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RESEARCH PAPER

The additional ACh binding site at the α4(+)/α4(–) interface of the $(\alpha4\beta2)_2\alpha4$ nicotinic ACh receptor contributes to desensitization

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BACKGROUND AND PURPOSE

Nicotinic ACh $(\alpha4\beta2)_2\alpha4$ receptors are highly prone to desensitization by prolonged exposure to low concentrations of agonist. Here, we report on the sensitivity of the three agonist sites of the $(\alpha4\beta2)_2\alpha4$ to desensitization induced by prolonged exposure to ACh. We present electrophysiological data that show that the agonist sites of the $(\alpha4\beta2)_2\alpha4$ receptor have different sensitivity to desensitization and that full receptor occupation decreases sensitivity to desensitization.

EXPERIMENTAL APPROACH

Two-electrode voltage-clamp electrophysiology was used to study the desensitization of concatenated $(\alpha4\beta2)_2\alpha4$ receptors expressed heterologously in *Xenopus* oocytes. Desensitization was assessed by measuring the degree of functional inhibition caused by prolonged exposure to ACh, as measured under equilibrium conditions. We used the single-point mutation α4W182A to measure the contribution of individual agonist sites to desensitization.

KEY RESULTS

$(\alpha4\beta2)_2\alpha4$ receptors are less sensitive to activation and desensitization by ACh than $(\alpha4\beta2)_2\beta2$ receptors. Incorporation of α4W182A into any of the agonist sites of concatenated $(\alpha4\beta2)_2\alpha4$ receptors decreased sensitivity to activation and desensitization but the effects were more pronounced when the mutation was introduced into the α4(+)/α4(–) interface.

CONCLUSIONS AND IMPLICATIONS

The findings suggest that the agonist sites in $(\alpha4\beta2)_2\alpha4$ receptors are not functionally equivalent. The agonist site at the α4(+)/α4(–) interface defines the sensitivity of $(\alpha4\beta2)_2\alpha4$ receptors to agonist-induced activation and desensitization. Functional differences between $(\alpha4\beta2)_2\alpha4$ and $(\alpha4\beta2)_2\beta2$ receptors might shape the physiological and behavioural responses to nicotinic ligands when the receptors are exposed to nicotinic ligands for prolonged periods of times.

Abbreviations

nAChR, nicotinic ACh receptor

Introduction

α4β2 Nicotinic ACh receptors (nAChRs) belong to the family of pentameric ligand-gated ion channels, which includes muscle nAChRs, 5-HT type 3 receptors, GABA receptors types A and C, and glycine receptors (Taly *et al.*, 2009). α4 and β2 nAChR subunits assemble into alternate stoichiometries $(\alpha4\beta2)_2\alpha4$ and $(\alpha4\beta2)_2\beta2$ that display 100-fold difference in

sensitivity to activation by ACh (Nelson *et al.*, 2003; Moroni *et al.*, 2006; Carbone *et al.*, 2009). The alternate receptors also differ in sensitivity to α4β2-preferring ligands (Moroni *et al.*, 2006; Carbone *et al.*, 2009), unitary conductance (Nelson *et al.*, 2003), calcium permeability (Tapia *et al.*, 2007), sensitivity to modulation by Zn^{2+} (Moroni *et al.*, 2008) and the compound NS9283 (Timmermann *et al.*, 2012). Both receptor forms consist of two identical α4β2 subunit pairs and a fifth auxiliary α4 or β2 subunit, all arranged around a central cation pore (Taly *et al.*, 2009). Each α4β2 subunit pair harbours an identical ACh binding site formed at the interface between the two adjacent subunits. The principal or (+) face of the binding site at α4/β2 interfaces is contributed by α4 subunit, while β2 subunit contributes the complementary or (–) face (Taly *et al.*, 2009). Hence, structurally, the alternate α4β2 receptors are identical except for the auxiliary subunit, which is α4 in $(\alpha4\beta2)_2\alpha4$ nAChRs and β2 in $(\alpha4\beta2)_2\beta2$ nAChRs. That the auxiliary subunit can be either α4 or β2 leads to stoichiometry-specific interfaces. In the $(\alpha4\beta2)_2\beta2$ receptor there is a β2(+)/β2(–) interface, whereas in the $(\alpha4\beta2)_2\alpha4$ receptor there is a α4(+)/α4(–) interface (Figure 1A). Structural determinants bestowing functional signatures to $(\alpha4\beta2)_2\alpha4$ nAChRs have so far been identified only on the auxiliary subunit (Tapia *et al.*, 2007; Moroni *et al.*, 2008; Timmermann *et al.*, 2012). More recently, we (Mazzaferro *et al.*, 2011) and others (Harpsøe *et al.*, 2011) have shown an additional operational agonist site at the α4(+)/α4(–) interface that defines the agonist sensitivity of $(\alpha4\beta2)_2\alpha4$ nAChRs.

α4β2 nAChRs undergo acute and high-affinity desensitization. Acute desensitization refers to the loss in receptor response during a single exposure to high (~EC_{60}–EC_{100}) concentrations of agonist, whereas high-affinity desensitization is induced by chronic exposure to low or non-activating agonist concentrations (Steinbach, 1990; Gopalakrishnan *et al.*, 1996; Paradiso and Steinbach, 2003; Lester, 2004; Giniatullin *et al.*, 2005). The molecular mechanisms producing acute or high-affinity desensitization have not been fully identified. It is also unclear whether the two types of desensitization are the result of a single process. Mutations within the second transmembrane domain of the α4 subunit (S248F and 776ins3) that affect both the apparent affinity to ACh and acute and chronic desensitization (Bertrand *et al.*, 1998) suggest a single desensitization pathway. In addition, studies with chimeric nAChRs have shown that the N-terminal domain of the α4 subunit, along with the first three transmembrane domains, and the N-terminal domain of the β2 subunit are involved in high-affinity desensitization (Corringer *et al.*, 1998; Kuryatov *et al.*, 2000). However, other single-point mutations in the α4 subunit can cause changes in desensitization without effects on agonist binding (Matsushima *et al.*, 2002).

Here, we report on the contribution of the three agonist sites in $(\alpha4\beta2)_2\alpha4$ nAChRs to high-affinity desensitization. The sites were individually mutated, and consequences of these mutations on ACh-induced activation and high-affinity desensitization were assessed. We found that the additional agonist site decreases sensitivity to activation and high-affinity desensitization. Strikingly, we found that the agonist sites at the α4(+)/β2(–) and α4(+)/α4(–) interfaces are not functionally equivalent, which adds another level of complexity to the function of $(\alpha4\beta2)_2\alpha4$ nAChRs.

Methods

Materials

All chemicals used in the study were purchased from Sigma-Aldrich (Dorset, UK). Drug and molecular target nomenclature conforms to the *British Journal of Pharmacology* Guide to Receptors and Channels (Alexander *et al.*, 2011).

Mutagenesis and expression in oocytes

β2_α4_β2_α4_β2 or β2_α4_β2_α4_α4 were engineered as described by Carbone *et al.* (2009). All constructs were assayed for integrity using the LT reporter mutation (L9′T in the second transmembrane domain; Mantione *et al.*, 2012) as described by Mazzaferro *et al.* (2011). For clarity, α4W182A is shown as superscript positioned in the (+) or (–) face of the mutated subunit (e.g. in $\beta2\_^{W182A}\alpha4\_\beta2\_\alpha4\_\alpha4$ the mutation W182A is located in the (+) face of the α4 subunit occupying the second position of the linear sequence of the concatamer; Mazzaferro *et al.*, 2011). The residue numbering used later includes the signal sequence. Concatenated α4β2 nAChRs were expressed in *Xenopus laevis* oocytes as previously described (Carbone *et al.*, 2009; Mazzaferro *et al.*, 2011). Concatamer cRNAs were synthesized *in vitro* (mMESSAGE mMACHINE T7, Ambion, Paisley, UK) from linearized plasmid templates (Carbone *et al.*, 2009).

Oocyte electrophysiology

Two-electrode voltage-clamp recordings on oocytes were carried out as previously described (Carbone *et al.*, 2009; Mazzaferro *et al.*, 2011). Activation concentration–response curves for ACh were obtained as described by Mazzaferro *et al.* (2011). Concentration–response curves were plotted using Prism v.5.0 (Graph Pad Software, San Diego, CA, USA). The ACh activation concentration–response curve data were first fitted to the one-component Hill equation, $I = I_{max}/[1 + (EC_{50}/x)^{nH}]$, where EC_{50} represents the concentration of agonist inducing 50% of the maximal response (I_{max}); x is the agonist concentration; and nH the Hill coefficient. When ACh induced biphasic receptor activation, the concentration–response curve data were analysed using the equation Y = Bottom + Section 1 + Section 2 that fits the sum of two one-component Hill equations (Prism v.5.0), where both responses are excitatory. In this equation, Section 1 is equal to Span × Frac/{1 + 10^[(LogEC_{50}_1 – X) × nH_1]}, while Section 2 represents Span × (1 – Frac) / {1 + 10^[(LogEC_{50}_2 – X) × nH_2]}. Span is Top – Bottom. LogEC_{50_1} and LogEC_{50_2} are the concentrations that give half-maximal stimulatory effects in the same units as X. Bottom and Top are the plateaus at the left and right ends of the curve, in the same units as Y. nH_1 and nH_2 are the unitless Hill coefficients. Frac represents the proportion of the maximal response due to the more sensitive component. For wild-type receptors, the ACh responses were normalized to the responses elicited by 1 mM ACh, whereas for mutant receptors the activation concentration–response curves were constructed by normalizing the data to the responses stimulated by 5 mM ACh.

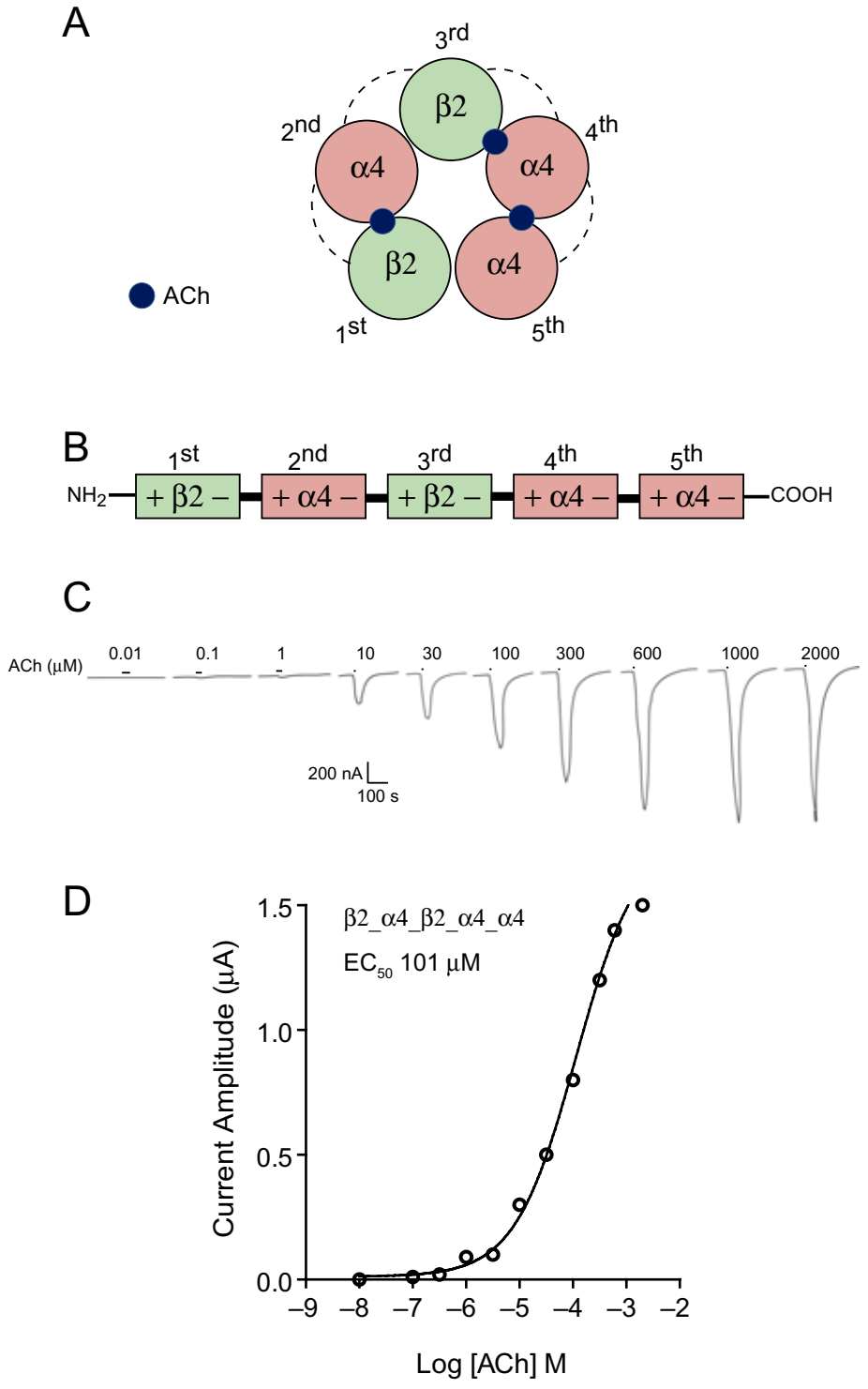

Figure 1

Diagram and ACh responses of β2_α4_β2_α4_α4 nAChRs. (A) A cartoon of the subunit arrangement of the concatenated form of $(\alpha4\beta2)_2\alpha4$ receptors (β2_α4_β2_α4_α4) showing ACh binding sites (circled ACh) at the α4(+)/β2(–) and α4(+)/α4(–) interfaces. (B) Linear sequence of the concatenated $(\alpha4\beta2)_2\alpha4$ receptor showing orientation of (+) and (–) faces of each subunit. See Mazzaferro *et al.* (2011) for details on the experimental strategy used to define the orientation of the subunits in β2_α4_β2_α4_α4 receptors. (C) Responses of β2_α4_β2_α4_α4 receptors expressed heterologously by an oocyte injected with β2_α4_β2_α4_α4 cRNA to a range of ACh concentrations. The numbers above the traces show the concentration of the ACh pulses. (D) Concentration–response curve for the activation data shown in C. The responses to the ACh concentrations shown above the traces in C were normalized to the responses elicited by a maximal concentration of ACh (1 mM). The concentration–response curve data were best fitted to a monophasic Hill model as described in Methods.

Receptor desensitization was assessed by determining the degree of functional inhibition caused by prolonged exposure to ACh as measured under equilibrium conditions. The protocol used was based on that designed by Corringer *et al.* (1998) to measure desensitization of α7-5HT3 chimeric receptors. Oocytes injected with nAChR cRNAs were challenged approximately every 90 s by short pulses (2 s) of ACh at a concentration near the ACh EC_{50} (test pulses) first in the absence and then during a prolonged perfusion with Ringer solution containing low concentrations of ACh (prepulse). Prepulses were applied until the amplitude of the test pulses was constant. Although the extent and rate of inhibition varied depending on the ACh concentration of the prepulse and the nature of the receptor under study, a 10 min prepulse period was sufficient to reach steady-state level of desensitization for all ACh concentrations used. Desensitization data were fitted using non-linear regression to the one-component Hill equation: $I = I_{min} + (I_{max} - I_{min}) / \{1 + 10\text{^}[(\text{Log}IC_{50} - X) \times nH]\}$, where IC_{50} is the concentration of drug that inhibits 50% of maximum response. The amplitude of steady-state currents was used for data analysis.

Statistical analysis

Data analyses were performed using GraphPad Prism software (GraphPad, San Diego, CA, USA). Data were pooled from at least three different batches of oocytes. An *F*-test determined whether the one-site or biphasic model best fits the data; the simpler one-component model was preferred unless the extra sum-of-squares *F*-test had a value of *P* less than 0.05. Log EC_{50} values for ACh, and changes in current response amplitudes in response to mutations were analysed using one-way ANOVA with a Dunnett or Bonferroni *post hoc* correction for the comparison of all mutated receptors, to determine significance between wild-type and mutant receptors. Significance levels between mutant receptors were determined using unpaired *t*-tests. Differences were considered significant at the level $P < 0.05$. Data are plotted as mean ± SEM. Fit parameter values are the best fitting values with the SEM values estimated from the fit.

Results

Desensitization of $(\alpha4\beta2)_2\alpha4$ receptors

We examined the contribution of the ACh binding sites of $(\alpha4\beta2)_2\alpha4$ receptors to desensitization by measuring the fraction of activatable receptors remaining after prolonged exposure to a range of ACh concentrations. The studies were carried out on *Xenopus* oocytes expressing β2_α4_β2_α4_α4 receptors, the concatenated version of $(\alpha4\beta2)_2\alpha4$ nAChRs. α4(+)/β2(−) subunit interfaces located between the first and second and between the third and fourth subunit positions of the β2_α4_β2_α4_α4 concatamer house prototype heteromeric nAChR agonist sites (Figure 1A, B; Mazzaferro *et al.*, 2011). The additional agonist site is located at the interface between the α4 subunits located in the fourth and fifth subunit positions of the concatenated receptor (Figure 1A, B; Mazzaferro *et al.*, 2011).

Typical responses to several concentrations of ACh from β2_α4_β2_α4_α4 receptors are shown in Figure 1C, and the concentration–response curve for the data is shown in Figure 1D. Mean EC_{50} and Hill coefficient estimates from 10 independent experiments are shown in Table 1. ACh responses were first detected at concentrations higher than 10 nM, and maximal responses were evoked by ACh concentrations of 1–2 mM. Higher ACh concentrations elicited smaller responses (Figure 1C), which could reflect acute desensitization and blocking action by ACh. However, under our perfusion conditions, we did not observe rebound currents upon the removal of ACh. Rebound currents may indicate agonist-dependent open-channel block in nAChRs (Bertrand *et al.*, 1992). The fit to the data provided the following parameter estimates: ACh EC_{50} 99 ± 12 μM and Hill coefficient 1.10 ± 0.09 (Table 1). These values are comparable with EC_{50} and Hill coefficient estimates previously reported for β2_α4_β2_α4_α4 receptors (Mazzaferro *et al.*, 2011) and for $(\alpha4\beta2)_2\alpha4$ receptors assembled from loose α4 and β2 subunits (Moroni *et al.*, 2006; Carbone *et al.*, 2009). The data were best fitted to a monophasic Hill equation. However, a higher sensitivity component could be present at low concentrations of ACh, as suggested by a slight plateauing of the response data at around 1–3 μM ACh (Figure 1D), consistent with the findings of Harpsøe *et al.* (2011) on the function of $(\alpha4\beta2)_2\alpha4$ nAChRs.

Next, we determined the sensitivity of β2_α4_β2_α4_α4 receptors to desensitization by prolonged exposure to a range of ACh concentrations. Representative traces of the responses of β2_α4_β2_α4_α4 receptors to short (2 s) ACh test pulses at a concentration near the EC_{50} ACh (100 μM) in the absence and during the ACh prepulse aplication are shown in Figure 2A. Exposure to ACh prepulses decreased the amplitude of the responses to EC_{50} ACh pulses in a concentration-dependent manner. Inhibition reached equilibrium within a few minutes following initiation of the prepulse application and some recovery was observed when going back to control conditions. Plots of peak ACh EC_{50} current responses measured at equilibrium conditions as a function of the concentration of the ACh prepulses produced monophasic sigmoidal inhibitory concentration–response curves (Figure 2B). The estimated IC_{50} was approximately 500-fold greater than the EC_{50} for ACh-induced activation ($P < 0.05$; $n = 8$; Table 1), and the Hill coefficient was less than 1. For comparison, we include in Table 1 the values for the ACh-induced functional inactivation of $(\alpha4\beta2)_2\alpha4$ receptors assembled from loose subunits. Desensitization of $(\alpha4\beta2)_2\alpha4$ by ACh was measured using the protocol described for β2_α4_β2_α4_α4 receptors (not shown). Fits to the data yielded IC_{50} and Hill coefficient values that were comparable with those estimated for β2_α4_β2_α4_α4 receptors (Table 1).

To test if the concentration of the ACh test pulse affected the degree of functional inhibition induced by prolonged exposure to ACh, we measured desensitization of β2_α4_β2_α4_α4 receptors using ACh test pulses of 4, 10, 30 or 300 μM. ACh prepulses higher than 0.01 μM produced inhibition of all the ACh test pulses studied. Figure 2C shows representative traces of the inactivation of current responses evoked by the various ACh test pulses studied by an ACh prepulse of 1 μM. The inhibitory concentration–response curves produced by the inhibition data are shown in Figure 2D. The estimated IC_{50} and Hill coefficient values for the desensitization of currents evoked by ACh test pulses of

Table 1

Summary of desensitization and activation of α4β2 nAChRs by ACh

Receptor	EC_{50} (μM)	nH	IC_{50} (μM)	nH	EC_{50}/IC_{50}
β2_α4_β2_α4_α4	99 ± 12	1.10 ± 0.09	0.032 ± 0.007^+	0.57 ± 0.06	3093+
			0.29 ± 0.08^^	0.54 ± 0.03	341
			0.23 ± 0.06^^^	0.56 ± 0.04	430
			0.22 ± 0.04^^^^	0.60 ± 0.06	450
			0.27 ± 0.04^^^^^	0.52 ± 0.09	367
$(α4β2)_2α4$	101 ± 21	0.95 ± 0.08	0.18 ± 0.01	0.61 ± 0.07	561
β2_α4_ β2_α4_ β2	2.8 ± 0.8*	1.05 ± 0.09	0.058 ± 0.001*	0.61 ± 0.02	48.28*
$(α4β2)_2β2$	2.2 ± 0.3*	0.79 ± 0.09	0.071 ± 0.001*	0.54 ± 0.01*	31.03*

EC_{50} values were determined by fitting the activation data to the monophasic Hill equation equations given in Methods. $(α4β2)_2α4$ and $(α4β2)_2β2$ nAChRs were expressed from individual subunits injected into oocytes as previously described (Moroni *et al.*, 2006). For all wild-type receptors, the concentration data points shown in the concentration–response curves were obtained by normalizing the responses to any given ACh concentration to the responses elicited by 1 mM ACh. Desensitization of concatenated β2_α4_β2_α4_α4 receptors was measured using ACh test pulses of of 4 μM (^), $n = 4$, 10 μM (^^), $n = 6$, 30 μM (^^^), $n = 4$, 100 μM (^^^^) $n = 8$ or 300 μM (^^^^^), $n = 4$. IC_{50} values were estimated from inhibition concentration–response curves obtained by fitting the data to the monophasic Hill equation described in Methods. IC_{50} and EC_{50}/IC_{50} values for $(α4β2)_2β2$ or β2_α4_ β2_α4_ β2 receptors were statistically different (noted by *) from those estimated for the desensitization of β2_α4_β2_α4_α4 receptors measured using ACh pulses of 100, 10, 30 or 300 μM. IC_{50} and EC_{50}/IC_{50} values estimated for the desensitization of the responses elicited by 4 μM ACh at β2_α4_β2_α4_α4 receptors were statistically different from the values estimated for the desensitization of the responses evoked by higher concentrations of ACh at β2_α4_β2_α4_α4 receptors (noted by +). For each receptor construct studied, EC_{50} values were estimated from data from 6 to 10 experiments (β2_α4_β2_α4_α4, $n = 10$; $(α4β2)_2α4$, $n = 10$; β2_α4_ β2_α4_ β2, $n = 8$; $(α4β2)_2β2$, $n = 6$). Statistical comparisons were carried out as described in Methods.

10, 30 or 300 μM were not different from each other, or from the IC_{50} and Hill coefficient values estimated from the inhibition of currents evoked by 100 μM ACh test pulses (Table 1). In contrast, when the ACh test pulse was 4 μM, a concentration of agonist that elicited less than 10% of the maximal ACh response, the estimated IC_{50} value was about ninefold lower ($P < 0.05$; $n = 4$; Figure 2D; Table 1). A contributor to the observed concentration-dependent effects could be procedural. Thus, desensitization may not have reached equilibrium at low concentrations of ACh, or processes such as ion channel blockade by ACh may have contributed to the observed functional inhibition at high concentrations of ACh test pulses (Bertrand *et al.*, 1992; Chavez-Noriega *et al.*, 1997). This is unlikely for the following reasons. Firstly, if at low concentrations of ACh test pulse desensitization had not completely attained steady-state conditions, the extent of desensitization elicited may not yet be complete. Under these conditions, the inhibition concentration–response curve would have shifted to the right, yielding higher IC_{50} values for desensitization. Clearly this was not what we observed. Secondly, if at higher concentrations of ACh test pulses, other inhibitory processes such as ion channel blockade by ACh added to the inhibitory process, sensitivity to desensitization would have increased, which is not the pattern we found.

$(α4β2)_2α4$ and $(α4β2)_2β2$ receptors may be expressed in the brain (Marks *et al.*, 1999; 2010; Gotti *et al.*, 2008; Rode *et al.*, 2012). Therefore, we compared the desensitization of β2_α4_β2_α4_α4 nAChRs with that of β2_α4_β2_α4_β2 nAChRs, the concatenated form of the $(α4β2)_2β2$ nAChR (Carbone *et al.*, 2009). The $(α4β2)_2β2$ nAChR displays 100-fold higher sensitivity for activation by ACh than the $(α4β2)_2α4$ isoform (Nelson *et al.*, 2003; Moroni *et al.*, 2006; Carbone *et al.*, 2009). Typical recordings obtained for the inhibition of EC_{50} (2 μM) ACh responses of β2_α4_β2_α4_β2 nAChRs are shown in Figure 3A. Inhibition was monophasic as shown for β2_α4_β2_α4_α4 receptors (Figure 3B). The EC_{50} and Hill coefficient values estimated from the fit are shown in Table 1. Desensitization of β2_α4_β2_α4_β2 receptors (IC_{50} 0.058 ± 0.001 μM) and $(α4β2)_2β2$ receptors (IC_{50} 0.071 ± 0.001 μM; not shown) were comparable (Table 1). The IC_{50} value obtained for β2_α4_β2_α4_β2 was about 3.8-fold lower, compared with the IC_{50} values estimated for the desensitization of β2_α4_β2_α4_α4 receptors stimulated by concentrations of ACh higher than 4 μM. However, we found no statistical differences between the IC_{50} values estimated for β2_α4_β2_α4_β2 receptors and for β2_α4_β2_α4_α4 receptors activated by ACh test pulses of 4 μM.

The window current of ligand-gated ion channels is the range of agonist concentrations at which receptors can be activated by agonists before desensitization is complete (Steinbach, 1990). To provide an estimate of the window current for each α4β2 receptor form, we replotted the desensitization concentration–response curves obtained for EC_{50} ACh test pulses and the activation concentration–response curve of each receptor type on the same axes (Figure 3B). The concentration range at which the activation and desensitization curves overlap indicates the window current of receptors (Steinbach, 1990; Lester, 2004). Figure 3B and C show that for β2_α4_β2_α4_β2 receptors the window current extended from about 0.1 nM to 300 μM Ach, with the peak occurring at 0.1 μM ACh. For β2_α4_β2_α4_α4 receptors stimulated by

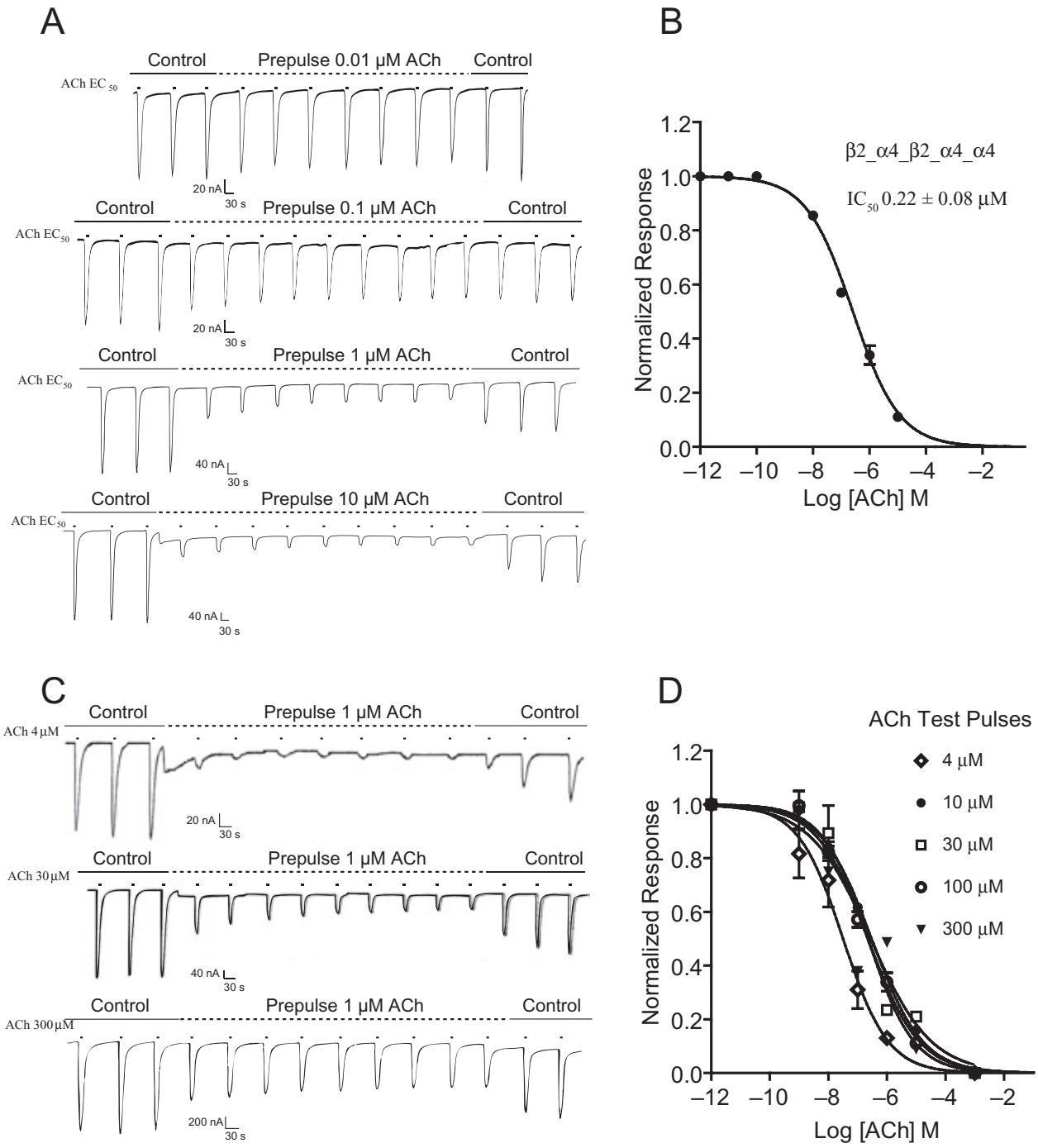

Figure 2

Functional inhibition of β2_α4_β2_α4_α4 nAChRs. (A) Typical traces of β2_α4_β2_α4_α4 nAChRs obtained by using the prolonged exposure to ACh protocol described in the Methods section. Impaled oocyte were challenged at regular intervals (90 s) by a 2 s pulse of 100 μM ACh, a concentration of ACh near the EC_{50}. Responses evoked prior to prepulses show no desensitization, but prepulses (ACh prepulse, dashed lines) produce a concentration-dependent decrease of the responses evoked by 100 μM ACh. On termination of the prepulse some recovery is observed. (B) Concentration–response curve for the desensitization of β2_α4_β2_α4_α4 nAChR produced by the protocol shown in A. The peak current of the EC_{50} ACh responses immediately before the end of the period of prolonged exposure to ACh was normalized to the control ACh responses. The data were best fitted to the monophasic Hill equation described in Methods. The estimated IC_{50} and Hill coefficient are summarized in Table 1. (C) Typical traces obtained from β2_α4_β2_α4_α4 nAChRs stimulated by 4, 30 or 300 μM ACh test pulses exposed to Ringer's solution containing 1 μM ACh. (D) Concentration–response curve for the desensitization of β2_α4_β2_α4_α4 nAChR currents stimulated by a range of ACh test pulses. The exposure protocol used to induce desensitization was identical to the one shown in A, except that the ACh test pulses were 4, 10, 30 or 300 μM. The peak current of the EC_{50} ACh responses immediately after the prepulses were normalized to the control ACh responses. The data were best fitted to the monophasic Hill equation described in Methods. The estimated IC_{50} and Hill coefficient are summarized in Table 1.

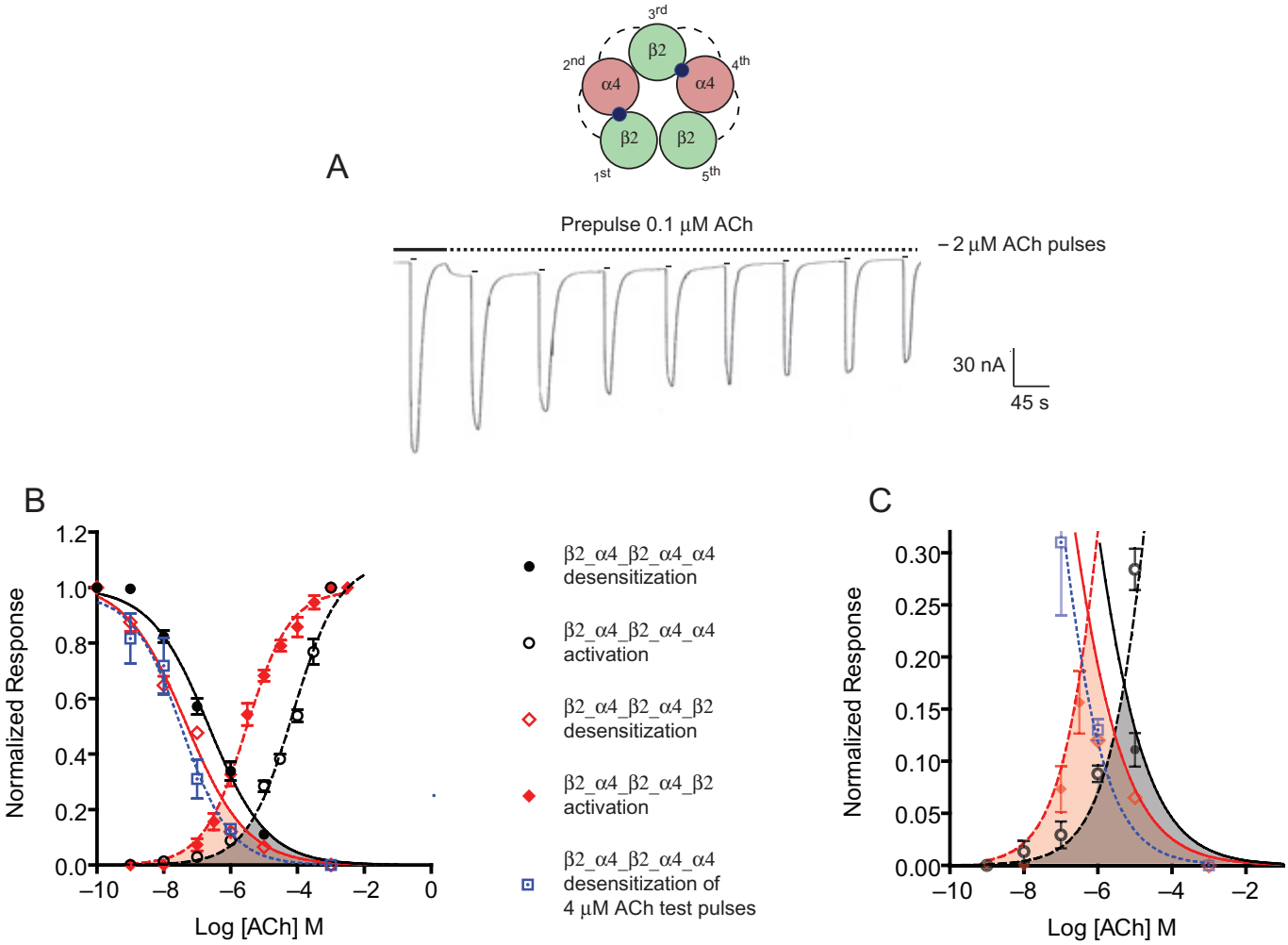

Figure 3

High-affinity desensitization in concatenated $(\alpha4\beta2)_2\beta2$ and $(\alpha4\beta2)_2\alpha4$ nAChRs. (A) Representative traces obtained from β2_α4_β2_α4_ β2 receptors using the desensitization protocol described in Methods. Oocytes expressing β2_α4_β2_α4_β2 receptors were challenged at regular intervals (90 s) by a 2 s ACh test pulse of 2 μM, a concentration of ACh near the ACh EC_{50} at this receptor type. The control responses decreased in amplitude upon chronic exposure to low concentrations of ACh. (B) Activation and desensitization concentration–response curves of β2_α4_β2_α4_α4 or β2_α4_β2_α4_β2 receptors. Activation and desensitization curves obtained with EC_{50} ACh test pulses are plotted on the same axis to reveal the window current for the α4β2 nAChR isoforms. The region where the activation and desensitization concentration–response curve overlap represents the window current of the receptors. We also include the data for the stimulation of β2_α4_β2_α4_α4 nAChRs with 4 μM ACh (blue dots). Data were best fitted to the monophasic Hill equation described in Methods ($P < 0.001$). The estimated EC_{50}, IC_{50}, Hill coefficient values and number of experiments carried out are summarized in Table 1. (C) Detail of plot in B, showing the overlapping regions of the activation and desensitization ACh concentration–response curve.

EC_{50} ACh the window current was between 10 nM and 500 μM ACh, with the peak occurring at about 10 μM ACh. At the peak of the window current only about 17% of the maximal response elicited by ACh at β2_α4_β2_α4_α4 receptors was activated, whereas for β2_α4_β2_α4_β2 receptors 24% of the maximal response was stimulated. The EC_{50} : IC_{50} ratio for the alternate receptors differed by about ninefold ($P < 0.05$; $n = 8$; Table 1). The value of the EC_{50} : IC_{50} ratio for β2_α4_β2_α4_α4 receptors varied depending on the concentration of the ACh test pulse used in the desensitization experiments. Thus, when the ACh test pulse was 4 μM the EC_{50} : IC_{50} ratio was sevenfold lower than the EC_{50} : IC_{50} values estimated for β2_α4_β2_α4_α4 receptors activated by higher concentrations of ACh test pulses (Figure 3B and C; Table 1). Also, compared with test pulses of higher ACh concentration, the peak of the window current obtained with 4 μM ACh test pulses was approximately sevenfold lower (1.5 μM), and at this point only 10% of the maximal ACh response was stimulated (Figure 3B, C).

The ACh binding site at the α4(+)/α4(–) interface contributes to desensitization

We introduced W182A into the fifth subunit of the concatamer to engineer β2_α4_β2_α4_^{W182A}α4 receptors to examine the contribution of the additional ACh binding site to desensitization. This mutant receptor has the agonist sites at the

α4(+)/β2(–) interfaces intact, while the ACh site at the α4(+)/α4(–) interface is impaired (Mazzaferro *et al.*, 2011). α4W182 is equivalent to W149 in muscle-type α1 subunit. α1W149 cation-π interacts with the quaternary ammonium group of ACh (Xiu *et al.*, 2009) and affects agonist-binding affinity as well as gating (Akk and Auerbach, 1999; Akk, 2001). Because agonist-induced desensitization occurs following occupation of agonist sites (Steinbach, 1990; Giniatullin *et al.*, 2005), we reasoned that impairment of the ACh binding site at the α4(+)/α4(–) interface by alanine substitution of α4W182 should affect desensitization of β2_α4_β2_α4_α4 receptors, if the additional ACh site contributed to it. To test the validity of this view, we first determined ACh-induced desensitization in β2_α4_β2_α4_α4 receptors with its three agonist sites impaired (i.e. β2_^{W182A}α4_β2_^{W182A}α4_^{W182A}α4). As previously reported, introducing W182A simultaneously into the three α4 subunits of β2_α4_β2_α4_α4 receptors produces monophasic sensitivity to activation by ACh and a sevenfold decrease in sensitivity to activation (Mazzaferro *et al.*, 2011; see Table 2). Chronic exposure to low concentrations of ACh desensitized the current responses evoked by 700 μM ACh (a concentration near the ACh EC_{50} at β2_^{W182A}α4_β2_^{W182A}α4_^{W182A}α4 receptors; Mazzaferro *et al.*, 2011). Compared with wild type, sensitivity to desensitization was reduced significantly from 0.22 ± 0.04 to 60 ± 2.1 μM ($P < 0.001$; $n = 6$; Figure 4A). Thus, impairment of agonist binding reduces sensitivity to desensitization. Accordingly, the window current of β2_^{W182A}α4_β2_^{W182A}α4_^{W182A}α4 was shifted to the right ($P < 0.001$; $n = 4$; Figure 4B). The findings indicated that α4W182A could be used to measure the contribution of individual agonist binding sites to desensitization.

The typical responses to a range of concentrations of ACh from β2_α4_β2_α4_^{W182A}α4 receptors are shown in Figure 5A. The mean activation concentration–response data from 10 independent experiments are shown in Figure 5B. In comparison with wild type, a clear biphasic behaviour was noted with a slight plateau in the amplitude of the current responses at around 1 mM. In contrast to wild type, maximal peak currents were elicited by 5 mM ACh. Concentrations of ACh of up to 10 mM did not increase further the amplitude of the responses. The data were best fitted to the biphasic Hill equation described in Methods ($P < 0.001$; $n = 10$; estimated EC_{50} and Hill coefficient values are shown in Table 2). These data confirm our previously reported findings that impairment of any one of the agonist sites in β2_α4_β2_α4_α4 receptors produces biphasic agonist-induced activation (Mazzaferro *et al.*, 2011). The high-sensitivity component of the curve displayed fourfold greater sensitivity to activation by ACh than wild type (Table 2). In contrast, the ACh sensitivity of the low-sensitivity component was approximately 35-fold lower than wild type ($P < 0.001$, $n = 10$). We have previously shown that binding of ACh to the unaltered binding sites at the α4(+)/β2(–) subunit interfaces gives rise to the higher sensitivity component of the concentration–response curve, while the mutated site at the α4(+)/α4(–) interface contributes mostly to the low-sensitivity component of the curve (Mazzaferro *et al.*, 2011). To measure desensitization of intact and impaired agonist sites, we measured the effects of prolonged exposure to ACh on the responses elicited by ACh test pulses of either $EC_{50\text{-}1}$ (mostly intact agonist sites) ACh or $EC_{50\text{-}2}$ (intact agonist sites plus mutated

Table 2

Summary of ACh-induced desensitization and activation of the three agonist sites of β2_α4_β2_α4_α4 receptors

Receptor	EC_{50_1} (μM)	nH_1	IC_{50_1} (μM)	nH_{in}	EC_{50}/IC_{50}	EC_{50_2} (μM)	nH_2	IC_{50_2} (μM)	nH_{in}	EC_{50}/IC_{50}
β2_^{W182A}α4_β2_^{W182A}α4_^{W182A}α4	684 ± 63	1.4 ± 0.1	60 ± 2	0.8 ± 0.01	–	–	–	–	–	11.4
β2_^{W182A}α4_β2_α4_α4	1.8 ± 0.02^{+}	0.78 ± 0.08	0.038 ± 0.009^{+}	0.54 ± 0.01	47^{+}	630 ± 71^{+}	1.3 ± 0.5	1.72 ± 0.2^{+}	0.6 ± 0.02	366
β2_α4_β2_^{W182A}α4_α4	1.20 ± 0.03^{+}	0.71 ± 0.06	0.025 ± 0.008^{+}	0.48 ± 0.08	48^{+}	640 ± 65^{+}	1.4 ± 0.7	1.68 ± 0.2^{+}	0.54 ± 0.8	376
β2_α4_β2_α4_^{W182A}α4	28 ± 2*$^{,+}$	0.74 ± 0.09	0.032 ± 0.009^{+}	0.5 ± 0.02	875^{+}	3483 ± 25*$^{,++}$	1.8 ± 1	9 ± 0.2*$^{,+}$	0.54 ± 0.02	387

EC_{50} values were determined by fitting the activation data to the monophasic or biphasic Hill equations given in Methods. EC_{50} values were estimated from data from 6 to 10 experiments: β2_^{W182A}α4_β2_^{W182A}α4_^{W182A}α4, $n = 6$; β2_^{W182A}α4_β2_α4_α4, $n = 10$; β2_α4_β2_^{W182A}α4_α4, $n = 9$; β2_α4_β2_α4_^{W182A}α4, $n = 10$. IC_{50} values were determined by fitting the inhibition data to the monophasic Hill equation described in Methods. IC_{50} values were estimated from data obtained from $n = 5$ (β2_^{W182A}α4_β2_^{W182A}α4_^{W182A}α4), $n = 7$ (β2_^{W182A}α4_β2_α4_α4) and $n = 8$ (β2_α4_β2_^{W182A}α4_α4 and β2_α4_β2_α4_^{W182A}α4) experiments. $IC_{50\text{-}1}$ values were estimated from data produced by desensitization experiments that used $EC_{50\text{-}1}$ ACh as test pulses, while $IC_{50\text{-}2}$ values were estimated from experimental data generated by $EC_{50\text{-}2}$ ACh test pulses. $EC_{50\text{-}1}$ and $EC_{50\text{-}2}$ values were estimated from the high-sensitivity and low-sensitivity components of the concentration–response curves, respectively. For biphasic data, Frac and Span were β2_^{W182A}α4_β2_α4_α4, 0.68 and 1.06; β2_α4_β2_^{W182A}α4_α4, 0.64 and 1.01; β2_α4_β2_α4_^{W182A}α4, 0.63 and 1.04, respectively. Note that for β2_^{W182A}α4_β2_^{W182A}α4_^{W182A}α4 receptors the stimulation and inhibition concentration–response data were best fitted ($P < 0.0001$) to the monophasic Hill equation described in Methods. Statistical differences between the values estimated for the agonist binding site at α4(+)/α4(–) and α4(+)/β2(–) interfaces were assessed as described in the Methods section. *denotes statistical comparisons between β2_α4_β2_α4_^{W182A}α4 and receptors with impaired α4(+)/β2(–) agonist sites. *$P < 0.05$. $^{+}$denotes statistical comparisons between mutant and wild-type receptors. $^{+}P < 0.05$; $^{++}P < 0.001$.

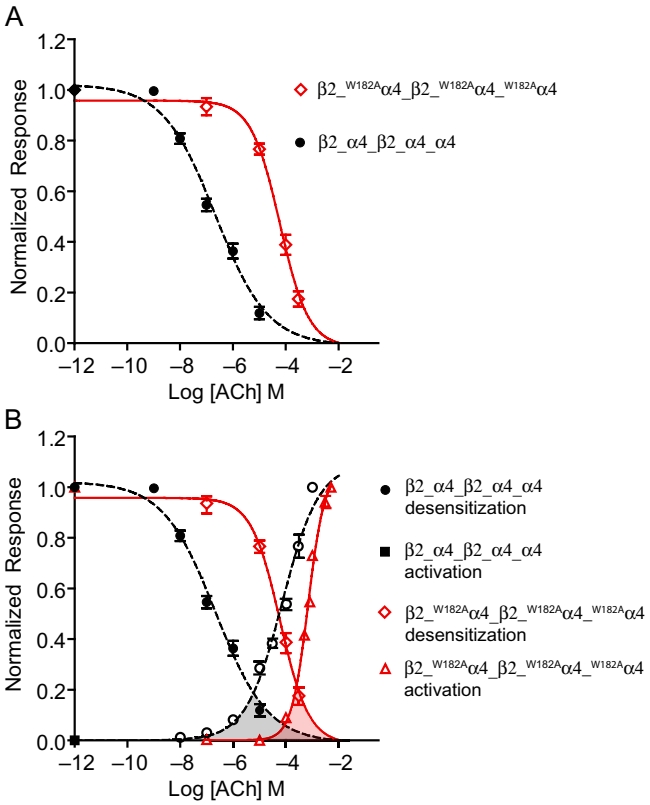

Figure 4

Effect of W182A on the activation and high-affinity desensitization of β2_α4_β2_α4_α4 receptors. (A) Wild type and mutant (β2_^{W182A}α4_β2_^{W182A}α4_^{W182A}α4 receptors) inhibition concentration–response curves. Desensitization was induced using a prolonged application of ACh test pulse protocol. The data were best fitted to the monophasic Hill model described in Methods. IC_{50} and Hill coefficient values are shown in Table 1 (wild-type receptors) or Table 2 (mutant receptor). (B) Plots of activation and inhibition concentration–response curve data plotted on the same axes. The area under the curves where the activation and desensitization concentration–response curve overlap represents the window current of the receptor.

agonist site) ACh. Representative responses obtained for the β2_α4_β2_α4_^{W182A}α4 receptors using this protocol are shown in Figure 5C. For both ACh test pulses, desensitization produced monophasic inhibition curves ($P < 0.001$; $n = 5$; Figure 5D). However, the IC_{50} values estimated from these curves differed by 280-fold ($P < 0.05$; $n = 5$; Table 2). The mutated site at the α4(+)/α4(–) interface was less sensitive to desensitization than wild-type agonist sites, which is consistent with our previous observation that impairment of ACh binding sites decreases sensitivity of α4β2 nAChRs to desensitization (Figure 4A). Also, the IC_{50} value estimated for the component with lower sensitivity to activation was about 41-fold greater compared with the IC_{50} estimated for currents evoked by EC_{50} ACh on wild-type β2_α4_β2_α4_α4 receptors. The estimated IC_{50} at intact agonist sites ($IC_{50\text{-}1}$) was not statistically different from that estimated for ACh-induced desensitization in β2_α4_β2_α4_β2 receptors (see Tables 1 and 2), or for desensitization of the responses of β2_α4_β2_α4_α4 receptors to 4 μM ACh pulses.

In order to determine whether the ACh sites at α4(+)/β2(–) interfaces desensitized similarly to the site at the α4(+)/α4(–) interface, we engineered receptors β2_^{W148A}α4_β2_α4_α4 and β2_α4_β2_^{W148A}α4_α4 and determined their sensitivity to high-affinity desensitization. β2_^{W148A}α4_β2_α4_α4 and β2_α4_β2_^{W148A}α4_α4 receptors displayed similar sensitivity to activation by ACh ($P < 0.001$; $n = 6$; Figure 6A, C; Table 2). As for β2_α4_β2_α4_^{W182A}α4 receptors, 5 mM ACh induced maximal responses, whose amplitude did not increase by application of higher concentrations of ACh. In agreement with previous findings (Mazzaferro *et al.*, 2011), the sensitivity to activation by ACh of intact agonist sites ($EC_{50\text{-}1}$) was no different from the ACh sensitivity of wild-type $(α4β2)_2β2$ or β2_α4_β2_α4_β2 receptors, while $EC_{50\text{-}2}$ values were sixfold lower than wild-type β2_α4_β2_α4_α4 receptors (Tables 1 and 2). Compared with β2_α4_β2_α4_^{W182A}α4, the estimated EC_{50} value for the lower affinity component of the curves was about 5.5-fold lower, showing that the effects of α4W182A is agonist-site specific. As for β2_α4_β2_α4_^{W182A}α4 receptors, desensitization associated to intact or mutant ACh sites was monitored by assaying the effects of ACh prepulses on currents evoked by either ACh $EC_{50\text{-}1}$ or ACh $EC_{50\text{-}2}$ (Figure 6B, D). Introduction of α4W182A into any of the α4(+)/β2(–) interfaces altered wild-type sensitivity to desensitization induced by ACh. Patterns of desensitization for intact or mutant agonist sites at β2_^{W148A}α4_β2_α4_α4 and β2_α4_β2_^{W148A}α4_α4 receptors were similar (Table 2). Comparisons of desensitization in β2_^{W148A}α4_β2_α4_α4, β2_α4_β2_^{W148A}α4_α4 and β2_α4_β2_α4_^{W148A}α4 receptors showed that although $IC_{50\text{-}1}$ values estimated for all three mutant receptors were not different from each other, $IC_{50\text{-}2}$ values for mutant agonist sites located at α4(+)/β2(–) interfaces were approximately sevenfold lower than the $IC_{50\text{-}2}$ value for mutant α4(+)/(–)α4 agonist site ($P < 0.05$; $n = 5$–8; Table 2).

Discussion and conclusions

All agonist sites in $(α4β2)_2α4$ receptors contribute to agonist-induced desensitization. The ACh site at the α4(+)/α4(–) interface endows $(α4β2)_2α4$ receptors their signature ACh sensitivity (Mazzaferro *et al.*, 2011), and decreases sensitivity to ACh-induced desensitization, compared with $(α4β2)_2β2$ receptors, which possesses an agonist site at each of its two α4(+)/β2(–) subunit interfaces. The α4(+)/α4(–) interface also bestows sensitivity to potentiation by Zn^{2+} (Moroni *et al.*, 2008) and NS9283, an allosteric modulator selective for nAChRs with a $(αβ)_2α$ stoichiometry (Timmermann *et al.*, 2012). Hence, the signature α4(+)/α4(–) interface defines both the competitive and allosteric ligand sensitivity of $(α4β2)_2α4$ receptors, compared with $(α4β2)_2β2$ receptors, which makes it a promising target for the development of α4β2-stoichiometry-specific compounds.

The desensitization of α4β2 nAChRs is a complex process. Previous studies on α4β2 nAChRs of unknown stoichiometry have shown the existence of two desensitized states (Fenster *et al.*, 1997; Buisson *et al.*, 2000; Paradiso and Steinbach, 2003). Recovery from desensitization is also biphasic, although a third desensitized state may also exist (Paradiso and Steinbach, 2003). High-affinity desensitization and

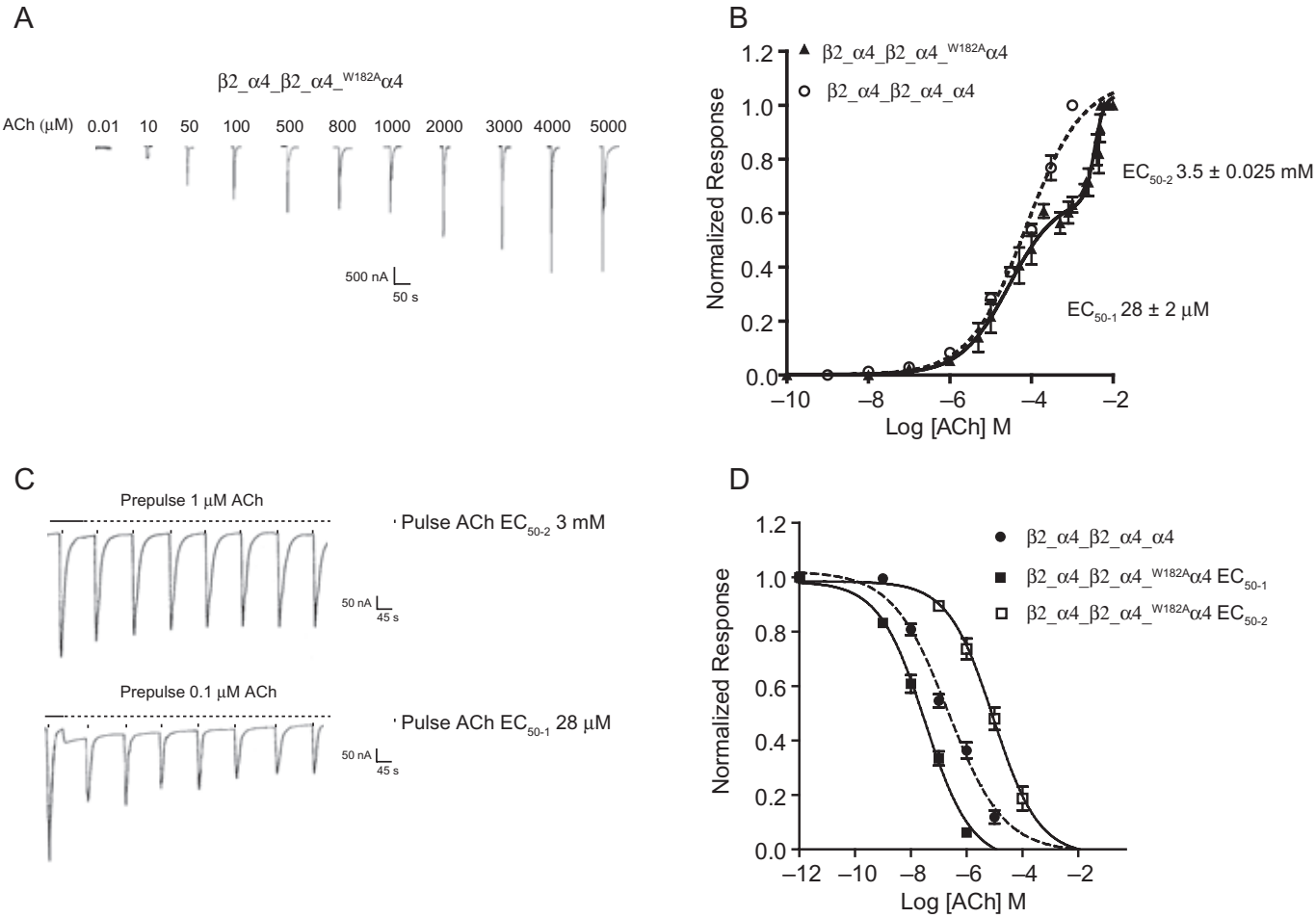

Figure 5

Effect of impairing the ACh binding site at the α4(+)/α4(–) interface of β2_α4_β2_α4_α4 receptors on high-affinity desensitization. (A) The response of β2_α4_β2_α4_^{W182A}α4 nAChRs to applications of ACh at 0.01 μM to 5 mM. (B) The responses of β2_α4_β2_α4_^{W182A}α4 receptors to ACh (1–5 mM) were best fitted to the biphasic Hill equation described in Methods ($P < 0.001$; $n = 10$). The biphasic curve comprises a component with comparatively greater sensitivity to ACh ($EC_{50\text{-}1}$ 28 ± 2 μM) and a component with about 124-fold lower sensitivity to ACh ($EC_{50\text{-}2}$ 3483 ± 2 μM). We have shown previously that the component with greater ACh sensitivity is contributed mostly by the non-mutated agonist sites at the α4(+)/β2(–) interface, while the lower sensitivity component is contributed mostly by the mutated site at the α4(+)/α4(–) interface (Mazzaferro *et al.*, 2011). For comparison wild-type ACh concentration–response curve is included (dashed lines). (C) Representative traces showing the desensitization of ACh currents evoked by ACh $EC_{50\text{-}1}$ (non-mutated agonist sites) or ACh $EC_{50\text{-}2}$ (mutated α4(+)/α4(–) site). ACh prepulses were applied by adding ACh to the perfusing Ringer solution (solid lines). (D) Desensitization concentration–response curves of ACh EC_{50} responses of mutated α4(+)/α4(–) interface or intact α4(+)/β2(–) interfaces. The peak currents evoked by ACh $EC_{50\text{-}1}$ or ACh $EC_{50\text{-}2}$ measured at the end of the ACh prepulses were normalized to control ACh currents (ACh currents evoked prior to application of prepulses). The inhibition data were best fitted to the monophasic Hill equation described in Methods ($P < 0.001$; $n = 8$). For comparison, the wild-type desensitization concentration–response curve is included (dashed lines).

recovery from desensitization of α4β2 nAChRs are both agonist dependent, which likely reflects differential affinity of agonists for the desensitized states (Marks *et al.*, 2010; Paradiso and Steinbach, 2003). Our findings add further complexity to the activation/desensitization of α4β2 nAChRs by showing that the effects of α4W182A on desensitization (and activation) are agonist-site specific. This strongly suggests that the agonist sites of $(α4β2)_2α4$ receptors have different sensitivity to both stimulation by ACh and high-affinity desensitization. Moreover, the observation that impairment of any of the agonist sites by α4W182A produced biphasic sensitivity to activation and that a small fraction (less than 10%) of the maximal ACh response desensitized with higher sensitivity suggests that two agonist sites, or perhaps one, when engaged by agonist, can lead to activation and then desensitization with a sensitivity that is different from that observed with full occupancy. Together, these findings suggest that the agonist sites in $(α4β2)_2α4$ receptors are not functionally equivalent.

Functional differences between the agonist sites in $(α4β2)_2α4$ nAChRs might arise from differences in the structure and downstream coupling/gating pathways (Auerbach, 2010). The primary face in the agonist site at α4(+)/α4(–) and α4(+)/β2(–) interfaces are identical but the complementary

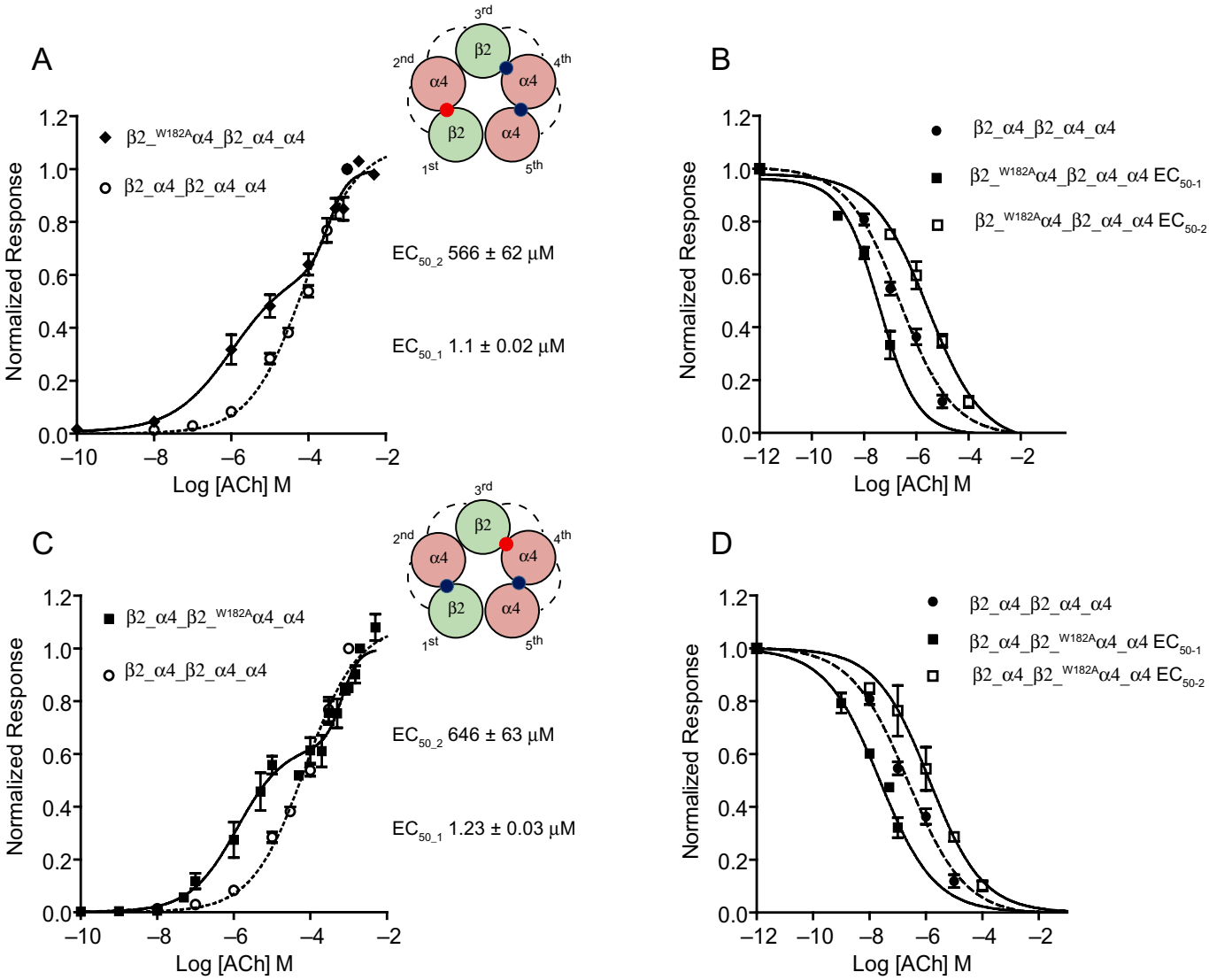

Figure 6

Mutant ACh agonist sites at α4(+)/β2(–) interfaces affect activation and high-affinity desensitization of β2_α4_β2_α4_α4 nAChRs. (A, C) Activation ACh concentration–response curve for β2_^{W182A}α4_β2_α4_α4 or β2_α4_β2_^{W182A}α4_α4 receptors. The sensitivity of β2_^{W182A}α4_β2_α4_α4 or β2_α4_β2_^{W182A}α4_α4 receptors to activation by ACh is biphasic in comparison to wild type (dashed lines; $P < 0.001$). Non-mutated agonist sites have higher sensitivity to ACh (EC_{50-1}) than the impaired ACh site (EC_{50-2}). Insets show the position of the mutated agonist sites in the concatamers. (B, D) Desensitization concentration–response curves of ACh responses of mutated α/β and intact agonist sites in β2_^{W182A}α4_β2_α4_α4 or β2_α4_β2_^{W182A}α4_α4 receptors. The peak currents evoked by ACh EC_{50-1} or ACh EC_{50-2} measured at the end of ACh prepulses were normalized to control ACh currents (ACh currents evoked prior to application of prepulses). The inhibition data were best fitted to the monophasic Hill equation described in Methods ($P < 0.002$; $n = 5$ for β2_^{W182A}α4_β2_α4_α4 and $n = 8$ for β2_α4_β2_^{W182A}α4_α4 receptors). For comparison, the wild-type desensitization concentration–response curve is included (dashed lines). There is no significant difference in the activation or high-affinity desensitization of β2_^{W182A}α4_β2_α4_α4 and β2_α4_β2_^{W182A}α4_α4 receptors (see Table 2).

face is not. In the case of the agonist site at the α4(+)/α4(–) interface, the complementary face is contributed by the (–) face of the adjacent α4 subunit. Studies of $GABA_A$ receptors, which are endowed with two structurally identical agonist sites, have shown that structurally identical agonist sites may function non-equivalently (Baumann *et al.*, 2003). In the case of $GABA_A$ receptors, non-equivalency may arise from differences in the subunits flanking each agonist site, which could produce agonist-site specific conformational transitions, and hence, agonist-site specific activation and/or desensitization states (Baumann *et al.*, 2003). However, previous studies of muscle nAChRs have shown that even though the two agonist sites have different complementary subunits (δ or γ/ε), they are functionally comparable, with each having approximately the same affinity for ACh in the open and closed states of the receptor (Jha and Auerbach, 2010).

Incorporation of α4W182A into any of the agonist binding sites decreased sensitivity of the site to both activation and desensitization, but the effects were more striking on activation, consistent with the key role of α4W182 in ACh

binding (Corringer *et al.*, 1998; Mazzaferro *et al.*, 2011). The effect of α4W182 on desensitization confirms earlier findings that high-affinity desensitization is partly influenced by amino acid residues in the N-terminal domain of the α4 subunit. Other regions that have also been implicated in high-affinity desensitization are transmembrane domain M2 (Bertrand *et al.*, 1998), and trasmembrane M1 and M3 (Kuryatov *et al.*, 2000). The mechanism connecting the open receptor states to the desensitized states are not fully understood (Auerbach, 2010). Recent studies on the α7 nAChR have suggested that desensitization of nAChRs may be governed by the balance between agonist binding, coupling and gating (Zhang *et al.*, 2011). According to this proposal the energy from agonist binding induces conformational changes in the coupling region (N-terminal loops 2 and 7 and M2–M3 linker from the transmembrane domain) that eventually result in channel opening. However, the coupling region tends to uncouple due to instability of the open state, as compared with the closed state, triggering desensitization (Zhang *et al.*, 2011). This possibility is consistent with our finding that weakening agonist binding by incorporation of W182A in β2_α4_β2_α4_α4 receptors decreases sensitivity to desensitization. Presumably, W182A decreases coupling strength, which would affect the critical balance between coupling and gating towards a decrease in sensitivity to desensitization.

Considering the function of $(\alpha4\beta2)_2\alpha4$ nAChRs in comparison to $(\alpha4\beta2)_2\beta2$ nAChRs, it seems that full agonist occupation in $(\alpha4\beta2)_2\alpha4$ nAChRs decreases sensitivity to both activation and high-affinity desensitization. Recent studies on α7 nAChRs assembled from PNU-120596-sensitive and PNU-120596-resistant α7 subunits have suggested that heteromeric receptor desensitization is determined by the fastest desensitizing subunit (daCosta and Sine, 2013). Because our data suggest that engagement of two binding sites by agonist activate and desensitize $(\alpha4\beta2)_2\alpha4$ nAChRs with sensitivities typical of $(\alpha4\beta2)_2\beta2$ nAChRs, it seems that for $(\alpha4\beta2)_2\alpha4$ nAChRs occupation of the third agonist site modulates the function of the two other agonist sites by decreasing their sensitivity to high-affinity desensitization. Modulation could be exerted at the gate or could be transmitted to the adjacent binding sites to affect agonist binding, and hence, the energy transitions leading to gating. Modulation of the function of two agonist sites by a third agonist site has been previously observed in homomeric 5HT3-α7 chimeric receptors, although in this receptor full occupancy increases sensitivity to activation and possibly to acute desensitization (Rayes *et al.*, 2009).

We do not have a kinetic scheme that could represent adequately the activation and desensitization of the $(\alpha4\beta2)_2\alpha4$ receptors because we cannot estimate from our data the affinity of each type of agonist site for the various states of the receptors, including closed, open and desensitized or how these various states are connected. Single-channel analysis of the function of this receptor type should help to address this issue. Notwithstanding this lack of detail, our data strongly suggest that occupancy of two agonist sites is sufficient to activate and desensitize the $(\alpha4\beta2)_2\alpha4$ nAChR and that full occupation adds an additional active and desensitized state. From our data, these additional states are likely to be reached at concentrations of ACh producing more than 10% stimulation of the maximal response, from which point receptor function appears to be shaped by the modulatory activity exerted by the third agonist site.

Finally, studies of mouse thalamic α4β2 nAChR receptors, which show biphasic agonist concentration–response curves for stimulation of putative $(\alpha4\beta2)_2\alpha4$ and $(\alpha4\beta2)_2\beta2$ nAChR function, have suggested that $(\alpha4\beta2)_2\alpha4$ and $(\alpha4\beta2)_2\beta2$ receptors have similar sensitivity to high-affinity desensitization, although the receptors differ in their window current due to differences in agonist sensitivity (Marks *et al.*, 2010). We found that the receptors desensitize with different sensitivities. Although possible artefacts introduced by concatenation cannot be discarded, our findings may differ from those reported by Marks *et al.* (2010) because native α4β2 nAChR function reflects stimulation of α4β2 and α4β2α5 nAChRs (Gotti *et al.*, 2008; Marks *et al.*, 2010; Grady *et al.*, 2012).

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Statement of conflict of interests

No competing interests.

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