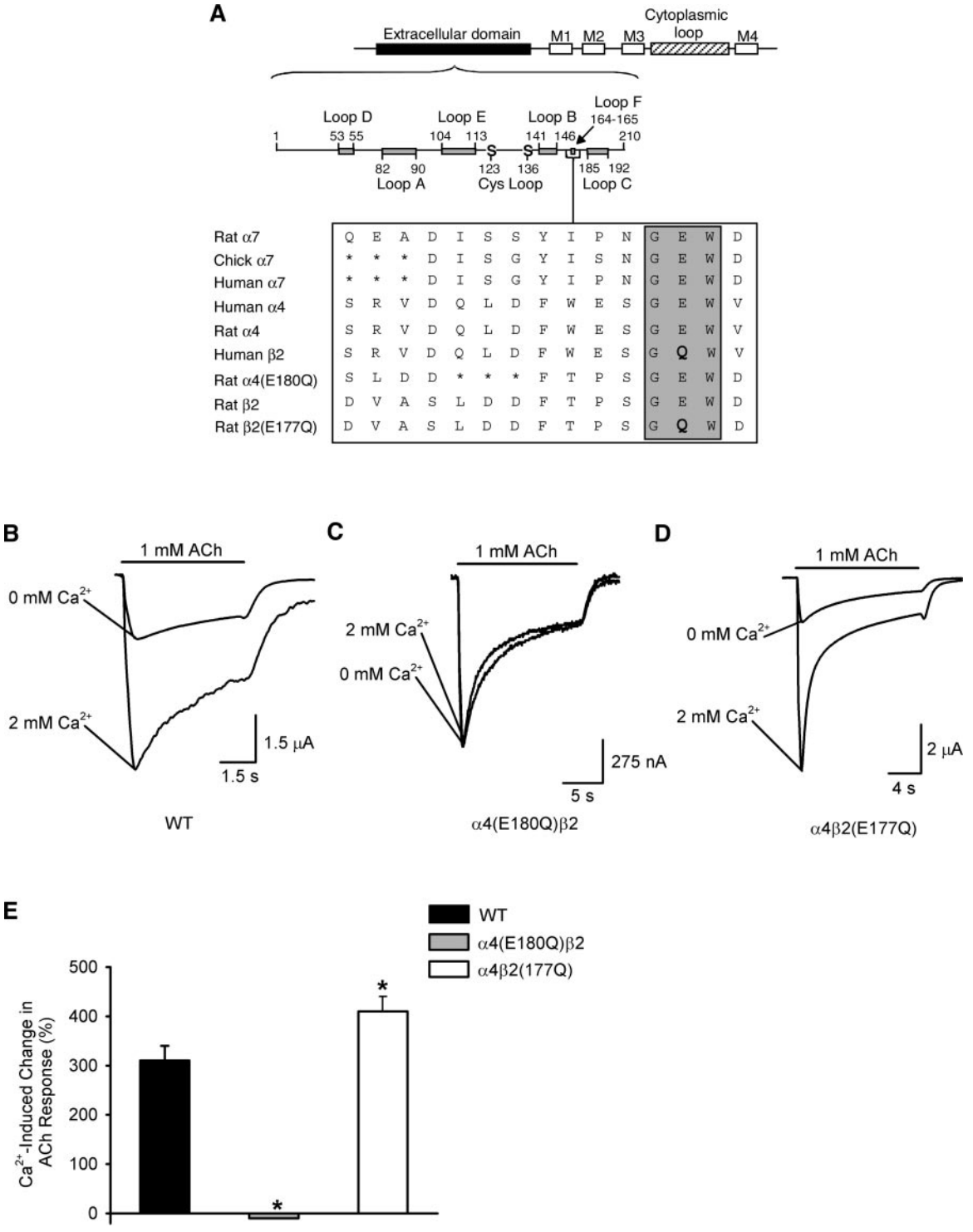

Fig. 1. The α4(E180Q) mutation abolishes 2 mM Ca^{2+} potentiation of the rat wild-type (WT) α4β2 1 mM acetylcholine (ACh) response. A, location of the α4(E180Q) mutation in the α4 N-terminal domain (top). The linear map above the aligned sequences shows the major domains of the nicotinic subunit and the location of loops A to F in the N terminus. The aligned amino acid sequences (bottom) show that the Glu at α4(E180) is conserved across different species (rat, human, chick) and neuronal subunits. Bold letters denote the positions of the α4(E180Q) and β2(E177Q) mutations. Identical residues in the wild-type subunits are highlighted in light gray. B–D, voltage-clamped responses of the wild-type, α4(E180Q)β2, and α4β2(E177Q) receptors to 1 mM acetylcholine in 0 and 2 mM added extracellular Ca^{2+}. The lines above the traces show the acetylcholine applications. V = −50 mV. E, the bars show the Ca^{2+}-induced changes in the peak wild-type, α4(E180Q)β2, and α4β2(E177Q) responses. The error bars are ± S.E.M. (n = 4–8 oocytes). Asterisks denote significant differences from the wild-type ($P < 0.05$).

i.d.; Garner Glass Company, Claremont, CA) using a modified Kopf electrode puller (Hamill et al., 1981). To patch onto the oocytes, we removed the vitelline membrane with forceps manually after incubating the oocytes in a hypertonic solution (ND98 plus 100 mM NaCl) for 20 to 30 min on a rotating shaker. The ionic composition of the pipette-filling and bathing solution used for the recordings was 98 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.4. The patch electrode resistance was 10 to 30 MΩ. Because the extracellular K^+ concentration was 98 mM, the oocyte resting potentials in the patch experiments were near 0 mV. We added 10 nM acetylcholine to the pipette-filling solution to activate the wild-type and mutant channels with minimal desensitization. To measure the effects of Ca^{2+} on the single-channel current, we added 2 mM $CaCl_2$ to the pipette-filling and bathing solution. Single-channel currents were recorded digitally using the Digidata 1322A acquisition system and pCLAMP version 8 software. The single-channel data were sampled at 10 kHz and low-pass filtered at 2 kHz before digitization. We used pCLAMP version 9 to construct and fit all-points histograms of the single-channel current to the sum of two or more Gaussian components. The single-channel current amplitudes were obtained from the differences between the means of these Gaussian components. We used a two-way analysis of variance with conductance state and Ca^{2+} concentration as factors to determine 1) whether the amplitudes of the small and large single-channel currents were significantly different and 2) whether Ca^{2+} affected the single-channel currents. Post hoc comparisons were carried out using the Student-Newman-Keuls test.

Results

The α4(E180Q) Mutation Abolishes Ca^{2+} Potentiation of the α4β2 Acetylcholine Response. To ensure that the α4(E180Q) mutation eliminated allosteric Ca^{2+} activation of the α4β2 receptor, we measured its effects on 2 mM Ca^{2+} potentiation of the α4β2 1 mM acetylcholine response. We used 1 mM acetylcholine for these experiments instead of the 30 μM concentration used previously (Rodrigues-Pinguet et al., 2003) to maximize any potential open-channel Ca^{2+} block of the ADNFLE mutant receptors and to minimize any effects that mutant-induced shifts in acetylcholine potency might have on Ca^{2+} potentiation of the acetylcholine response. Adding 2 mM Ca^{2+} to a nominally Ca^{2+}-free saline (ND98; see *Materials and Methods*) increased the peak 1 mM wild-type acetylcholine response by 310 ± 30% (mean ± S.E.M.) at −50 mV (Fig. 1B; Table 1). In contrast, adding 2 mM Ca^{2+} reduced the α4(E180Q)β2 1 mM acetylcholine response by 10 ± 1% (Fig. 1, C and E; Table 1). Thus, consistent with previous experiments using 30 μM acetylcholine (Rodrigues-Pinguet et al., 2003), the α4(E180Q) mutation completely abolished 2 mM Ca^{2+} potentiation of the α4β2 1 mM acetylcholine response (Fig. 1; Table 1). It is interesting that a β2 mutation—β2(E177Q)—homologous to the α4(E180Q) mutation did not reduce α4β2 Ca^{2+} potentiation (Fig. 1, D and E; Table 1).

The α4(S252F) and α4(S256L) Mutations Obviously Alter Allosteric Ca^{2+} Activation. To determine whether the ADNFLE mutations reduced Ca^{2+} potentiation by enhancing Ca^{2+} block of the receptor or by altering allosteric Ca^{2+} activation, we compared the effects of 2 mM Ca^{2+} on the ADNFLE single- and α4(E180Q):ADNFLE double-mutant acetylcholine responses. Ca^{2+} affected the double mutants with ADNFLE mutations closer to the intracellular end of the M2 region—α4(E180Q:S252F)β2 and α4(E180Q:S256L)β2—differently from those with ADNFLE mutations near the extracellular end of M2—α4(E180Q:+L264)β2, α4(E180Q)β2(V262M), and α4(E180Q)β2(V262L). We shall discuss the results for the ADNFLE mutations closer to the intracellular end first. Consistent with their previously reported effects on Ca^{2+} potentiation of the 30 μM acetylcholine response (Rodrigues-Pinguet et al., 2003), the single α4(S252F) and α4(S256L) mutations significantly reduced 2 mM Ca^{2+} potentiation of the α4β2 1 mM acetylcholine response (Fig. 2, A and B; Table 1). Adding 2 mM Ca^{2+} increased the α4(S256L)β2 and α4(S252F)β2 1 mM acetylcholine responses by only ~40% (Fig. 2, A, B, and E) as opposed to a 310% increase for the wild-type (Fig. 1, B and E). Thus, the Ca^{2+}-induced mutant increases were ~60% less than that of the wild-type. If enhanced Ca^{2+} block accounted entirely for the effects of the α4(S252F) and α4(S256L) mutations on Ca^{2+} potentiation, then 2 mM Ca^{2+} should have also reduced the α4(E180Q:S252F)β2 and α4(E180Q:S256L)β2 1 mM acetylcholine responses by ~60%. However, 2 mM Ca^{2+} had little effect on the α4(E180Q:S252F)β2 and α4(E180Q:S256L)β2 responses (Fig. 2, C, D, and F; Table 1). In fact, it reduced the α4(E180Q:S252F)β2 1 mM acetylcholine response by only 10 ± 1%, and it increased the α4(E180Q:S256L)β2 1 mM acetylcholine response by 10 ± 4%. Therefore, the α4(S252F) and α4(S256L) mutations seem to reduce α4β2 Ca^{2+} potentiation by diminishing allosteric Ca^{2+} activation of the α4β2 receptor rather than by enhancing Ca^{2+} block.

The α4(S256L) and α4(S252F) mutations did not significantly affect the amplitude of the 1 mM acetylcholine response in the absence of Ca^{2+} ($P > 0.05$). In 0 mM Ca^{2+}, the peak amplitudes of the wild-type, α4(S256L)β2, and α4(S252F)β2 1 mM acetylcholine responses were 1400 ± 550 ($n = 10$), 830 ± 280 ($n = 4$), and 1000 ± 460 nA ($n = 4$), respectively.

The Effects of the α4(+L264), β2(V262M), and β2(V262L) Mutations Are More Complex. The single α4(+L264), β2(V262M), and β2(V262L) ADNFLE mutations reduced Ca^{2+} potentiation of the acetylcholine response by an amount similar to that of the α4(S252F) and α4(S256L) mutations (Fig. 3, A, C, E, and G; Table 1) but Ca^{2+} inhibi-

TABLE 1
Effect of 2 mM Ca^{2+} on the peak 1 mM acetylcholine response at −50 mV
Values are presented as mean ± S.E.M.

Receptor	Number of Oocytes	Ca^{2+}-Induced Change in Acetylcholine Response
		%
α4β2	4	310 ± 30
α4(E180Q)β2	4	−10.0 ± 0.4
α4β2(E177Q)	8	410 ± 30*
α4(S252F)β2	4	30 ± 10*
α4(S256L)β2	4	40 ± 4*
α4(+L264)β2	4	10 ± 2*
α4β2(V262L)	4	−20 ± 4*
α4β2(V262M)	5	40 ± 4*
α4(E245Q)β2	5	90 ± 30*
α4(E266Q)β2	4	300 ± 10
α4(E180Q:S252F)β2	8	−10 ± 4
α4(E180Q:S256L)β2	5	10 ± 4†
α4(E180Q:+L264)β2	4	−50 ± 2†
α4(E180Q)β2(V262L)	6	−30 ± 5†
α4(E180Q)β2(V262M)	4	−40 ± 6†
α4(E245Q)β2(V262L)	5	−50 ± 10
α4(E266Q)β2(V262L)	4	−40 ± 2

* Significantly different ($P < 0.05$) from the wild-type (α4β2) using Dunnett's test.
† Significantly different ($P < 0.05$) from α4(E180Q)β2 using Dunnett's test.

ted the α4(E180Q:+L264)β2, α4(E180Q)β2(V262M), and α4(E180Q)β2(V262L) double-mutant receptors more than the α4(E180Q)β2 receptor. Adding 2 mM Ca^{2+} to the extracellular solution increased the α4(+L264)β2 1 mM acetylcholine response by only 10 ± 2%, it increased the α4β2(V262M) response by only 40 ± 4%, and it actually reduced the α4β2(V262L) response by 20 ± 4% (Fig. 3G and Table 1). Thus, similar to α4(S252F) and α4(S256L) mutations, the α4(+L264), β2(V262M), and β2(V262L) mutations reduced 2 mM Ca^{2+} potentiation of the acetylcholine response by 55 to 75% compared with the wild-type. However, in contrast to the α4(S252F) and α4(S256L) mutations, adding 2 mM Ca^{2+} reduced the α4(E180Q:+L264)β2, α4(E180Q)β2(V262M), and α4(E180Q)β2(V262L) double-mutant 1 mM acetylcholine responses by 30 to 50% (Fig. 3, B, D, F, and H; Table 1). Thus, Ca^{2+} inhibited the α4(E180Q:+L264)β2, α4(E180Q)-β2(V262M), and α4(E180Q)β2(V262L) responses significantly ($P < 0.05$) more than the α4(E180Q)β2 response (Fig. 3H; Table

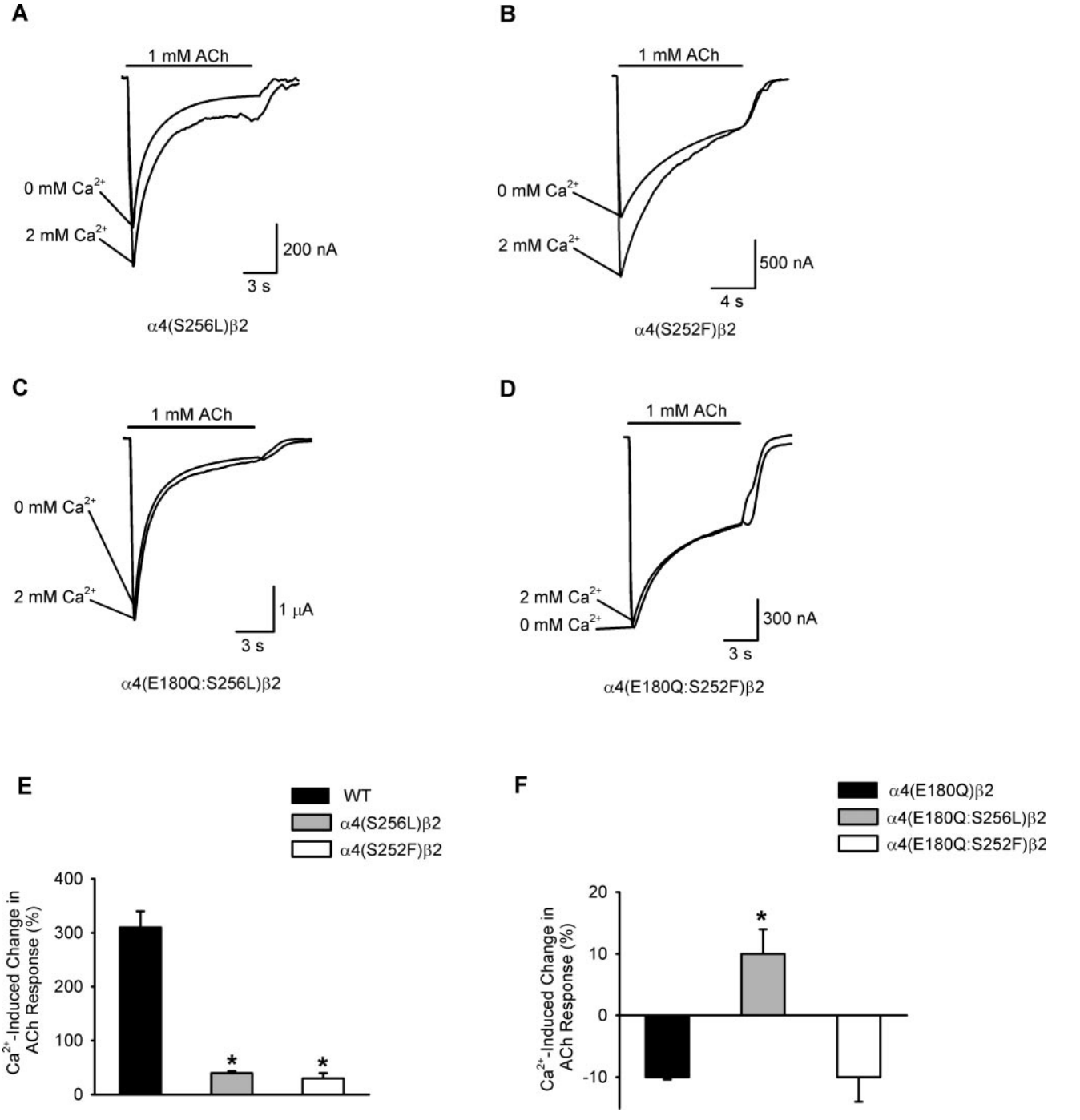

Fig. 2. Combining the α4(S252F) or α4(S256L) mutation with the α4(E180Q) mutation does not enhance 2 mM Ca^{2+} inhibition of the α4(E180Q)β2 acetylcholine response. A–D, voltage-clamped 1 mM acetylcholine responses of the α4(S256L)β2, α4(S252F)β2, α4(E180Q:S256L)β2, and α4(E180Q:S252F)β2 receptors in 0 and 2 mM added extracellular Ca^{2+}. V = −50 mV. E and F, the bars show the Ca^{2+}-induced changes in the peak wild-type, α4(S256L)β2, α4(S252F)β2, α4(E180Q)β2, α4(E180Q:S256L)β2, and α4(E180Q:S252F)β2 responses. The error bars are ± S.E.M. (n = 4–8 oocytes). Asterisks denote significant ($P < 0.05$) differences from the wild-type (E) or α4(E180Q)β2 receptors (F).

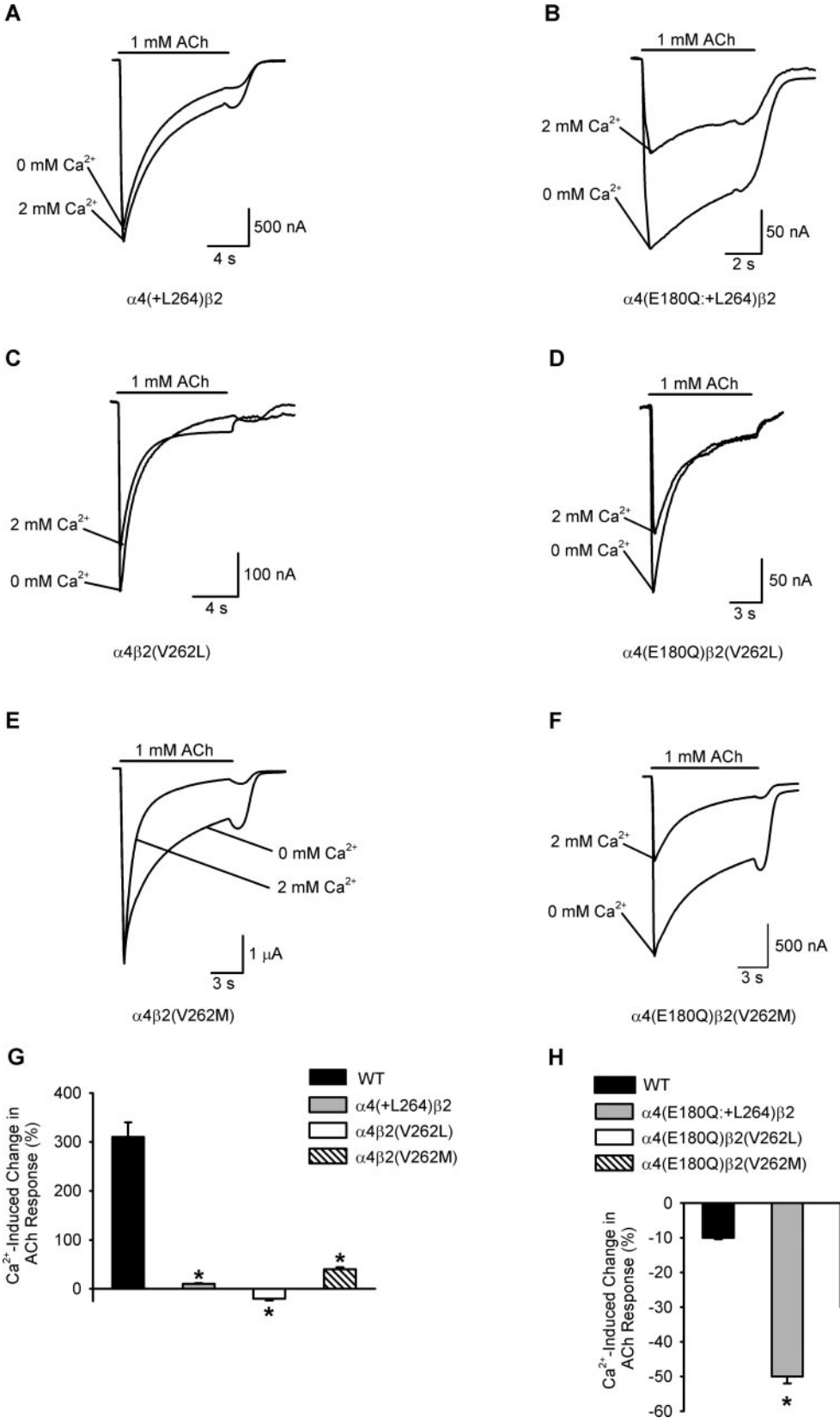

Fig. 3. Combining the α4(+L264), β2(V262L), or β2(V262M) mutation with the α4(E180Q) mutation does enhance 2 mM Ca^{2+} inhibition of the α4(E180Q)β2 acetylcholine response. A–F, voltage-clamped 1 mM acetylcholine responses of the α4(+L264)β2, α4β2(V262L), α4β2(V262M), α4(E180Q:+L264)β2, α4(E180Q)β2(V262L), and α4(E180Q)β2(V262M) receptors in 0 and 2 mM added extracellular Ca^{2+}. V = −50 mV. G and H, the bars show the Ca^{2+}-induced change in the peak wild-type, α4(+L264)β2, α4β2(V262L), α4β2(V262M), α4(E180Q)β2, α4(E180Q:+L264)β2, α4(E180Q)β2(V262L), and α4(E180Q)β2(V262M) responses. The error bars are ± S.E.M. (n = 4–6 oocytes). Asterisks denote significant ($P < 0.05$) differences from the wild-type (G) or α4(E180Q)β2 receptors (H).

1). Nevertheless, Ca^{2+} inhibition of the double-mutant responses was less than that predicted from the corresponding single-mutant reductions in Ca^{2+} potentiation by a pure blocking mechanism (Fig. 4).

The α4(+L264) and β2(V262L) mutations did not significantly affect the peak amplitude of 1 mM acetylcholine response in 0 mM Ca^{2+} ($P > 0.05$). However, the β2(V262M) mutation significantly ($P < 0.05$) increased it. The peak amplitudes of the α4β2(V262M), α4(+L264)β2, and α4β2(V262L) 1 mM acetylcholine responses in 0 mM Ca^{2+} were 4000 ± 2000 ($n = 5$), 3000 ± 2000 ($n = 4$), and 500 ± 240 nA ($n = 4$), respectively. Thus, the α4β2, α4(+L264), β2(V262L), and (V262M) mutations did not reduce the amplitude of the 1 mM acetylcholine response in 0 mM Ca^{2+}.

Altered Allosteric Ca^{2+} Activation Explains the Effects of the ADNFLE Mutations on the Ca^{2+} Concentration-Potentiation Relation Better than Enhanced Ca^{2+} Block. There are two possible interpretations of the effects of Ca^{2+} on the α4(E180Q:+L264)β2, α4(E180Q)β2(V262M), and α4(E180Q)β2(V262L) acetylcholine responses. First, the α4(+L264), β2(V262M), and β2(V262L) mutations could reduce α4β2 Ca^{2+} potentiation by both enhancing Ca^{2+} block and inhibiting allosteric Ca^{2+} activation of the receptor. Second, the α4(E180Q) mutation could interact with these particular ADNFLE mutations to convert the allosteric Ca^{2+} binding site from a positive to a negative site. To decide between these two alternatives, we compared the effects of two of the ADNFLE mutations on the Ca^{2+} concentration-potentiation relation and asked whether they were better explained by a mutant-induced change in allosteric Ca^{2+} activation of the receptor or enhanced Ca^{2+} block. We chose the α4(S256L) mutation as representative of the ADNFLE mutations closer to the intracellular end of M2 and, the α4(+L264) mutation as representative of those closer to the extracellular end. We used 30 μM acetylcholine for these experiments rather than 1 mM to avoid cumulative receptor desensitization by repeated acetylcholine applications.

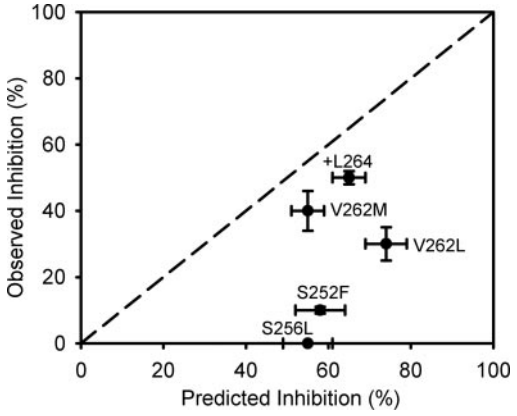

Fig. 4. Ca^{2+} inhibition of the E180Q:ADNFLE double-mutant acetylcholine responses is less than that expected from a noncompetitive Ca^{2+} blocking model. *Y* axis, the percentage inhibition of the peak E180Q:ADNFLE double-mutant 1 mM acetylcholine response by 2 mM added extracellular Ca^{2+} (Observed Inhibition). *X* axis, the percentage by which the corresponding single ADNFLE mutations reduced 2 mM Ca^{2+} potentiation of the wild-type 1 mM acetylcholine response (Predicted Inhibition). The dashed line has a slope of unity and denotes equal observed and predicted inhibition. The horizontal and vertical error bars are ± S.E.M. ($n = 4–8$ oocytes).

Consistent with a small or negligible Ca^{2+} block of the wild-type receptor between 0.1 and 2 mM Ca^{2+}, a simple hyperbolic binding function (eq. 1) fit the wild-type data well ($R^2 = 0.98$; Fig. 5, A and B):

$$\frac{I_{Ca}}{I_0} = 1 + \frac{P_{max} - 1}{1 + \frac{EC_{50}}{[Ca^{2+}]_0}} \quad (1)$$

where the I_{Ca}/I_0 is the ratio of the peak 30 μM acetylcholine response with added extracellular Ca^{2+} to that in 0 mM added Ca^{2+}, the $[Ca^{2+}]_o$ is the added extracellular Ca^{2+} concentration, the EC_{50} is the half-maximal $[Ca^{2+}]_o$ for potentiation of the acetylcholine response, and the P_{max} is the maximum value of the I_{Ca}/I_0. The fitted wild-type P_{max} and EC_{50} were 3.6 ± 0.1 and 0.4 ± 0.1 mM (df = 6), respectively. To determine whether enhanced Ca^{2+} block could account for the effects of the ADNFLE mutations on the Ca^{2+} concentration-potentiation relation, we multiplied the wild-type hyperbolic binding function (eq. 1) by a simple inhibitory binding isotherm and fit this function (eq. 2) to the α4(S256L)β2 and α4(+L264)β2 Ca^{2+} concentration-potentiation relations (Fig. 5, A and B):

$$\frac{I_{Ca}}{I_0} = \left(1 + \frac{2.6}{1 + \frac{0.4\ mM}{[Ca^{2+}]_0}}\right)\left(\frac{1}{1 + \frac{[Ca^{2+}]_0}{IC_{50}}}\right) \quad (2)$$

where the IC_{50} is the half-maximal inhibitory concentration for Ca^{2+} block of the mutant receptor and the expression,

$$\left(1 + \frac{2.6}{1 + \frac{0.4\ mM}{[Ca^{2+}]_0}}\right)$$

describes allosteric Ca^{2+} activation of the receptor (assuming that it is unaffected by the mutations). This function fit the α4(S256L)β2 and α4(+L264)β2 Ca^{2+} concentration-potentiation data poorly ($R^2 = 0$; Fig. 5, A and B) because it predicted a relief from Ca^{2+} block; thus, a relief from the mutant-mediated reductions in Ca^{2+} potentiation as the $[Ca^{2+}]_o$ approached zero. The fitted IC_{50} values for Ca^{2+} block of the α4(S256L)β2 and α4(+L264)β2 receptors were 0.8 ± 0.3 mM (df = 4) and 1.27 ± 0.03 mM (df = 4), respectively. Thus, enhanced Ca^{2+} block cannot account for the effects of the α4(S256L) and α4(+L264) mutations on the α4β2 Ca^{2+} concentration-potentiation relation.

A model (eq. 3, below) assuming that the mutations altered allosteric Ca^{2+} activation of receptor fit the data better (Fig. 5, C and D),

$$\frac{I_{Ca}}{I_0} = \frac{1 + \frac{[Ca^{2+}]_0}{K_{Ca}}}{1 + \frac{[Ca^{2+}]_0}{K_{Ca}P_{max}}} \quad (3)$$

where the K_{Ca} is the equilibrium constant for Ca^{2+} binding to the open receptor state (see the online Supplement for the derivation of eq. 3). Because the parameters K_{Ca} and P_{max} were highly covariant, it was appropriate to fix the P_{max} beforehand and to let only the K_{Ca} vary during the fit. To fit eq. 3 to the wild-type data, we fixed the P_{max} at 3.6 (estimated from fitting eq. 1 to the wild-type data). With this

constraint, eq. 3 fit the wild-type data as well as eq. 1 (R^2 = 0.98; Fig. 5, C and D) and yielded a K_{Ca} of 108 ± 3 μM (df = 7). Visual inspection of the α4(S256L)β2 and α4(+L264)β2 data indicated that both mutations reduced the P_{max} for Ca^{2+} potentation (Fig. 5, C and D). Fixing the P_{max} at 1.6 gave reasonable fits to the α4(S256L)β2 and α4(+L264)β2 data (R^2 = 0.75, 0.56, respectively; Fig. 5, C and D) and yielded K_{Ca} values of 1.2 ± 0.4 mM and 200 ± 100 μM (df = 4), respectively. Thus, a mutant-induced change in allosteric Ca^{2+} activation accounts for the effects of ADNFLE mutations on the Ca^{2+} concentration-potentiation relation better than enhanced Ca^{2+} block. Therefore, an interaction between the ADNFLE mutations closer to the extracellular end of M2 and the α4(E180Q) mutation that creates a negative Ca^{2+} allosteric site may account for their more complex effects.

The ADNFLE Mutations Enhance Steady-State Desensitization in 2 mM Ca^{2+}. In the absence of any added extracellular Ca^{2+}, previous results show that the five ADNFLE mutations we studied do not consistently affect acetylcholine-induced desensitization (Figl et al., 1998; Rodrigues-Pinguet et al., 2003). However, we only tested the effects of three of the mutations—α4(S256L), β2(V262L), and β2(V262L)—in 2 mM Ca^{2+} previously. In 2 mM Ca^{2+}, these three mutations increase steady-state desensitization of the 30 μM acetylcholine response (Rodrigues-Pinguet et al., 2003). The effects of the α4(S252F) and α4(+L264) mutations on desensitization in 2 mM Ca^{2+} have not previously been reported. To determine whether all five mutations affect desensitization in 2 mM Ca^{2+}, we analyzed desensitization of the 1 mM acetylcholine responses obtained in the Ca^{2+} potentiation experiments above. The desensitizing phase of these responses primarily reflects the fast component of desensitization because we only applied acetylcholine for 5 to 16 s to minimize cumulative receptor desensitization by repeated agonist applications. The time course of desensitization of these responses was adequately fit by the sum of a

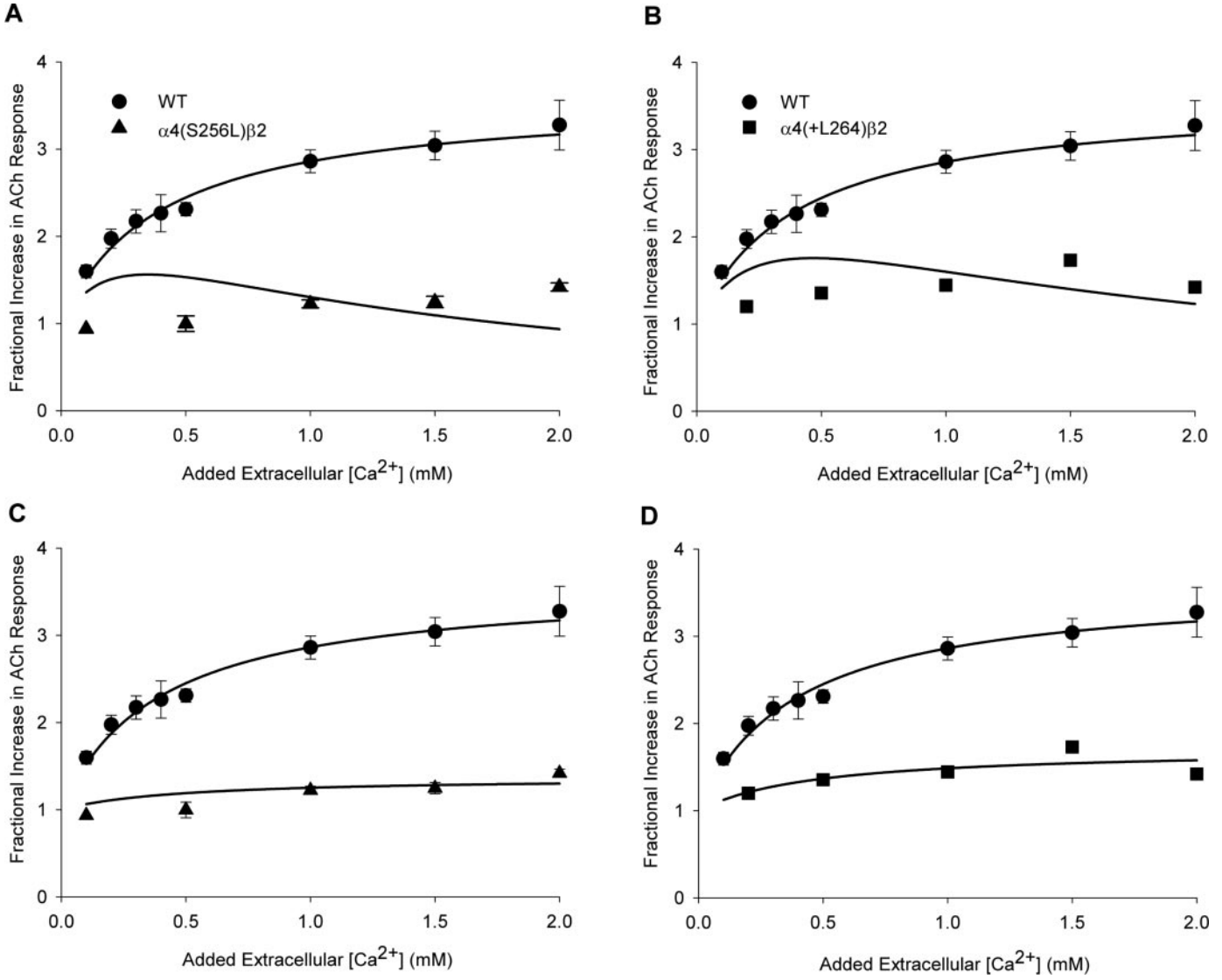

Fig. 5. Altered allosteric Ca^{2+} activation accounts for the effects of the α4(S256L) and α4(+L264) mutations on the Ca^{2+} concentration-potentiation relation better than enhanced Ca^{2+} block. A and B, fits of the wild-type Ca^{2+} concentration-potentiation data to a hyperbolic binding function (eq. 1 under *Results*) and the α4(S256L)β2 and α4(+L264)β2 Ca^{2+} concentration-potentiation data to the product of this hyperbolic function and a Ca^{2+} inhibitory isotherm (eq. 2 under *Results*). C and D, fits of the wild-type, α4(S256L)β2, and α4(+L264)β2 data to an allosteric Ca^{2+} activation model (eq. 3 under *Results*). Eqs. 1 to 3 were fit to the data using the nonlinear least-squares regression routine implemented in SigmaPlot version 8 (SPSS, Inc.). See the text for the fitted parameter values. The error bars are ± S.E.M. (n = 3–8 oocytes). V = −50 mV.

single exponential component and a constant term (Fig. 6, A and B). As in previous studies (Figl et al., 1998; Rodrigues-Pinguet et al., 2003), the ADNFLE mutations did not consistently affect the *SSD* or τ_D of desensitization (see *Materials and Methods*) in 0 mM Ca^{2+}. However, all five mutations significantly ($P < 0.05$) increased the *SSD* in 2 mM Ca^{2+} (Fig. 6C; Table 2). In 2 mM Ca^{2+}, the *SSD* of the mutant receptors was 38 to 77% larger than that of the wild-type. Thus, the ADNFLE mutations increased steady-state desensitization in 2 mM Ca^{2+}. The mutations did not consistently affect the τ_D in 0 or 2 mM Ca^{2+} (Fig. 6D; Table 2).

Consistent with previous results (Rodrigues-Pinguet et al., 2003), Ca^{2+} did not affect the *SSD* of the wild-type 1 mM acetylcholine response. However, it significantly ($P < 0.05$) reduced the τ_D by 51% (Fig. 6, C and D; Table 2). Thus, Ca^{2+} increased the speed, but not the depth, of fast wild-type desensitization. It is interesting, the α4(E180Q) mutation eliminated this effect (Table 2).

Combining the α4(E180Q) and ADNFLE mutations only significantly affected desensitization of the α4β2(V262M) and α4(+L264)β2 responses (Table 2). Combination with the α4(E180Q) mutation significantly ($P < 0.05$) reduced the *SSD* of the α4β2(V262M) response in 0 and 2 mM Ca^{2+} and brought the mutant *SSD*s closer to the wild-type values (Table 2). It also significantly ($P < 0.05$) increased the τ_D in 2 mM Ca^{2+} but it did not eliminate the effects of Ca^{2+} on the mutant τ_D (Table 2). Thus, the α4(E180Q) mutation reduced steady-state desensitization of the α4β2(V262M) response in both 0 and 2 mM Ca^{2+} and, reduced the effects of Ca^{2+} on its time course of desensitization. Combining the α4(E180Q) with the α4(+L264) mutation significantly ($P < 0.05$) reduced the α4(+L264)β2 *SSD* in 0 mM Ca^{2+} but, did not significantly affect the *SSD* in 2 mM Ca^{2+} or, the τ_D in 0 or 2 mM Ca^{2+} (Table 2). Thus, the α4(E180Q) mutation did not consistently affect desensitization of the ADNFLE mutant responses.

The β2(V262L)-Induced Reduction in Ca^{2+} Potentiation Is Voltage-Independent. Another way to test the hypothesis that ADNFLE mutations near the extracellular end of M2 enhance Ca^{2+} block of the α4β2 channel is to examine

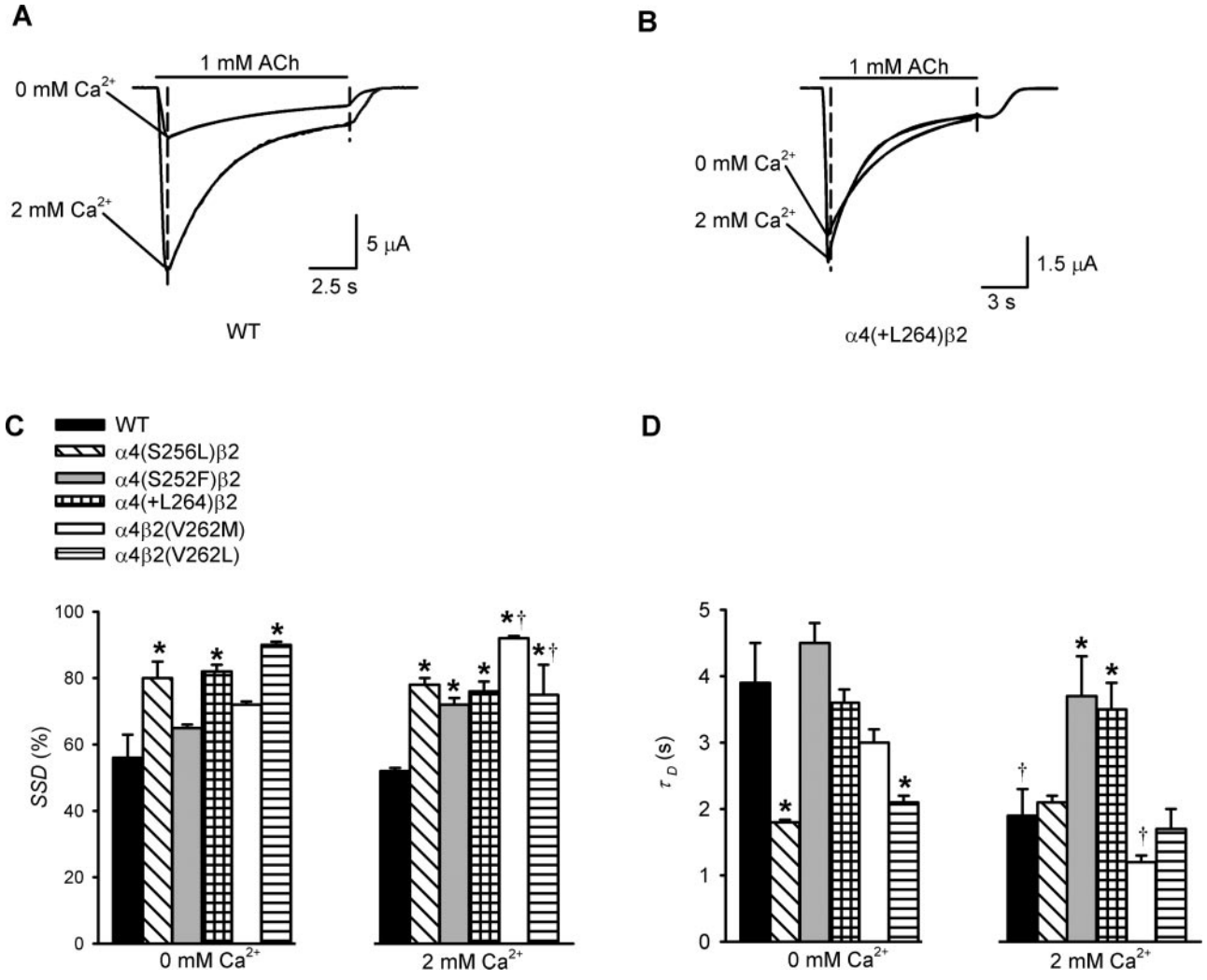

Fig. 6. The ADNFLE mutations enhance the *SSD* of the acetylcholine response in 2 mM Ca^{2+}. A and B, fits of the desensitizing phases of the wild-type (A) and α4(+L264)β2 (B) 1 mM acetylcholine responses in 0 and 2 mM Ca^{2+} to the sum of an exponential component and a constant term. The vertical dashed lines show the extent of the fitted region. The fits are superimposed on the traces over this region. C and D, the bars show the *SSD*s (C) and desensitization time constants (τ_D) (D) of the wild-type and ADNFLE mutant 1 mM acetylcholine responses in 0 and 2 mM Ca^{2+}. The error bars are ± S.E.M. ($n = 4–8$ oocytes). Asterisks denote a significant ($P < 0.05$) difference from the wild-type receptor. The crosses denote a significant effect of Ca^{2+} on the *SSD* (C) or τ_D (D).

the voltage dependence of their effects on Ca^{2+} potentiation. If the mutations enhance Ca^{2+} block at a site within the membrane electric field, then their effects on Ca^{2+} potentiation should be voltage-dependent. To measure the effects of the β2(V262L) mutation on Ca^{2+} potentiation at positive (+30 mV) and negative (−50 mV) membrane potentials, we used a second mutation—α4(E245Q)—expected to relieve inward rectification of the $\alpha4\beta2$ acetylcholine response. Severe inward rectification of the wild-type acetylcholine response precludes accurate measurements of Ca^{2+} potentiation at positive membrane potentials. To overcome this limitation, we used the α4(E245Q) mutation. Similar to the previously reported α4(E245A) mutation (Haghighi and Cooper, 2000), the α4(E245Q) mutation relieved inward rectification of the $\alpha4\beta2$ acetylcholine-induced current-voltage relation ($n = 5$; Fig. 7A), allowing us to easily measure responses at positive potentials. It is interesting that the α4(E245Q) mutation also significantly reduced 2 mM Ca^{2+} potentiation of the $\alpha4\beta2$ 1 mM acetylcholine response at −50 mV ($P < 0.05$; Fig. 7B), but its effects were less severe than the ADNFLE mutations (Table 1). Ca^{2+} increased the α4(E245Q)$\beta2$ 1 mM acetylcholine response by 90 ± 30% ($n = 5$) at −50 mV (Fig. 7B) and by 80 ± 20% ($n = 10$) at +30 mV (Fig. 7, D and F). Thus, consistent with previous results for native neuronal nicotinic receptors (Amador and Dani, 1995), Ca^{2+} potentiation of the α4(E245Q)$\beta2$ acetylcholine response was voltage-independent. Coexpression of the α4(E245Q) and β2(V262L) mutations yielded a double-mutant receptor that responded robustly to 1 mM acetylcholine at both negative and positive membrane potentials (Fig. 7, C, E, and F). If the β2(V262L) mutation reduces Ca^{2+} potentiation by enhancing Ca^{2+} block at a site within the membrane electric field, then positive membrane potentials should relieve the block and thus relieve the β2(V262L)-mediated reduction in α4(E245Q)$\beta2$ Ca^{2+} potentiation. However, the Ca^{2+}-induced change in the α4(E245Q)β2(V262L) acetylcholine response at −50 mV (−50 ± 10%, $n = 5$) was not significantly ($P > 0.05$) different from that at +30 mV (−30 ± 10%, $n = 13$) (Fig. 7, C, E, and F), consistent with previous results showing that the α4(S252F) and α4(+L264) ADNFLE mutations similarly affect Ca^{2+} potentiation at membrane potentials of −100 and −50 mV (Figl et al., 1998). Thus, enhanced Ca^{2+} block of the α4(E245Q)β2(V262L) receptor at a site inside the membrane electric field cannot account for the reduced Ca^{2+} potentiation of this receptor.

TABLE 2

Percent steady-state desensitization (*SSD*) and desensitization time constant (τ_D) of the 1 mM acetylcholine responses in 0 and 2 mM Ca^{2+}. The *SSD* and τ_D values are presented as means ± S.E.M.

Receptor	Number of Oocytes	$[Ca^{2+}]$	*SSD*	τ_D
		mM	%	*s*
$\alpha4\beta2$	4	0	56 ± 7	3.9 ± 0.6
α4(S256L)$\beta2$	4	0	80 ± 5*	1.80 ± 0.04*
α4(S252F)$\beta2$	4	0	65 ± 1	4.5 ± 0.3
α4(+L264)$\beta2$	4	0	82 ± 2*	3.6 ± 0.2
$\alpha4\beta2$(V262M)	4	0	72 ± 1	3.0 ± 0.2
$\alpha4\beta2$(V262L)	4	0	90 ± 1*	2.1 ± 0.1*
α4(E180Q)$\beta2$	4	0	66 ± 3	3.1 ± 0.4
α4(E180Q:S256L)$\beta2$	5	0	83 ± 1*‡	1.6 ± 0.1*‡
α4(E180Q:S252F)$\beta2$	8	0	61 ± 3	3.0 ± 0.2
α4(E180Q:+L264)$\beta2$	4	0	49 ± 2§	4.4 ± 0.3
α4(E180Q)β2(V262M)	8	0	62 ± 4§	3.6 ± 0.2
α4(E180Q)β2(V262L)	5	0	79 ± 5*	2.1 ± 0.1*‡
$\alpha4\beta2$	4	2	52 ± 1	1.9 ± 0.4†
α4(S256L)$\beta2$	4	2	78 ± 2*	2.1 ± 0.1
α4(S252F)$\beta2$	4	2	72 ± 2*	3.7 ± 0.6*
α4(+L264)$\beta2$	4	2	76 ± 3*	3.5 ± 0.4*
$\alpha4\beta2$(V262M)	4	2	92 ± 0.7*†	1.2 ± 0.1†
$\alpha4\beta2$(V262L)	4	2	75 ± 9*†	1.7 ± 0.3
α4(E180Q)$\beta2$	4	2	68 ± 2	2.8 ± 0.1
α4(E180Q:S256L)$\beta2$	5	2	85 ± 1*‡	1.6 ± 0.1‡
α4(E180Q:S252F)$\beta2$	7	2	60 ± 2	3.0 ± 0.1
α4(E180Q:+L264)$\beta2$	4	2	58 ± 10	3.9 ± 1.3†
α4(E180Q)β2(V262M)	8	2	67 ± 3§	2.8 ± 0.1†§
α4(E180Q)β2(V262L)	5	2	76 ± 3*	2.0 ± 0.2

* Significantly ($P < 0.05$) different from the wild-type.
† Significantly ($P < 0.05$) different from the 0 mM Ca^{2+} value.
‡ Significantly ($P < 0.05$) different from the α4(E180Q)$\beta2$ receptor.
§ Significantly ($P < 0.05$) different from the corresponding single ADNFLE mutation.

Eliminating Negative Charges in the Extracellular Ring Does Not Prevent the β2(V262L) Mutation from Reducing Ca^{2+} Potentiation. A ring of negatively charged residues located at the extracellular entrance to the channel pore (the extracellular ring) mediates external block of the muscle nicotinic receptor by the divalent cation Mg^{2+} (Imoto et al., 1988). The aligning residues in the rat $\alpha4\beta2$ nicotinic receptor are a negatively charged Glu in the α4 subunit—α4(E266)—(Goldman et al., 1987) and a positively charged Lys in the β2 subunit—β2(K260)—(Deneris et al., 1988). Because α4(E266) lies near the extracellular entrance to the channel pore, enhanced Ca^{2+} binding to this negatively charged residue could potentially mediate a voltage-independent Ca^{2+} block of the ADNFLE mutant receptors. To test this hypothesis, we mutated the Glu at position 266 in the α4 subunit to an uncharged Gln [α4(E266Q)], coexpressed this mutation with either the wild-type β2 or β2(V262L) subunit, and compared the effects of 2 mM Ca^{2+} on the α4(E266Q)$\beta2$ and α4(E266Q)β2(V262L) 1 mM acetylcholine responses. Similar to its effects on the wild-type receptor, 2 mM Ca^{2+} increased the α4(E266Q)$\beta2$ 1 mM acetylcholine response by 300 ± 10% ($n = 4$) (Fig. 8, A and C; Table 1). Hence, not all the $\alpha4\beta2$ mutations in and near M2 significantly reduce Ca^{2+} potentiation. If enhanced Ca^{2+} block at the extracellular ring mediates the effects of the ADNFLE mutations on Ca^{2+} potentiation, then we would expect the α4(E266Q) mutation to relieve the β2(V262L)-mediated reduction in Ca^{2+} potentiation. However, coexpression of the β2(V262L) and α4(E266Q) mutations did not relieve the β2(V262L)-mediated reduction in Ca^{2+} potentiation (Fig. 8, B and C; Table 1). Similar to the $\alpha4\beta2$(V262L) single-mutant receptor, 2 mM Ca^{2+} reduced the α4(E266Q)β2(V262L) double-mutant 1 mM acetylcholine response by 40 ± 2% ($n = 4$) (Table 1). Thus, the ADNFLE mutations do not seem to reduce Ca^{2+} potentiation by enhancing Ca^{2+} block at the $\alpha4\beta2$ extracellular ring.

Ca^{2+} Does Not Reduce the Mutant Single-Channel Currents More Than the Wild-Type Currents. The ADNFLE mutations reduce 2 mM Ca^{2+} potentiation of the 1 mM acetylcholine response by 55 to 74% (Table 1). If uncompetitive or noncompetitive Ca^{2+} inhibition of the mutant receptors mediates this effect, then the expected K_i for Ca^{2+} binding to its inhibitory site on the mutant receptors is ≥700 μM. If diffusion limits Ca^{2+} binding to this inhibitory site (i.e., a forward rate constant for Ca^{2+} binding of 10^{-8} ms^{-1}), then Ca^{2+} remains bound to this site for ≤14 μs (Hille, 2001). This brief residency time implies that if the ADNFLE mutations reduce Ca^{2+} potentiation by enhancing Ca^{2+} block of the receptor, then Ca^{2+} is expected to reduce the apparent

single-channel conductance (g_s) of the mutant receptors by 55 to 74%. To test this hypothesis, we measured the amplitudes of wild-type and mutant single-channel currents (i_s) in cell-attached patches at a pipette potential of +100 mV with and without 2 mM added Ca^{2+} in the pipette-filling solution (Fig. 9, A–J; Table 3). Consistent with previous reports (Charnet et al., 1992; Kuryatov et al., 1997; Figl et al., 1998), the rat α4β2 wild-type channels displayed multiple conductance states in 0 mM added Ca^{2+} (Fig. 9, A–D; Table 3). At a driving potential of −100 mV (pipette potential of + 100 mV), the i_s of the smaller conductance state (2.6 ± 0.1 pA, n = 5 patches) was significantly ($P < 0.01$) less than that of the larger state (4.4 ± 0.1 pA, n = 5) (Table 3). The internal and external K^+ concentrations for the oocytes in these experiments were nearly equal (assuming an internal K^+ concentration of ~100 mM). Thus, the oocyte resting potential and α4β2 reversal potential was near 0 mV, and the calculated g_s values for the two wild-type conductance states were 26 ± 1 and 44 ± 1 pS. These conductances closely matched those previously reported (34 ± 2 and 49 ± 1 pS) for rat α4β2 channels in outside-out patches in symmetrical 100 mM K^+ (Figl et al., 1998). In contrast, the α4(+L264)β2 channels displayed a single conductance state with an i_s of 1.8 ± 0.1 pA (n = 6) in 0 mM Ca^{2+} (Fig. 9, I and J; Table 3). The corresponding g_s (18 ± 1 pS) was similar to the value previously reported (14.7 ± 1 pS) for the small-conductance rat α4(+L264)β2 channels in outside-out patches with symmetrical 100 mM K^+ (Figl et al., 1998). Similar to the wild-type channels, the α4β2(V262L) channels also displayed two conductance states in 0 mM added Ca^{2+}. The i_s values associated with these two states at −100 mV (2.2 ± 0.1 pA, n = 4 and 3.3 ± 2 pA, n = 5) were significantly different ($P < 0.01$; Fig. 9, E–H; Table 3).

Previous results show that 10 to 20 mM added extracellular Ca^{2+} reduces the conductance of neuronal nicotinic channels (Vernino et al., 1992; Amador and Dani, 1995; Buisson et al., 1996). Adding 2 mM Ca^{2+} to the pipette-filling solution reduced the i_s values for the two wild-type states by a small, but significant ($P < 0.01$), amount (Fig. 9, A–D; Table 3). It reduced the small i_s by 19 ± 5% and the large wild-type i_s by 9 ± 2%. The g_s for the large wild-type conductance state in 2 mM Ca^{2+} (40 ± 4 pS, n = 6) (Table 3) was similar to that reported previously (~46 pS) for the predominant conductance state of human α4β2 nicotinic receptors expressed in human embryonic kidney cells and measured in outside-out patches using 120 mM external Na^+, 2 mM external Ca^{2+}, and 120 mM internal K^+ (Buisson et al., 1996). The addition of 2 mM Ca^{2+} also slightly reduced the i_s values for the

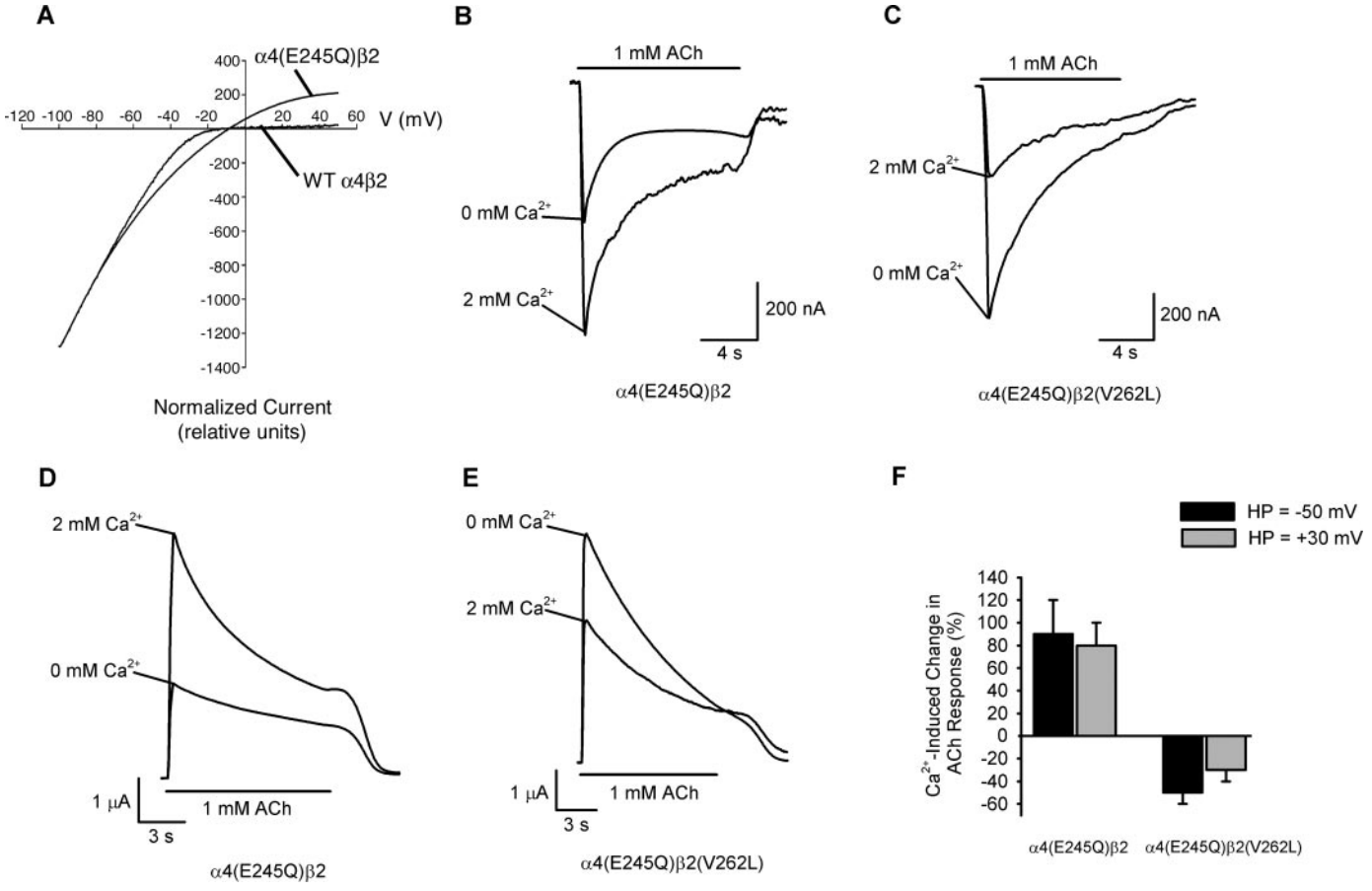

Fig. 7. The β2(V262L) mutation reduces Ca^{2+} potentiation of the α4(E245Q)β2 response almost equally well at negative (−50 mV) and positive (+30 mV) membrane potentials. A, acetylcholine-induced current-voltage relations for the wild-type and α4(E245Q)β2 receptors in 0 mM added Ca^{2+}, generated by ramping the membrane potential from −120 to +50 mV. To facilitate comparison, the α4(E245Q)β2 ACh-induced current was scaled up to match the value of the wild-type current at −120 mV. The wild-type and mutant current-voltage relations are from individual oocytes and represent typical results for five oocytes in each case. [acetylcholine] = 1 μM. B and C, voltage-clamped 1 mM ACh responses of the α4(E245Q)β2 and α4(E245Q)β2(V262L) receptors in 0 and 2 mM added extracellular Ca^{2+} at −50 mV. D and E, records at +30 mV. F, the bars show the Ca^{2+}-induced changes in the α4(E245Q)β2 and α4(E145Q)β2(V262L) responses at −50 and +30 mV. The error bars are ± S.E.M. (n = 5–13 oocytes).

mutant conductance states (Fig. 9, E–J; Table 3), but the reductions were not significant ($P > 0.05$). Thus, the $\alpha4(+L264)$ and $\beta2(V262L)$ mutations do not enhance Ca^{2+}-induced reductions in the $\alpha4\beta2$ single-channel current.

Discussion

Less effective Ca^{2+} potentiation of the acetylcholine response is a common feature of all the ADNFLE mutations tested so far. Consistent with previous results for the 30 μM acetylcholine response (Steinlein et al., 1997; Figl et al., 1998; Rodrigues-Pinguet et al., 2003), rat orthologs of five of the six reported ADNFLE mutations reduce 2 mM Ca^{2+} potentiation of the 1 mM acetylcholine response by 55 to 74%. The effect of the sixth ADNFLE mutation—$\alpha4(T265I)$—on Ca^{2+} potentiation has not been reported (Leniger et al., 2003). Contrary to previous suggestions (Rodrigues-Pinguet et al., 2003), a change in allosteric Ca^{2+} activation, rather than enhanced Ca^{2+} block, seems to be responsible for the ADNFLE mutant-induced reductions in Ca^{2+} potentiation. Several lines of evidence support this conclusion. Ca^{2+} at a concentration of 2 mM does not inhibit the $\alpha4(E180Q:S252F)\beta2$ and $\alpha4(E180Q:S256L)\beta2$ responses any more than it inhibits the $\alpha4(E180Q)\beta2$ response. The effects of the $\alpha4(S256L)$ and $\alpha4(+L264)$ mutations on the Ca^{2+} concentration-potentiation relation are more consistent with altered allosteric Ca^{2+} activation than enhanced Ca^{2+} block. The effects of the $\beta2(V262L)$ mutation on $\alpha4(E245Q)\beta2$ Ca^{2+} potentiation are voltage-independent. The $\alpha4(E266Q)$ mutation also fails to relieve the $\beta2(V262L)$-mediated reduction in Ca^{2+} potentiation. Finally, 2 mM Ca^{2+} does not significantly reduce the $\alpha4(+L264)\beta2$ and $\alpha4\beta2(V262L)$ single-channel conductances.

It is interesting that the five ADNFLE mutations we tested also increased the apparent steady-state desensitization of the 1 mM acetylcholine response in 2 mM Ca^{2+}. Because our acetylcholine applications lasted only 5 to 16 s, this increase could reflect either a real increase in steady-state desensitization or a decrease in the relative amplitude of the slow component of desensitization. Regardless of its origin, this effect does not seem to be a common feature of the mutations because the sixth reported ADNFLE mutation—$\alpha4(T265I)$—does not seem to affect desensitization of the acetylcholine response in Ca^{2+} (Leniger et al., 2003).

The effects of Ca^{2+} on the $\alpha4(E180Q:+L264)\beta2$, $\alpha4(E180Q)\beta2(V262M)$, and $\alpha4(E180Q)\beta2(V262L)$ double-mutant responses allow two possible interpretations. The $\alpha4(+L264)$, $\beta2(V262M)$, and $\beta2(V262L)$ mutations could reduce Ca^{2+} potentiation of the acetylcholine response by a mixed mechanism involving both altered allosteric Ca^{2+} activation and enhanced Ca^{2+} block. On the other hand, these mutations could interact with the $\alpha4(E180Q)$ mutation to change the allosteric Ca^{2+} site from a positive to a negative one. The effects of the $\alpha4(+L264)$ mutation on the Ca^{2+} concentration-potentiation relation, and the failure of 2 mM Ca^{2+} to reduce the $\alpha4(+L264)\beta2$ and $\alpha4\beta2(V262L)$ single-channel conductance more than the wild-type, support the latter interpretation. The voltage independence of the $\beta2(V262L)$-mediated reduction in $\alpha4(E245Q)\beta2$ Ca^{2+} potentiation also shows that the $\beta2(V262L)$ mutation does not

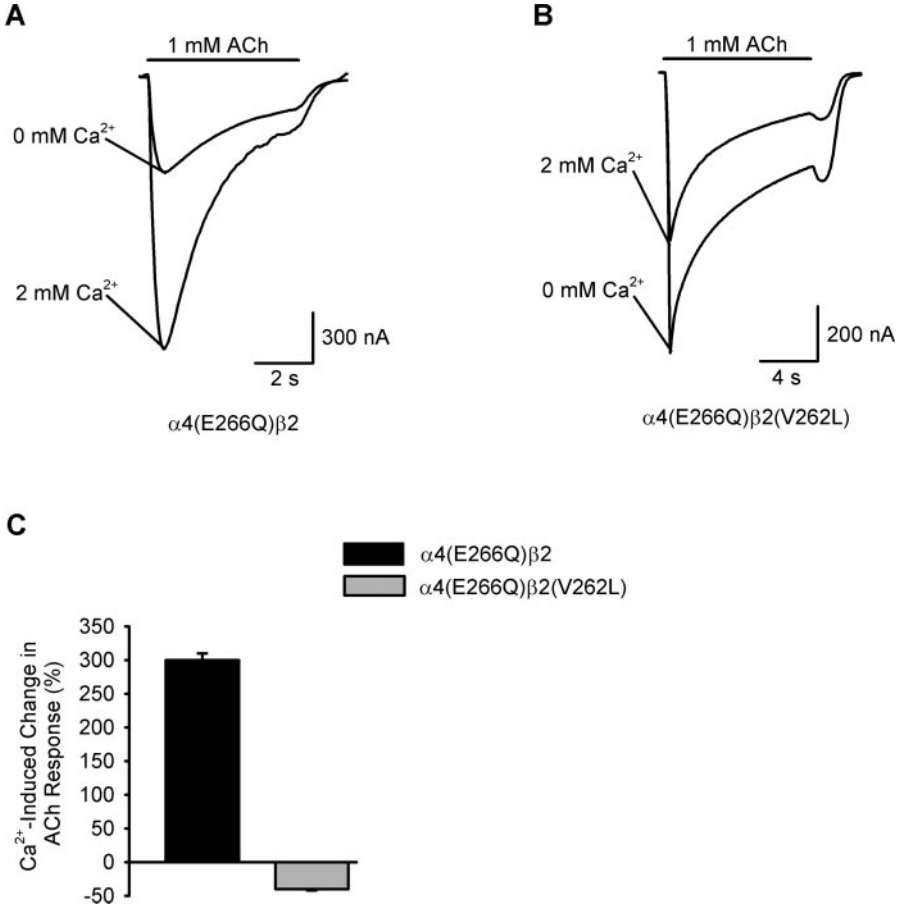

Fig. 8. The $\alpha4(E266Q)$ mutation eliminates the fixed negative charge in the extracellular ring of the $\alpha4\beta2$ receptor but does not relieve the effects of the $\beta2(V262L)$ mutation on 2 mM Ca^{2+} potentiation. A and B, voltage-clamped 1 mM acetylcholine responses of the $\alpha4(E266Q)\beta2$ and $\alpha4(E266Q)\beta2(V262L)$ receptors in 0 and 2 mM added extracellular Ca^{2+}. V = −50 mV. C, The bars show the Ca^{2+}-induced changes in the $\alpha4(E266Q)\beta2$ and $\alpha4(E266Q)\beta2(V262L)$ responses. The error bars are ± S.E.M. (n = 4 oocytes).

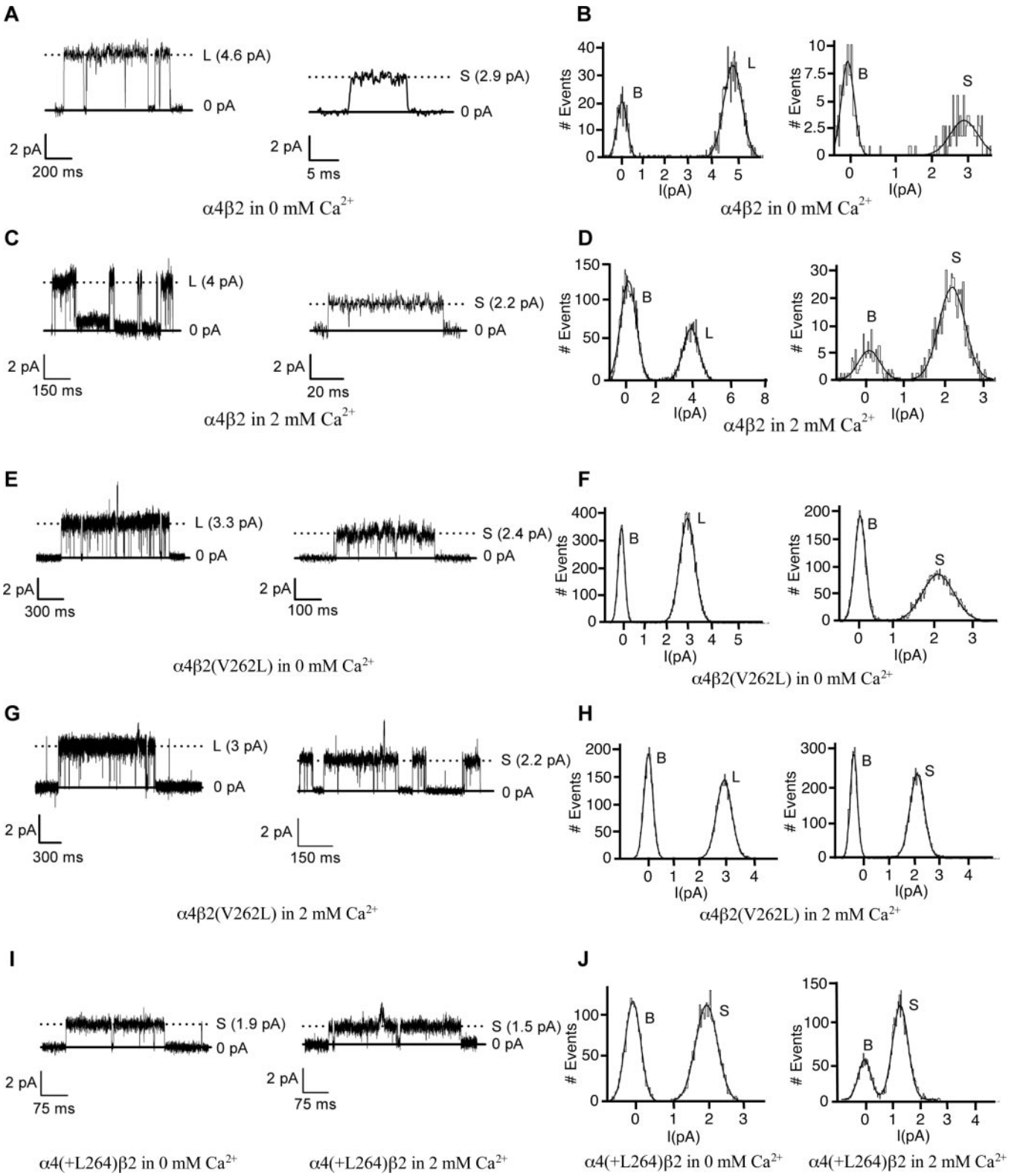

Fig. 9. Adding 2 mM Ca^{2+} to the pipette-filling solution (98 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4) significantly ($P < 0.01$) reduces the wild-type, but not the α4(+L264)β2 and α4β2(V262L), single-channel current in cell-attached patches. A, large (L) and small (S) wild-type single-channel currents in 0 mM added extracellular Ca^{2+}. The solid line is the mean baseline (closed-channel) current (0 pA). The dotted lines show the mean current amplitudes of the large and small wild-type openings. The values of the mean amplitudes are given in parentheses. B, all-points amplitude histograms for the single-channel currents in A. The ordinate is the number of points (# Events). The abscissa is the current (*I*) in picoamperes. The smooth lines are fits to the sum of two Gaussian components. Letters denote the mean baseline (B), large-amplitude (L), and small-amplitude (S) currents. C to D, wild-type single-channel currents in 2 mM added extracellular Ca^{2+} and their amplitude histograms. E and F, the data for the α4β2(V262L) single-channel currents in 0 mM added extracellular Ca^{2+}. G and H, the data for the α4β2(V262L) single-channel currents in 2 mM added extracellular Ca^{2+}. I and J, α4(+L264)β2 single-channel currents in 0 and 2 mM added extracellular Ca^{2+} and their amplitude histograms. The pipette voltage was +100 mV (driving potential of −100 mV).

enhance Ca^{2+} block at a site within the membrane electric field, and the failure of the α4(E266Q) mutation to relieve the β2(V262L)-mediated reduction in Ca^{2+} potentiation shows that the β2(V262L) mutation does not enhance Ca^{2+} block at negatively charged residues in the extracellular ring.

Consistent with previous results (Galzi et al., 1996; Rodrigues-Pinguet et al., 2003), the α4(E180Q) mutation abolishes Ca^{2+} potentiation of the α4β2 1 mM acetylcholine response. The homologous β2 mutation—β2(E177Q)—does not. The results for both mutations agree with a previous model of the α4β2 allosteric Ca^{2+} binding site (Le Novere et al., 2002) in which the Glu at α4(E180), but not that at β2(E177), directly contributes to Ca^{2+} binding. However, if combining the α4(E180Q) mutation with the ADNFLE mutations near the extracellular end of M2 produces a negative allosteric Ca^{2+} binding site, then the α4(E180Q) mutation cannot prevent Ca^{2+} binding to the α4β2 receptor.

Similar to α7 nicotinic receptors (Galzi et al., 1996), 2 mM Ca^{2+} increases the maximum α4β2 acetylcholine response ~3-fold. If we assume that 1) Ca^{2+} does not amplify the neuronal nicotinic acetylcholine response by removing receptor inactivation (Amador and Dani, 1995) and 2) the ADNFLE mutations do not enhance Ca^{2+} block of the receptor, then two mechanisms could account for the effects of the ADNFLE mutations on the Ca^{2+}-induced increase in acetylcholine efficacy. First, the ADNFLE mutations could increase acetylcholine efficacy in the absence of Ca^{2+} (decrease K_2 in Scheme 1; see the online Supplement), thereby reducing the number of available receptors that could be opened by adding extracellular Ca^{2+}. Second, the mutations could reduce the Ca^{2+} affinities of the closed, agonist-bound (K_3^{-1} in Scheme 1; see the online Supplement) and/or open α4β2 receptors (mK_3^{-1} in Scheme 1; see the online Supplement), thereby reducing the ability of Ca^{2+} to increase acetylcholine efficacy. Because the rat α4(S252F) and α4(+L264) ADNFLE mutations do not affect surface antibody binding or increase the maximum (500 μM) acetylcholine response in 0 mM added Ca^{2+} (Figl et al., 1998), these mutations at least do not seem to reduce Ca^{2+} potentiation by increasing acetylcholine efficacy in the absence of Ca^{2+}. Comparable data are not available for the α4(S256L), β2(V262M), and β2(V262L) mutations. However, our results show that adding 2 mM Ca^{2+} reduces the α4β2(V262L) 1 mM acetylcholine response by 20 ± 4%. At most, increasing the acetylcholine efficacy in the absence of Ca^{2+} could eliminate Ca^{2+} potentiation. It could not produce a Ca^{2+}-induced decrease in the maximum acetylcholine response, as observed for the β2(V262L) mutation.

TABLE 3

Single-channel currents (i_s) at a driving potential of −100 mV in 0 and 2 mM Ca^{2+}

The values are mean ± S.E.M. The numbers in parentheses indicate the number of patches.

Receptor	Conductance State	i_s	
		0 mM Ca^{2+}	2 mM Ca^{2+}
		pA	
α4β2	Large	4.4 ± 0.1 (5)	4.00 ± 0.04 (6)*
α4β2	Small	2.6 ± 0.1 (5)†	2.1 ± 0.1 (6)*†
α4(+L264)β2		1.8 ± 0.1 (6)	1.5 ± 0.1 (4)
α4β2(V262L)	Large	3.3 ± 0.2 (5)	3.0 ± 0.1 (6)
α4β2(V262L)	Small	2.2 ± 0.2 (4)†	2.0 ± 0.1 (4)†

* Significantly ($P < 0.01$) different from the 0 mM Ca^{2+} value.
† Significantly ($P < 0.01$) different from the large conductance state.

Thus, this mutation also does not seem to reduce Ca^{2+} potentiation by increasing acetylcholine efficacy in 0 mM added Ca^{2+}. However, we cannot rule out this mechanism for the α4(S256L) and β2(V262M) mutations.

Previous results show that three rat ADNFLE mutations—α4(S256L), β2(V262M), and β2(V262L)—reduce α4β2 Ca^{2+} potentiation in an acetylcholine concentration-dependent manner (Rodrigues-Pinguet et al., 2003). These mutations significantly reduce Ca^{2+} potentiation of the 30 μM and 1 mM acetylcholine responses but not that at lower acetylcholine concentrations (10 or 50 nM). In contrast, the α4(S252F) and α4(+L264) mutations reduce Ca^{2+} potentiation of the 10 and 50 nM and 30 μM acetylcholine responses equally well. Although uncompetitive Ca^{2+} inhibition, at a site independent of the one mediating allosteric Ca^{2+} activation, could explain the acetylcholine concentration dependence of the α4(S256L), β2(V262M), and β2(V262L) effects on Ca^{2+} potentiation, the evidence above argues against this explanation. An alternative explanation is receptor heterogeneity. There are least two distinct α4β2 subtypes with different pharmacological properties and subunit stoichiometries (Zwart and Vijverberg, 1998; Buisson and Bertrand, 2001; Nelson et al., 2003; Zhou et al., 2003). The α4(S256L), β2(V262M), and β2(V262L) mutations may selectively reduce Ca^{2+} potentiation of the low-affinity, but not the high-affinity, α4β2 subtypes. A third possible explanation, at least for the effects of the α4(S256L) and β2(V262M) mutations, is that these mutations reduce Ca^{2+} potentiation by increasing acetylcholine efficacy in the absence of Ca^{2+}. Such an increase would not affect Ca^{2+} potentiation at the foot of the acetylcholine concentration-potentiation relation (where a large fraction of additional receptors are available for opening), but it would reduce Ca^{2+} potentiation at higher acetylcholine concentrations, where a smaller fraction of additional receptors is available for opening.

Mechanism of Seizure Generation. The effects of the ADNFLE mutations on Ca^{2+} potentiation could facilitate seizure generation in two ways. If the mutations do not affect surface receptor expression or acetylcholine efficacy in 0 mM Ca^{2+}, then their effects on Ca^{2+} potentiation should reduce α4β2-mediated inhibitory release in vivo. This loss could facilitate seizure generation by reducing α4β2-mediated lateral inhibition in the cortex (McNamara, 1999). The rat α4(S252F) and α4(+L264) mutations do not affect surface anti-α4 or -β2 antibody binding and do not increase the 500 μM acetylcholine response in the absence of added Ca^{2+} (Figl et al., 1998). Thus, these two mutations could reduce the peak acetylcholine response under physiological Ca^{2+} concentrations. Consistent with this prediction, the human α4(S248F), α4(S252L), and β2(V287M) mutations—orthologous to the rat α4(S252F), α4(S256L), and β2(V262M) mutations—reduce the maximum acetylcholine response in 2.5 mM added extracellular Ca^{2+} by ~50% (Bertrand et al., 1998; Bertrand et al., 2002). However, these three mutations do not reduce the maximum acetylcholine response of mock heterozygous receptors (created by injecting *X. laevis* oocytes with a mixture of wild-type and mutant cRNA) (Bertrand et al., 2002), and ADNFLE patients are heterozygous for the mutant alleles. Cellular mRNA and protein regulation could also affect ADNFLE receptor expression in vivo. Hence, determining whether the ADNFLE mutations reduce the α4β2

acetylcholine response in vivo will ultimately require measurements in knock-in mice.

The ADNFLE mutant-mediated reductions in Ca^{2+} potentiation could also alter the balance between $\alpha4\beta2$-mediated excitatory and inhibitory transmitter release during bouts of synchronized, high-frequency firing in the cortex (Rodrigues-Pinguet et al., 2003). Presynaptic nicotinic receptors facilitate both excitatory and inhibitory neurotransmitter release in the central nervous system (Wonnacott, 1997; Alkondon et al., 2000). Because of the limited extracellular space in the brain, synchronous repetitive firing may selectively deplete Ca^{2+} from the extracellular space around excitatory synapses (Vassilev et al., 1997; Egelman and Montague, 1999; Rusakov and Fine, 2003). Ca^{2+} regulation of the neuronal nicotinic agonist response may act as a negative feedback mechanism to down-regulate nicotinic receptor-mediated excitatory transmitter release during high-frequency synaptic firing (Amador and Dani, 1995). In general, Ca^{2+} depletion at excitatory synapses may protect against $\alpha4\beta2$-initiated seizures by ensuring that $\alpha4\beta2$-mediated inhibitory transmitter dominates $\alpha4\beta2$-mediated excitatory transmitter release during bouts of synchronized, high-frequency cortical firing, such as sleep spindles. Reducing $\alpha4\beta2$ Ca^{2+} potentiation could impair the ability of $\alpha4\beta2$-mediated lateral inhibition to contain the spread of $\alpha4\beta2$-mediated excitation in the cortex and thus allow $\alpha4\beta2$-mediated excitation to initiate a seizure.

References

Alkondon M, Pereira EF, Eisenberg HM, and Albuquerque EX (2000) Nicotinic receptor activation in human cerebral cortical interneurons: a mechanism for inhibition and disinhibition of neuronal networks. *J Neurosci* **20:**66–75.

Amador M and Dani JA (1995) Mechanism for modulation of nicotinic acetylcholine receptors that can influence synaptic transmission. *J Neurosci* **15:**4525–4532.

Bertrand D, Picard F, Le HS, Weiland S, Favre I, Phillips H, Bertrand S, Berkovic SF, Malafosse A, and Mulley J (2002) How mutations in the nicotinic acetylcholine receptor can cause ADNFLE epilepsy. *Epilepsia* **43:**112–122.

Bertrand S, Weiland S, Berkovic SF, Steinlein OK, and Bertrand D (1998) Properties of neuronal nicotinic acetylcholine receptor mutants from humans suffering from autosomal dominant nocturnal frontal lobe epilepsy. *Br J Pharmacol* **125:**751–760.

Buisson B and Bertrand D (2001) Chronic exposure to nicotine upregulates the human $\alpha4\beta2$ nicotinic acetylcholine receptor function. *J Neurosci* **21:**1819–1829.

Buisson B, Gopalakrishnan M, Arneric SP, Sullivan JP, and Bertrand D (1996) Human $\alpha4\beta2$ neuronal nicotinic acetylcholine receptor in HEK 293 cells: A patch-clamp study. *J Neurosci* **16:**7880–7891.

Charnet P, Labarca C, Cohen BN, Davidson N, Lester HA, and Pilar G (1992) Pharmacological and kinetic properties of $\alpha4\beta2$ neuronal nicotinic acetylcholine receptors expressed in *Xenopus* oocytes. *J Physiol* **450:**375–394.

Cohen BN, Figl A, Quick MW, Labarca C, Davidson N, and Lester HA (1995) Region of $\beta2$ and $\beta4$ responsible for differences between the steady-state dose-response relationships of the $\alpha3\beta2$ and $\alpha3\beta4$ neuronal nicotinic receptors. *J Gen Physiol* **105:**745–764.

De Fusco M, Becchetti A, Patrignani A, Annesi G, Gambardella A, Quattrone A, Ballabio A, Wanke E, and Casari G (2000) The nicotinic receptor $\beta2$ subunit is mutant in nocturnal frontal lobe epilepsy. *Nat Genet* **26:**275–276.

Deneris ES, Connolly J, Boulter J, Wada E, Wada K, Swanson LW, Patrick J, and Heinemann S (1988) Primary structure and expression of $\beta2$: a novel subunit of neuronal acetylcholine receptors. *Neuron* **1:**45–54.

Egelman DM and Montague PR (1999) Calcium dynamics in the extracellular space of mammalian neural tissue. *Biophys J* **76:**1856–1867.

Figl A, Viseshakul N, Shafaee N, Forsayeth J, and Cohen BN (1998) Two mutations linked to nocturnal frontal lobe epilepsy cause use-dependent potentiation of the nicotinic acetylcholine response. *J Physiol* **513.3:**655–670.

Galzi JL, Bertrand S, Corringer JP, Changeux JP, and Bertrand D (1996) Identification of calcium binding sites that regulate potentiation of a neuronal nicotinic acetylcholine receptor. *EMBO (Eur Mol Biol Organ) J* **15:**5824–5832.

Goldman D, Deneris E, Luyten W, Kochhar A, Patrick J, and Heinemann S (1987) Member of a nicotinic acetylcholine receptor gene family are expressed in different regions of the mammalian central nervous system. *Cell* **48:**965–973.

Haghighi AP and Cooper E (2000) A molecular link between inward rectification and calcium permeability of neuronal nicotinic acetylcholine $\alpha3\beta4$ and $\alpha4\beta2$ receptors. *J Neurosci* **20:**529–541.

Hamill OP, Marty A, Neher E, Sakmann B, and Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflueg Arch Eur J Physiol* **391:**85–100.

Hille B (2001) *Ion Channels of excitable membranes.* Sinauer Associates, Inc., Sunderland, MA.

Hirose S, Iwata H, Akiyoshi H, Kobayashi K, Ito M, Wada K, Kaneko S, and Mitsudome A (1999) A novel mutation of CHRNA4 responsible for autosomal dominant nocturnal frontal lobe epilepsy. *Neurology* **53:**1749–1753.

Imoto K, Busch C, Sakmann B, Mishina M, Konno T, Nakai J, Bujo H, Mori Y, Fukuda K, and Numa S (1988) Rings of negatively charged amino acids determine the acetylcholine receptor channel conductance. *Nature (Lond)* **335:**645–648.

Kuryatov A, Gerzanich V, Nelson M, Olale F, and Lindstrom J (1997) Mutation causing autosomal dominant nocturnal frontal lobe epilepsy alters Ca^{2+} permeability, conductance and gating of human $\alpha4\beta2$ nicotinic acetylcholine receptors. *J Neurosci* **17:**9035–9047.

Leniger T, Kananura C, Hufnagel A, Bertrand S, Bertrand D, and Steinlein OK (2003) A new CHRNA4 mutation with low penetrance in nocturnal frontal lobe epilepsy. *Epilepsia* **44:**981–985.

Le Novere N, Grutter T, and Changeux JP (2002) Models of the extracellular domain of the nicotinic receptors and of agonist- and Ca^{2+}-binding sites. *Proc Natl Acad Sci USA* **99:**3210–3215.

Liu QS and Berg DK (1999) Extracellular calcium regulates responses of both $\alpha3$- and $\alpha7$-containing nicotinic receptors on chick ciliary ganglion neurons. *J Neurophysiol* **82:**1124–1132.

McNamara JO (1999) Emerging insights into the genesis of epilepsy. *Nature (Lond)* **399:**A15–22.

Mood AM, Graybill FA, and Boes DC (1974) *Introduction to the Theory of Statistics.* McGraw-Hill Book Company, New York.

Mulle C, Léna C, and Changeux JP (1992) Potentiation of nicotinic receptor response by external calcium in rat central neurons. *Neuron* **8:**937–945.

Nelson ME, Kuryatov A, Choi CH, Zhou Y, and Lindstrom J (2003) Alternate stoichiometries of $\alpha4\beta2$ nicotinic acetylcholine receptors. *Mol Pharmacol* **63:**332–341.

Phillips HA, Favre I, Kirkpatrick M, Zuberi SM, Goudie D, Heron SE, Scheffer IE, Sutherland GR, Berkovic SF, Bertrand D, and Mulley JC (2001) CHRNB2 is the second acetylcholine receptor subunit associated with autosomal dominant nocturnal frontal lobe epilepsy. *Am J Hum Genet* **68:**225–231.

Quick MW and Lester HA (1994) Methods of expression of excitability proteins in *Xenopus* oocytes, in *Methods in Neuroscience* (PM C ed) pp 261–279, Academic Press.

Rodrigues-Pinguet N, Jia L, Li M, Figl A, Klaassen A, Truong A, Lester HA, and Cohen BN (2003) Five ADNFLE mutations reduce the Ca^{2+} dependence of the mammalian $\alpha4\beta2$ acetylcholine response. *J Physiol* **550:**11–26.

Rusakov DA and Fine A (2003) Extracellular calcium depletion contributes to fast activity-dependent modulation of synaptic transmission in the brain. *Neuron* **37:**287–297.

Scheffer IE, Bhatia KP, Lopes-Cendes I, Fish DR, Marsden CD, Andermann E, Andermann F, Desbiens R, Keene D, and Cendes F (1995) Autosomal dominant nocturnal frontal lobe epilepsy: a distinctive clinical disorder. *Brain* **118:**61–73.

Steinlein OK, Magnusson A, Stoodt J, Betrand S, Weiland S, Berkovic SF, Nakken KO, Propping P, and Bertrand D (1997) An insertion mutation of the CHRNA4 gene in a family with autosomal dominant nocturnal frontal lobe epilepsy. *Hum Mol Genet* **6:**943–947.

Steinlein OK, Mulley JC, Propping P, Wallace RH, Phillips HA, Sutherland GR, Scheffer IE, and Berkovic SF (1995) A missense mutation in neuronal nicotinic acetylcholine receptor $\alpha4$ subunit is associated with autosomal dominant nocturnal frontal lobe epilepsy. *Nat Genet* **11:**201–203.

Vassilev PM, Mitchel J, Vassilev M, Kanazirska M, and Brown EM (1997) Assessment of frequency-dependent alterations in the level of extracellular Ca^{2+} in the synaptic cleft. *Biophys J* **72:**2103–2116.

Vernino S, Amador M, Leutje CW, Patrick J, and Dani JA (1992) Calcium modulation and high calcium permeability of neuronal nicotinic acetylcholine receptors. *Neuron* **8:**127–134.

Wonnacott S (1997) Presynaptic acetylcholine receptors. *Trends Neurol Sci* **20:**92–98.

Zhou Y, Nelson ME, Kuryatov A, Choi C, Cooper J, and Lindstrom J (2003) Human $\alpha4\beta2$ acetylcholine receptors formed from linked subunits. *J Neurosci* **23:**9004–9015.

Zwart R and Vijverberg HP (1998) Four pharmacologically distinct subtypes of $\alpha4\beta2$ nicotinic acetylcholine receptor expressed in *Xenopus laevis* oocytes. *Mol Pharmacol* **54:**1124–1131.

Address correspondence to: Bruce N. Cohen, Division of Biology 156–29, California Institute of Technology, Pasadena, CA 91125. E-mail: bncohen@caltech.edu