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The Nicotinic $\alpha5$ Subunit Can Replace Either an Acetylcholine-Binding or Nonbinding Subunit in the $\alpha4\beta2$* Neuronal Nicotinic Receptor

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ABSTRACT

Heteropentameric neuronal nicotinic receptors assemble so that the canonical acetylcholine-binding sites are located at the interfaces between two pairs of subunits, while the fifth subunit does not participate in a canonical transmitter-binding site. Several subunits are considered to be unable to participate in forming a functional receptor when they occupy a position that would contribute to such a site, including the $\alpha5$ subunit. The $\alpha5$ subunit is of interest because of its apparent involvement in nicotine dependence and in the control of dopamine release. We have examined this question using $\alpha4$ and $\beta2$ subunits in concatemeric constructs with the $\alpha5$ subunit, expressed in *Xenopus* oocytes. Using dimeric constructs of $\alpha4$ and $\beta2$ subunits expressed with free $\alpha5$ and pentameric constructs incorporating a single copy of $\alpha5$, we find that the $\alpha5$ subunit can occupy the position of a nonbinding subunit, or replace a $\beta2$ subunit participating in a canonical binding site. The resulting receptors functionally resemble pentamers assembled with two copies of $\alpha4$ and three copies of $\beta2$. Functional receptors apparently cannot be formed with $\alpha5$ subunits in both canonical binding sites. These observations extend the present ideas on the possible positions in the pentamer that may be occupied by the $\alpha5$ subunit, and suggest that additional physiologic or pharmacological subtypes of neuronal nicotinic receptors may be present in neurons.

Introduction

Pentameric ligand-gated ion channels are members of a gene family including the nicotinic, $GABA_A$, glycine, and serotonin type A receptors in vertebrates and a number of related channels in invertebrates and prokaryotes. The neuronal nicotinic receptors can form as pentamers of a single subunit (the $\alpha7$ subunit), but many are heteropentameric and contain both α ($\alpha2$–$\alpha6$) and β ($\beta2$–$\beta4$) subunits in a variety of stoichiometries (Gotti et al., 2007). Recently the $\alpha5$ subunit has been of particular interest since it was found that a nonsynonymous coding variant of this subunit is significantly associated with an increased risk of developing nicotine dependence (Bierut et al., 2008). Further, the level of $\alpha5$ in the medial habenula determines the aversive response to nicotine (Frahm et al., 2011), and receptors containing the $\alpha5$ subunit along with $\alpha4$ and $\beta2$ are critical in regulating dopamine release in the dorsal striatum (Exley et al., 2012). In heteropentameric receptors the canonical acetylcholine (ACh) binding sites are located at the interface between an α and a β subunit, in which the α subunit contributes the principal (or +) face and the β subunit the complementary (or −) face (Gotti et al., 2007; Mazzaferro et al., 2011; see Fig. 1). This means that there is one subunit in the heteropentamer that does not contribute to such a canonical site (the fifth subunit). It has been proposed that the $\alpha5$ subunit, in particular, cannot incorporate into a functional receptor in a position at which it contributes to a canonical binding site (Brown et al., 2007; Kuryatov et al., 2008). However, it can incorporate efficiently into the fifth subunit position and thereby affect the properties of the pentameric receptor (Gerzanich et al., 1998; Groot-Kormelink et al., 2001; Kuryatov et al., 2008). It should be noted that recent studies have shown that the fifth subunit in $\alpha4\beta2$ receptors can participate in a novel agonist-binding site; in particular, adjacent $\alpha4$ subunits form an ACh-binding site in which the fifth subunit contributes the principal face (Harpsøe et al., 2011; Mazzaferro et al., 2011). This expands the possible physiologic importance of incorporating the $\alpha5$ subunit.

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ABBREVIATIONS: 5I A85380, 5-iodo-3-[2(*S*)-azetidinylmethoxy]pyridine; ACh, acetylcholine.

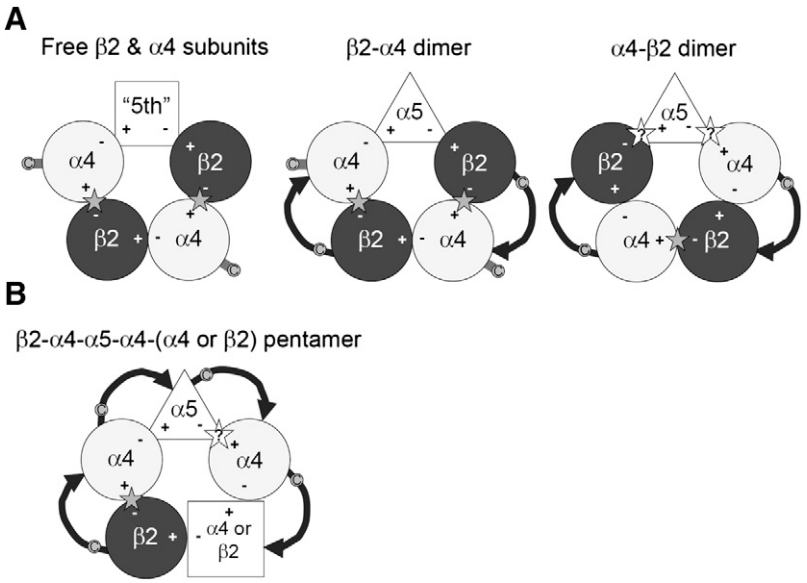

Fig. 1. Structure of $\alpha4\beta2$ receptor pentamers. (A) Receptors composed of free $\alpha4$ and $\beta2$ subunits (left) and receptors expressed when a dimeric concatemer is expressed with a free $\alpha5$ subunit (middle and right). The receptors are diagrammed as viewed from the extracellular space. The subunits are arranged in a rosette around the ion channel. Canonical ACh-binding sites (indicated by stars) are located at the interface between the $\alpha4$ (contributing the principal or + face) and the $\beta2$ subunit. The fifth subunit (indicated by the square) does not participate in an interface contributing to a canonical site. The middle panel shows the pentamer formed when $\beta2$-$\alpha4$ dimers are expressed with a free $\alpha5$ subunit (indicated by a triangle). The free subunit occupies the position of the fifth subunit. The right panel shows the pentamer formed when $\beta\alpha4$-$\beta2$ dimers are expressed with a free subunit. The free subunit occupies a position participating in a canonical binding interface, indicated by stars with question marks. The dimers are shown with the linking sequence as a curved line with the arrowhead indicating the N- to C-terminal direction. (B) The $\beta2$-$\alpha4$-$\alpha5$-$\alpha4$-($\alpha4$ or $\beta2$) pentameric concatemer containing the $\alpha5$ subunit in the middle position. In this construct one $\alpha4/\beta2$ interface is formed (indicated by the stars with dashed outline) and an $\alpha4/\alpha5$ interface. The subunit order is as proposed in Mazzaferro et al. (2011), but because the $\alpha5$ has an $\alpha4$ subunit on either side, the same $\alpha4/\alpha5$ interface would be formed irrespective of a reversal of order.

We examined the ability of the $\alpha5$ subunit to form functional receptors with the $\alpha4$ and $\beta2$ subunits, using concatemeric constructs of subunits to define the number and position of subunits in the pentamer. The results indicate that the $\alpha5$ subunit can assemble in the place of a $\beta2$ subunit at a position that would form a canonical ACh-binding site.

Materials and Methods

Constructs and Expression. We used human $\alpha4$ (NM000744), $\beta2$ (NM000748), and $\alpha5$ (NM000745) subunits. The generation of the dimeric constructs $\alpha4$-$\beta2$ and $\beta2$-$\alpha4$ is described in Jin and Steinbach (2011). The pentameric constructs $\beta2$-$\alpha4$-$\beta2$-$\alpha4$-$\alpha4$ and $\beta2$-$\alpha4$-$\beta2$-$\alpha4$-$\beta2$ are described in Carbone et al. (2009). The $\alpha5$ subunit was inserted into a pentamer by mutational insertion of the appropriate linker sequences: the sequences for the signal peptide at the 5′ end and the stop codon at the 3′ end were removed and replaced by the linker. This construct was then restriction digested and inserted into the position of the middle $\beta2$ subunit in the pentamers. All constructs were fully sequenced through the inserted receptor sequence. In the pentamers, subunits were excised using the appropriate restriction enzymes and sequenced independently to verify that each copy was intact. RNA was synthesized using the mMessage mMachine T7 kit (Ambion, Austin, TX). The concentration of RNA was estimated from the optical density measured at 260 nm.

Xenopus oocytes were prepared in Dr. C. Zorumski's laboratory (Washington University, St. Louis, MO) using an approved protocol. Oocytes were injected with 12–20 ng of cRNA in a volume of 18–23 nl. Oocytes were maintained at 18°C for 2–7 days before physiologic study.

Electrophysiology. Standard methods were used for two-electrode voltage clamp of *Xenopus* oocytes (Jin and Steinbach, 2011), using an OC-725C voltage clamp (Warner Instruments, Hamden, CT). Oocytes were clamped at −50 mV, and all recordings were made at room temperature (23–25°C). Currents were filtered at 20 Hz, then digitized at 50 Hz (Digidata 1200 interface; Molecular Devices, Sunnyvale, CA) and stored using pClamp 8.0 (Molecular Devices). Transients were analyzed with Clampfit (Molecular Devices). Oocyte recordings were performed in a small chamber that was continuously perfused with saline. Drug applications were made using a manually controlled perfusion system. The system was made with glass, stainless steel, or Teflon components to reduce steroid adsorption. The applications were relatively slow, with bath exchange times of ~1 second. The external solution contained (in mM): 96 NaCl, 2 KCl, 1.8 $BaCl_2$, 1 $MgCl_2$, and 10 HEPES; pH 7.3. External Ca^{2+} was replaced with Ba^{2+} to avoid activation of Ca^{2+}-activated channels. We did not use atropine to block muscarinic receptors, as it potentiates $\alpha4\beta2$ receptors (Zwart and Vijverberg, 1997). Occasional oocytes showed delayed responses to ACh; these oocytes were discarded.

The concentration-response relationship for activation by ACh was characterized for data from each cell using nonlinear regression in SigmaPlot (Systat Software, Chicago, IL) by fitting the Hill equation:

$$\left(Y([\mathrm{ACh}]) = Y_{\max}\left(\frac{1}{1+\left(\mathrm{EC}_{50}/[\mathrm{ACh}]\right)^{n_{\mathrm{Hill}}}}\right)\right)$$

where Y is the response to a concentration of ACh, Y_{max} is the maximal response, EC_{50} is the concentration producing half-maximal activation, and n_{Hill} is the Hill coefficient. Concentration-response data were collected for an individual cell, and data were normalized to the response to 1 mM ACh. The fit was rejected if the estimated error in any fit parameter was >60% of the fit value, and all parameter estimates for that fit were discarded. The relationship was analyzed for each cell, and overall mean values were then calculated for oocytes injected with that set of constructs.

Potentiation by 17β-estradiol or physostigmine is strongest for low concentrations of ACh (Paradiso et al., 2001; Curtis et al., 2002; Smulders et al., 2005). Because the EC_{50} for activation by ACh depends on the subunit combinations expressed (see *Results and Discussion*), each oocyte was tested with 1 mM ACh to estimate the maximal response. A low concentration of ACh, chosen to be able to evoke <20% of the maximal current, was then applied. After the response to ACh had reached a stable level, the application was switched to ACh plus 10 μM drug. The application was switched to bathing solution, followed by repeat of the control low concentration. The relative response in the presence of drug to that in the absence of drug was then calculated. Drug was not preapplied. ACh or ACh plus drug was applied for 10–20 seconds, and applications were separated by 3–4 minutes to allow full washout.

Values are presented as arithmetic mean ± S.E. (number of observations).

Drugs. 17β-Estradiol and ACh were purchased from Sigma-Aldrich (St. Louis, MO). 5I A85380 (5-iodo-3-[2(*S*)-azetidinylmethoxy]pyridine) and physostigmine hemisulfate (physostigmine) were purchased from Tocris (Ellisville, MO). 17β-Estradiol was prepared as a 20 mM stock solution in dimethylsulfoxide and diluted into external solution on the day of an experiment. ACh was prepared as a 1 M stock solution in bath solution and stored frozen at −20°C. 5I A85380 was prepared as a 50 μM stock solution in bath solution and stored frozen at −20°C. Physostigmine was prepared as a 10 mM stock in deionized water and stored frozen at −20°C. Working solutions were prepared on the day of experiments.

Results and Discussion

We used concatemeric constructs to express receptors containing α5 subunits in a defined stoichiometry and position in nicotinic α4β2* receptors. Receptors were expressed in *Xenopus* oocytes and responses were determined using two-electrode voltage clamp.

Initially we used dimeric concatemers containing a single α4 and a single β2 subunit, in either α4-β2 or β2-α4 orientation. Previous work (Zhou et al., 2003; Jin and Steinbach, 2011) has shown that these concatemers assemble in the pentamer in a counterclockwise fashion (Fig. 1), so that a free additional subunit takes the position either of the fifth (nonbinding) subunit when expressed with the β2-α4 concatemer or of

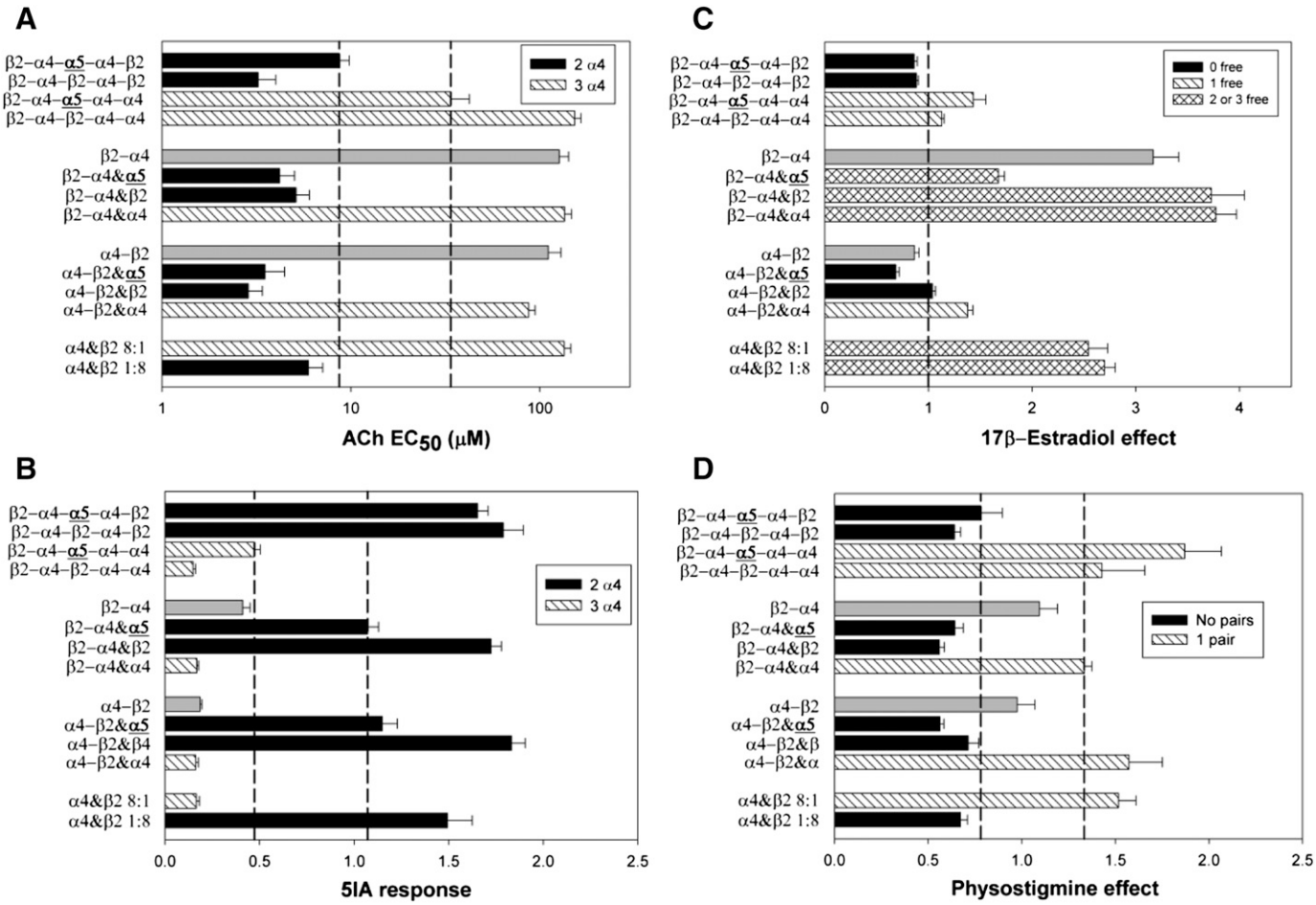

Fig. 2. Graphical depiction of the pharmacological profiles. (A and B) Responses to agonists [(A) ACh EC_{50} (note logarithmic abscissa); (B) response to 1 μM 5I A85380 relative to 1 mM ACh], while C and D show the effects of two modulating drugs [(C) effect of 10 μM 17β-estradiol; (D) 10 μM physostigmine]. The panels are formatted in the same way: at the bottom are free subunits at two different cRNA ratios, then a group of four receptors containing the α4-β2 concatemer, four receptors containing the β2-α4 concatemer, and four pentamers. The position of the α5 subunit is emphasized in bold and underlined. The bars are filled to indicate predicted structural features. In each panel, the bars corresponding to dimers expressed without a free subunit are filled with gray. In A and B, the solid fill indicates receptor predicted to have two copies of α4 and diagonal hatching indicates three copies of α4. In C, solid fill indicates no free (untethered) α4 C termini, diagonal hatching one free C terminus, and cross-hatching two or three free C termini. In D, solid fill indicates no adjacent α4 subunits, while diagonal hatching indicates a pair of adjacent α4 subunits. The bars in the panels show mean values ± S.E. Data values are presented in Table 1.

a subunit contributing to the canonical ACh-binding site interface when expressed with the $\alpha4$-$\beta2$ concatemer. To assess the number and position of subunits in the receptor pentamer, we used pharmacological tests. We determined the concentration of ACh that produced a half-maximal response (ACh EC_{50}), the relative current elicited by a 1 μM concentration of 5I 85380 (compared with 1 mM ACh), and the ability of 17β-estradiol to potentiate responses to a low concentration of ACh. Previous work has shown that receptors containing three copies of $\alpha4$ have an ACh EC_{50} near 100 μM while those with two copies have an ACh EC_{50} of $<$10 μM (Nelson et al., 2003; Moroni et al., 2006; Jin and Steinbach, 2011). 5I A85380 is a more efficacious agonist on receptors containing three copies of $\beta2$, with the response to 1 μM 5I A85380 larger than that to high ACh (~1.4×), while for receptors with three copies of $\alpha4$ the response is less (~0.3×) (Zwart et al., 2006; Jin and Steinbach, 2011). Finally, for 17β-estradiol to potentiate responses there must be a free (untethered) C terminus for an $\alpha4$ subunit (Paradiso et al., 2001; Zhou et al., 2003; Jin and Steinbach, 2011). In addition, we tested the ability of physostigmine to potentiate responses to a low concentration of ACh. As shown in Fig. 2 and Table 1, potentiation by physostigmine is seen when there are two adjacent $\alpha4$ subunits. We also determined the response to a high concentration of ACh (1 mM) as an estimate of the level of expression of functional receptors. The results are presented in Fig. 2 and Table 1.

We will use abbreviations for the constructs studied. Concatemers are named with subunits in order from the N terminus, so $\alpha4$-$\beta2$ indicates that the dimer begins with $\alpha4$ subunit sequence. When more than one construct was used, the constructs are separated by "&," so $\alpha4\&\beta2$ 1:8 indicates that both $\alpha4$ and $\beta2$ subunit cRNA were injected, at a 1:8 mass ratio.

The inclusion of three copies of $\alpha4$ ($\alpha4\&\beta2$ 8:1, $\alpha4$-$\beta2\&\alpha4$, $\beta2$-$\alpha4\&\alpha4$, or $\beta2$-$\alpha4$-$\beta2$-$\alpha4$-$\alpha4$) resulted in receptors that had a large EC_{50} for ACh (~100 μM), had a small relative response to 5I A85380 ($<$0.3), and showed potentiation by physostigmine (ratio $>$ 1.2). In addition, the response to 17β-estradiol reflected whether there was a free (untethered) C terminus for at least one $\alpha4$ subunit (e.g., comparing $\alpha4$-$\beta2\&\beta2$ to $\beta2$-$\alpha4\&\beta2$). In contrast, inclusion of three copies of $\beta2$ ($\alpha4\&\beta2$ 1:8, $\alpha4$-$\beta2\&\beta2$, $\beta2$-$\alpha4\&\beta2$, or $\beta2$-$\alpha4$-$\beta2$-$\alpha4$-$\beta2$) resulted in receptors that had a small EC_{50} for ACh ($<$10 μM), had a large relative response to 5I A85380 ($>$1.0), and showed block by physostigmine ($<$0.7). We also tested responses to the dimeric concatemers injected alone. Overall, these properties were more similar to receptors containing three copies of $\alpha4$ (Fig. 2; Table 1), but clearly distinct from either dimer expressed with free $\beta2$ subunits.

We then expressed dimeric constructs with free $\alpha5$ subunits. We tested both wild-type $\alpha5$ and $\alpha5$(D398N), as the $\alpha5$(D398N) variant is associated with an increased risk of developing nicotine dependence (Bierut et al., 2008). However, we saw no difference between these constructs in any of the parameters measured, so the results have been pooled. Injected oocytes expressed functional receptors, although the response to saturating concentrations of ACh was reduced compared with when $\alpha4$ was used as the free subunit (Table 1). In this case the pharmacological properties of the receptors were very similar to those of receptors containing three copies of $\beta2$ (Fig. 2; Table 1). The properties of the receptors containing free $\alpha5$ subunits are clearly distinct from those of receptors when the dimers are expressed in the absence of a free subunit, as shown in Fig. 2 and Table 1. Accordingly, even though the dimeric constructs can assemble to produce functional surface receptors, in the presence of free $\alpha5$ subunits the majority of the functional receptors contain the free subunit in addition to the dimer. These results suggest that the $\alpha5$ subunit can replace either the subunit that does not contribute to a canonical binding site (when expressed with the $\beta2$-$\alpha4$ dimer) or a subunit contributing to a binding site (when expressed with the $\alpha4$-$\beta2$ dimer). However, it is also possible that the presence of the $\alpha5$ subunit caused the dimers to assemble in different orientations, so that in association with the $\beta2$-$\alpha4$ dimer the $\alpha5$ subunit actually occupied the position that does not contribute to a canonical binding site.

TABLE 1

Summary of results

The first column gives the subunits injected. The first set of four rows gives results for receptors predicted to contain three copies of $\alpha4$ and two copies of $\beta2$, while the second set gives results for two copies of $\alpha4$ and three of $\beta2$. The third set of four rows shows data for receptors containing the $\alpha5$ subunit, again ordered based on the predicted number of copies of $\alpha4$. The final set of two rows shows data for dimeric concatemers expressed in the absence of a free subunit; the pattern of properties is distinct from a dimer expressed with a free subunit (although closest to receptors formed with a free $\alpha4$ subunit). The second column gives the number of $\alpha4$ subunits predicted to be in the receptor, while the third and fourth columns give the ACh EC_{50} and the response to 1 μM 5I A85380 relative to the response of the same oocyte to 1 mM ACh. The fifth and sixth columns give whether the predicted receptor has adjacent $\alpha4$ subunits and the observed effect of physostigmine. The seventh and eighth columns give the predicted number of untethered $\alpha4$ C termini in the receptor and the observed effect of 17β-estradiol. The final column gives the response to 1 mM ACh. All data are mean ± S.E. (number of observations).

Subunits	No. of $\alpha4$ Subunits	ACh EC_{50}	5I A85380 Relative Response	Adjacent $\alpha4$?	Physostigmine Effect	No. of Free $\alpha4$ C Termini	17 β-Estradiol Effect	Maximal Response
		μM	*relative to ACh*		*fold*		*fold*	*−nA*
$\alpha4\&\beta2$ 8:1	3	135 ± 11 (32)	0.17 ± 0.02 (17)	Yes	1.52 ± 0.09 (18)	3	2.54 ± 0.18 (23)	13,451 ± 1459 (45)
$\alpha4$-$\beta2\&\alpha4$	3	87 ± 7 (46)	0.16 ± 0.01 (41)	Yes	1.57 ± 0.18 (21)	1	1.38 ± 0.05 (35)	7238 ± 1179 (68)
$\beta2$-$\alpha4\&\alpha4$	3	135 ± 12 (52)	0.17 ± 0.01 (56)	Yes	1.33 ± 0.04 (34)	3	3.77 ± 0.20 (11)	14,454 ± 1301 (77)
$\beta2$-$\alpha4$-$\beta2$-$\alpha4$-$\alpha4$	3	153 ± 12 (9)	0.15 ± 0.01 (12)	Yes	1.43 ± 0.23 (8)	1	1.13 ± 0.02 (20)	2020 ± 265 (25)
$\alpha4\&\beta2$ 1:8	2	6 ± 1 (18)	1.49 ± 0.13 (18)	No	0.67 ± 0.04 (11)	2	2.70 ± 0.11 (4)	1103 ± 208 (43)
$\alpha4$-$\beta2\&\beta2$	2	3 ± 1 (14)	1.83 ± 0.07 (19)	No	0.71 ± 0.06 (6)	0	1.04 ± 0.03 (5)	341 ± 98 (32)
$\beta2$-$\alpha4\&\beta2$	2	5 ± 1 (40)	1.72 ± 0.05 (60)	No	0.56 ± 0.03 (15)	2	3.73 ± 0.32 (7)	819 ± 114 (73)
$\beta2$-$\alpha4$-$\beta2$-$\alpha4$-$\beta2$	2	3 ± 1 (16)	1.79 ± 0.11 (7)	No	0.64 ± 0.03 (14)	0	0.88 ± 0.02 (8)	110 ± 23 (20)
$\beta2$-$\alpha4$-$\alpha5$-$\alpha4$-$\alpha4$	3	34 ± 9 (2)	0.47 ± 0.03 (8)	Yes	1.87 ± 0.20 (6)	1	1.43 ± 0.12 (12)	24 ± 5 (30)
$\beta2$-$\alpha4\&\alpha5$	2	4 ± 1 (20)	1.07 ± 0.06 (31)	No	0.64 ± 0.04 (20)	2	1.68 ± 0.06 (7)	399 ± 67 (45)
$\alpha4$-$\beta2\&\alpha5$	2	4 ± 1 (9)	1.15 ± 0.08 (15)	No	0.56 ± 0.02 (19)	0	0.69 ± 0.03 (5)	436 ± 124 (20)
$\beta2$-$\alpha4$-$\alpha5$-$\alpha4$-$\beta2$	2	9 ± 1 (13)	1.65 ± 0.06 (17)	No	0.78 ± 0.11 (9)	0	0.86 ± 0.03 (10)	27 ± 4 (36)
$\beta2$-$\alpha4$	?	127 ± 15 (52)	0.41 ± 0.04 (72)	No	1.09 ± 0.10 (13)	≥2	3.17 ± 0.25 (6)	5155 ± 781 (82)
$\alpha4$-$\beta2$	?	111 ± 18 (24)	0.19 ± 0.01 (24)	No	0.98 ± 0.09 (9)	0	0.87 ± 0.05 (8)	859 ± 160 (37)

To address this concern we used pentameric concatemers. We first confirmed that the $\beta2$-$\alpha4$-$\beta2$-$\alpha4$-$\beta2$ and $\beta2$-$\alpha4$-$\beta2$-$\alpha4$-$\alpha4$ constructs behaved as would be predicted from the expected structure (Carbone et al., 2009; Mazzaferro et al., 2011). With $\beta2$-$\alpha4$-$\beta2$-$\alpha4$-$\alpha4$ the properties were consistent with there being three copies of $\alpha4$ present: the ACh EC_{50} was high; the response to 1 μM 5I A85380 was low. Furthermore, 17β-estradiol potentiated the response, indicating that the C-terminal $\alpha4$ sequence was present, and physostigmine potentiation indicated that there were adjacent $\alpha4$ subunits. In contrast, with $\beta2$-$\alpha4$-$\beta2$-$\alpha4$-$\beta2$ the properties of the receptor were consistent with only two copies of $\alpha4$ being present (Fig. 2;

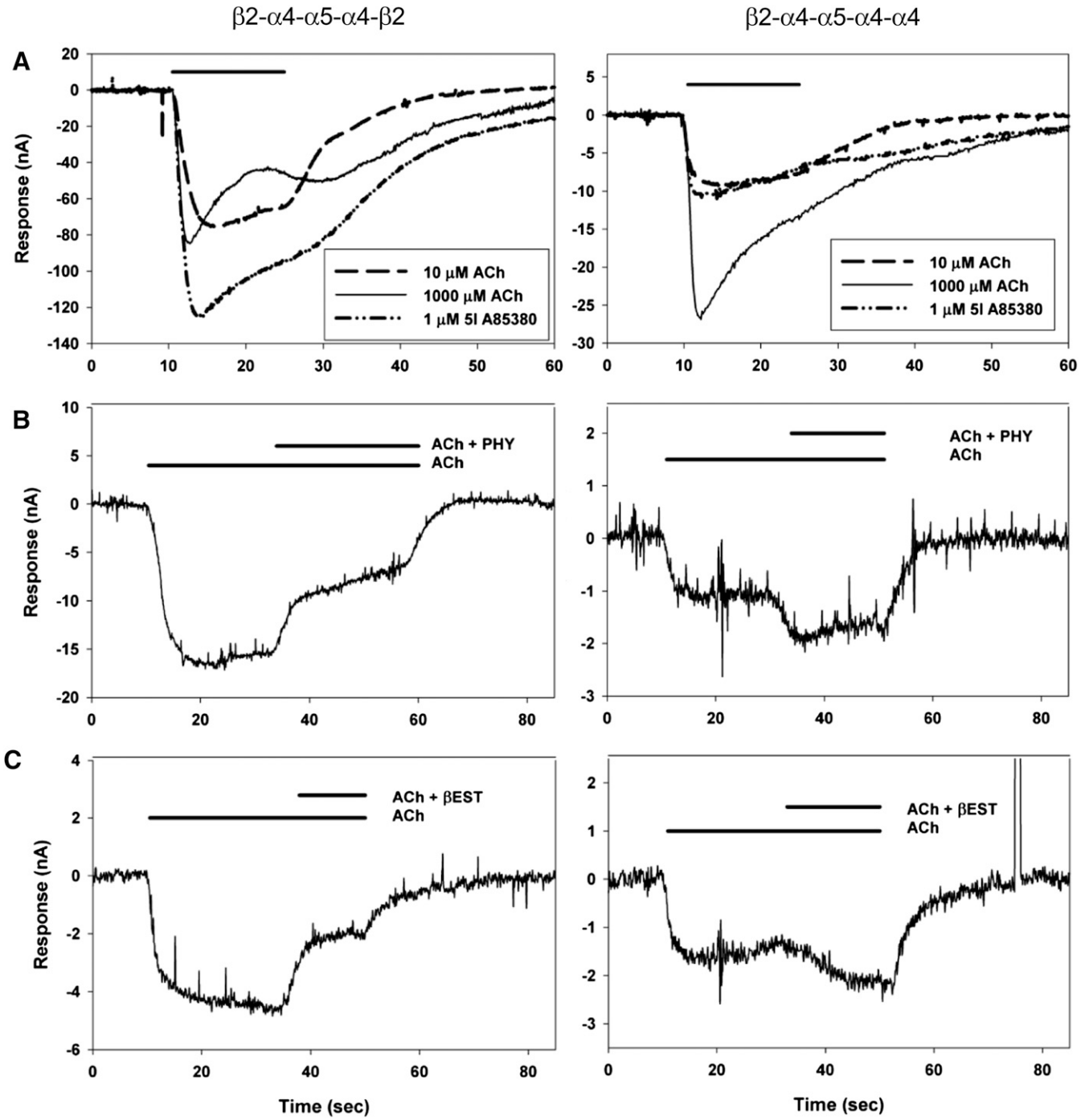

Fig. 3. Responses of pentamers containing $\alpha5$. The left column shows responses of receptors formed from $\beta2$-$\alpha4$-$\alpha5$-$\alpha4$-$\beta2$ pentamers, and the right column from $\beta2$-$\alpha4$-$\alpha5$-$\alpha4$-$\alpha4$. (A) Responses to agonists; note that in the left column 5I A85380 produces a larger response than 1 mM ACh, and that 10 μM ACh is clearly a more than half-maximal concentration. In contrast, in the right column 5I A85380 produces a smaller response than 1 mM ACh, and 10 μM ACh is clearly a less than half-maximal concentration. (B and C) Responses to 0.3 μM ACh; then the application is switched to 0.3 μM ACh plus 15 μM physostigmine (PHY; B) or 10 μM 17β-estradiol (βEST; C). Both drugs inhibit responses of $\beta2$-$\alpha4$-$\alpha5$-$\alpha4$-$\beta2$ pentamers but potentiate responses of $\beta2$-$\alpha4$-$\alpha5$-$\alpha4$-$\alpha4$. Each frame in A shows responses from one oocyte, while each frame in B and C shows responses from separate oocytes.

Table 1), without either a free α4 C terminus (indicated by the absence of potentiation by 17β-estradiol) or adjacent α4 subunits (indicated by the absence of potentiation by physostigmine). To examine the properties of receptors containing α5 we replaced the central β2 subunit in each pentamer so that α5 was flanked on both sides by an α4 subunit (Fig. 1). In these constructs α5 would occupy the position of β2 irrespective of whether the concatemer assembled in a clockwise direction (as found by Mazzaferro et al., 2011) or a counterclockwise direction in the pentameric receptor. As shown in Figs. 2 and 3 and Table 1, the inclusion of the α5 subunit resulted in receptors whose properties resemble those of the receptors formed by the original, β2-containing, pentameric concatemers. That is, the pharmacological properties of the receptors containing α5 in place of β2 support the idea that each receptor contains the subunits in a single pentameric construct. They are not the result of receptors assembled with contributions from multiple concatemers or concatemers with subunits derived from proteolysis of concatemers. These results strongly support the idea that the α5 subunit can assemble to produce functional receptors even when it occupies the position of a β2 subunit at a canonical ACh-binding site.

A concern in using subunit concatemers is that the linker sequences or the physical constraints imposed in linking subunits will alter receptor properties. In the case of these constructs, this does not seem to be the case. Examination of the data in Table 1 indicates that for receptors containing α4 and β2 subunits, the properties of receptors containing three copies of α4 are indistinguishable for receptors derived from free subunits, dimers, and pentamers except for the expected effects on potentiation by 17β-estradiol. Similarly, for receptors containing two copies of α4, the properties are indistinguishable (again, except for 17β-estradiol). Accordingly, there is no indication that the linkers per se affect properties for the present studies.

We also generated pentameric β2-α5-β2-α4-α4 concatemers in which the second position (occupied by α4) was replaced by α5. In this case the α5 subunit would be constrained to replace an α4 subunit that contributes to a canonical binding site. These constructs expressed so poorly that reliable data could not be obtained (data not shown). Previous work has shown that the α5 subunit does not assemble to produce functional receptors when expressed with the β2, β3, or β4 subunits (Boulter et al., 1990), so it seems unlikely that the α5 subunit can replace an α4 subunit at a position producing a canonical ACh-binding site. We found that no functional receptors were produced when we injected free α4 and free α5 subunits (data not shown), which indicates that there must be at least one α4/β2 interface in an α4β2α5 receptor.

The efficiency of expression of functional surface receptors containing the α5 subunit appears to be low. A previous study (Kuryatov et al., 2008) found that surface expression of α4β2* was reduced by α5, although total (surface plus intracellular) expression was actually increased. They proposed that the α5 subunit might enhance formation of unproductive oligomers that could not associate to form pentamers and successfully traffic to the cell surface (Kuryatov et al., 2008).

In sum, the α5 subunit is not restricted to occupying only the position in a receptor of the subunit that does not contribute to a canonical binding site. In the pentameric constructs we used, the α5 subunit contributes to both α4/α5 and α5/α4 interfaces, in the first case replacing a β2 subunit at an interface containing a canonical site and in the second case possibly contributing to a noncanonical (α4/α4) site (Harpsøe et al., 2011; Mazzaferro et al., 2011). When expressed with the α4-β2 dimeric construct, the α5 subunit would contribute to interfaces containing a canonical site, most likely replacing β2 to generate an α4/α5 site. However, it is not clear that the α4/α5 interface actually forms a functional ACh-binding site. Previous work has shown that it is possible to mutate residues in a canonical binding site to render that site ineffective, and still form functional (albeit impaired) receptors (Mazzaferro et al., 2011). Still, functional receptors can form with α5 replacing a subunit contributing to a canonical site. The physiologic consequences of an α4β2* receptor containing the α5 subunit in place of a β2 subunit at a canonical binding site are not yet known, since in the assays that we have used the α4β2* receptor containing the α5 subunit in place of a β2 subunit at a canonical binding site is indistinguishable from one with β2 or α5 in the fifth subunit position. Additional experiments will be required to define the consequences, including a possible effect on the ability of nicotine to upregulate the surface expression of the receptor (Kishi and Steinbach, 2006; Kuryatov et al., 2008; Mao et al., 2008).

Authorship Contributions

Participated in research design: Jin, Steinbach.
Conducted experiments: Jin.
Contributed new reagents or analytic tools: Bermudez, Jin.
Performed data analysis: Jin, Steinbach.
Wrote or contributed to the writing of the manuscript: Bermudez, Jin, Steinbach.

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