The E loop of the transmitter-binding site is a key determinant of the modulatory effects of physostigmine on neuronal nicotinic $\alpha_4\beta_2$ receptors

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Running title: The E loop is a key determinant for physostigmine action

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Number of text pages: 19
Number of Tables: 4
Number of Figures: 4
Number of references: 33
Number of words: Abstract: 233
Number of words: Introduction: 499
Number of words: Discussion: 1502

List of nonstandard abbreviations.

ACh: acetylcholine chloride, 2-Acetoxy-N,N,N-trimethylethanaminium, CAS 51-84-3; Zn\(^{2+}\): zinc chloride, CAS 7646-85-7; carbamylcholine: 2-[(Aminocarbonyl)oxy]-N,N,N-trimethylethanaminium chloride, CAS 51-83-2.
Abstract

Physostigmine is a well-known inhibitor of acetylcholinesterase, which can also activate, potentiate and inhibit acetylcholine receptors, including neuronal nicotinic receptors comprising α4 and β2 subunits. We have found that the two stoichiometric forms of this receptor differ in the effects of physostigmine. The form containing 3 copies of α4 and 2 of β2 was potentiated at low concentrations of ACh and physostigmine, while the form containing 2 copies of α4 and 3 of β2 was inhibited. Chimeric constructs of subunits indicated that the presence of inhibition or potentiation depended on the source of the extracellular ligand-binding domain of the subunit. Further sets of chimeric constructs demonstrated that a portion of the ACh-binding domain, the E loop, is a key determinant. Transferring the E loop from the β2 subunit to the α4 subunit resulted in strong inhibition, while the reciprocal transfer reduced inhibition. We expressed chimeric constructs with subunit dimers, to control the number and position of the incorporated chimeric subunits. Surprisingly, incorporation of a subunit with an altered E loop had similar effects whether it contributed either to an intersubunit interface containing a canonical ACh-binding site or to an alternative interface. The observation that the α4 E loop is involved suggests that physostigmine interacts with regions of subunits that contribute to the ACh-binding site, while the lack of interface specificity indicates that interaction with a particular ACh-binding site is not the critical factor.
Introduction

Physostigmine is best known as an inhibitor of the enzyme acetylcholinesterase (Triggle et al., 1998). In addition to its action on acetylcholinesterase, physostigmine was one of the first drugs characterized as an allosteric activator of neuronal nicotinic receptors (Storch et al., 1995) and as a potentiating drug for receptors containing the nicotinic α4 subunit (Zwart et al., 2000; Smulders et al., 2005). There has been recent interest in the ability of physostigmine and other drugs to act as “allosteric potentiating ligands” for neuronal nicotinic receptors, due to possible therapeutic uses in, for example, treatment of dementias including Alzheimer’s disease (Triggle et al., 1998; Maelicke and Albuquerque, 2000). Physostigmine can also inhibit nicotinic receptors, an action thought to result from block of open channels (Smulders et al., 2005).

We examined the actions of physostigmine on the neuronal nicotinic receptor containing the α4 and β2 subunits. This is the most prevalent type of nicotinic receptor in the brain (Dani and Bertrand, 2007; Gotti et al., 2007), where nicotinic receptors are thought to play an important, if subtle, role to modulate the release of transmitters (McGehee and Role, 1995; Dani and Bertrand, 2007). The α4β2 receptors can assemble in two functional stoichiometries, one containing 3 copies of α4 and 2 copies of β2 and the other 2 copies of α4 and 3 of β2. These two stoichiometric forms have distinct pharmacological properties, including the potency of ACh at activating the receptor (Zwart and Vijverberg, 1998; Zhou et al., 2003; Moroni et al., 2006). Previous studies of the action of physostigmine had utilized the form containing 3 copies of the α4 subunit (Zwart et al., 2000; Smulders et al., 2005), and found that low concentrations of physostigmine potentiated the response to low concentrations of acetylcholine. However, we reported that physostigmine potentiated the form containing 3 copies of α4, while inhibiting the form containing 2 copies (Jin et al., 2014). These results suggested that the actions of physostigmine may be more complex than appreciated.

To further examine the subunit dependence we studied the effects of physostigmine on receptors containing various chimeric constructs, expressed with concatemeric constructs to
allow us to control the number and position of the chimeric subunits in the assembled receptor. We find that the potentiating and inhibiting actions of physostigmine map to the amino-terminal, extracellular domain of the subunits, where the key determinant is “E loop” that forms part of the complementary side of the ACh-binding site. When the E loop of the \( \beta_2 \) subunit was transferred to the \( \alpha_4 \) subunit potentiation was lost and physostigmine inhibition was strong. Transferring the E loop from the \( \alpha_4 \) to the \( \beta_2 \) subunit reduced the extent of inhibition but did not confer potentiation. Surprisingly, the position of the transferred E loop does not appear to be critical: similar effects are seen when the transferred E loop is present at an interface formed by 2 \( \alpha_4 \) subunits (\( \alpha_4/\alpha_4^* \), where the * indicates a subunit with altered sequence) or a \( \beta_2 \) and an \( \alpha_4 \) subunit (\( \beta_2/\alpha_4^* \)).
Materials and Methods

Constructs and expression

We used human α4 (NM000744) and β2 (NM000748) subunits kindly provided by Dr. J. Lindstrom (University of Pennsylvania, Philadelphia PA). The generation of the dimeric constructs α4-β2 and β2-α4 has been described (Jin and Steinbach, 2011). The pentameric constructs β2-α4-β2-α4-α4 and β2-α4-β2-α4-β2 have also been described (Carbone et al., 2009). All constructs were fully sequenced through the subunit 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 mMachne T7 kit (Ambion, Austin TX). The concentration of RNA was estimated from the OD260 value.

Xenopus oocytes were prepared in Dr. C. Zorumski's laboratory (Washington University, St. Louis MO) using an approved protocol. Oocytes were injected with 12 to 20 ng of cRNA in a volume of 18 to 23 nL. Oocytes were maintained at 18 °C for 2 to 7 days before physiological 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 unless noted otherwise, 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 which was continuously perfused with external solution. Drug applications were made using a manually controlled perfusion system. The external solution contained (in mM): 96 NaCl, 2 KCl, 1.8 BaCl2, 1 MgCl2, and 10 HEPES, pH 7.3. External Ca2+ was replaced with Ba2+, to avoid activation of Ca2+ activated channels. We did not use atropine to block muscarinic receptors, as it potentiatates α4β2 receptors (see Results and Zwart and
Vijverberg, 1997). Occasional oocytes showed delayed responses to ACh; these oocytes were not studied.

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

\[
Y([ACh]) = \text{Ymax} \left(1 / \left(1 + \left(\text{EC}_{50}/[ACh]\right)^n\text{Hill}\right)\right),
\]

where \(Y\) is the response to a concentration of ACh, \(\text{Ymax}\) is the maximal response, \(\text{EC}_{50}\) is the concentration producing half-maximal activation, and \(n\text{Hill}\) 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 greater than 60% of the fit value, and all parameter estimates for that fit were discarded. The relationship was analyzed for each cell and then overall mean values were calculated for oocytes injected with that set of constructs.

Potentiation by physostigmine and other modulators is strongest for low concentrations of ACh (Smulders et al., 2005). Since the \(\text{EC}_{50}\) for activation by ACh depends on the subunit combinations expressed (see Results), a low concentration of ACh, chosen to be able to evoke less than 20% of the maximal current, was used. Each oocyte was tested with 1 mM ACh to estimate the maximal response. To examine the effects of modulators, the low concentration of ACh was applied. After the response to ACh had reached a stable level, the application was switched to ACh plus modulator. The application was then switched to bathing solution, followed by repeat of the control low concentration of ACh. 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 were applied for 10 to 20 seconds, until a response was stable, and applications were separated by 3 to 4 minutes to allow full washout.

Modulators were used at concentrations of 10-30 \(\mu\)M physostigmine (most often 15 \(\mu\)M), 100 \(\mu\)M atropine, 0.5 and 10 \(\mu\)M galantamine and 100 \(\mu\)M Zn\(^{2+}\) unless otherwise stated.
To examine the voltage-dependence of inhibition by physostigmine cells were held at -50 mV, then the potential was changed to -100 mV and a voltage ramp from -100 to +40 mV was applied over 4 sec (~35 mV/sec). Ramps were collected in the absence of ACh or physostigmine (background), then in the presence of 0.3 µM ACh alone and in the presence of 0.3 µM ACh + 15 µM physostigmine. The ramp currents in the presence of drug were corrected by subtracting the background ramp current, then the ramp current with ACh + physostigmine was normalized to the current with ACh alone to estimate the voltage-dependence of inhibition. At potentials more positive than about -50 mV the responses were quite small and the ratio showed a marked increase in variability.

Values are presented as arithmetic mean ± SEM (number of observations). The ability of a given receptor to be potentiated was assessed by a one sample t-test of the potentiation ratio to 1 (no effect). Comparison of potentiation between receptors was assessed by t-test (2-tailed with unequal variance). Comparisons among multiple types of receptor were done by ANOVA with Dunnett’s or Bonferroni’s correction, as described in Results. Fits of concentration-response relationships were made using SigmaPlot (Systat Software, Inc., San Jose, CA). Statistical tests were made using Excel (Microsoft, Redmond WA) or STATA (StataCorp LP, College Station, Texas).

Drugs

Acetylcholine chloride (ACh; CAS 60-31-1), was purchased from Sigma-Aldrich (St. Louis, MO). ACh was prepared as a 500 mM stock solution in bath solution and stored frozen at -20 °C. Physostigmine hemisulfate (physostigmine; CAS 64-47-1) was purchased from Tocris (Ellisville, MO). Physostigmine was prepared as a 10 mM stock in deionized water and stored frozen at -20 °C. Atropine (Atropine sulfate monohydrate; CAS 5908-99-6) was obtained from Sigma-Aldrich and prepared as a 10 mM stock in deionized water, stored frozen at -20 °C. Galantamine hydrobromide (CAS 1953-04-4) was obtained from Tocris and prepared as a 10 mM stock in deionized water, stored frozen at -20 °C. Zinc chloride (CAS 7646-85-7) was
obtained from Sigma-Aldrich and prepared as a 2 mM stock in bath solution, stored at 4 °C.

Working solutions were prepared on the day of experiments.
Results

Physostigmine potentiates receptors containing 3 copies of α4 and inhibits receptors containing 3 copies of β2.

Physostigmine can potentiate activation of nicotinic receptors containing the α4 and β2 subunits when both ACh and physostigmine are applied at low concentrations, as reported previously (Zwart et al., 2000; Smulders et al., 2005). These studies were performed using expression conditions in which the majority of the receptor population was the stoichiometric subtype containing 3 copies of the α4 subunit and 2 of the β2 subunit. We confirmed that responses of receptors containing 3 copies of α4 were well potentiated by injecting oocytes with cRNA at a ratio of 8:1 α4:β2 (Fig 1A). In contrast, when we repeated the applications on receptors largely of the subtype containing 2 copies of α4 and 3 of β2 we found that physostigmine was inhibitory to responses elicited by low concentrations of ACh (Fig 1B). The data are summarized in Table 1.

The dependence of the effects on the concentration of physostigmine was flat between 10 and 30 µM (Fig. 2) as reported previously for potentiation (Smulders et al., 2005). The responses of oocytes injected with α4 and β2 cRNA at a 1:1 ratio were similar to that reported previously showing a broad plateau of potentiation between 10 and 30 µM followed by a change to inhibition at 100 µM physostigmine (Smulders et al., 2005), although the extent of potentiation was lower than reported. For oocytes injected at an 8:1 ratio potentiation was greater. Inhibition of responses from oocytes injected at an 1:8 ratio appeared to be concentration-independent in this concentration range, which suggests that inhibition is only partial even at saturating concentrations.

Previous work has found that inhibition by higher concentrations of physostigmine is strongly voltage dependent (Zwart et al., 2000). However, the inhibition produced by 15 µM physostigmine on responses from oocytes injected at a 1:8 ratio was only weakly voltage
dependent between -50 and -100 mV (Fig. 2).

A residue in the α1 subunit of *Torpedo* nicotinic receptors can be labeled by physostigmine (α1K125; Schrattenholz et al., 1993) and has been proposed to form part of the physostigmine-binding site. However mutation of the homologous residue in the α4 subunit had no effect on potentiation. α4K130Q expressed with β2 at an 8:1 ratio had an EC$_{50}$ for ACh of 93 ± 15 μM (5 cells) and potentiation by physostigmine of 1.4 ± 0.1 (7), which did not differ significantly from wild-type values (see Table 1). This agrees with a more recent photolabeling study that found no incorporation of physostigmine at α1K125 (Hamouda et al., 2013), and the finding that α1K125Q and α1K125E mutations do not affect activation of the muscle-type receptor by physostigmine (Militante et al., 2008).

*Design of studies of chimeric subunits*

We wanted to test the consequences of alterations in the sequences of particular subunits. To do this, we expressed chimeric subunits with dimeric constructs composed of an α4 and a β2 linked together (Fig. 3). We wished to control the position of the free (chimeric) subunit in the pentameric receptor to obtain insights into the possible role for an effect at a particular interface in the receptor: canonical α4/β2 or alternative α4/α4 or β2/α4 interfaces (Seo et al., 2009; Harpsoe et al., 2011; Mazzaferro et al., 2011; Lucero et al., 2016).

The dimers assemble in a clockwise fashion (Jin and Steinbach, 2011), so the β2-α4 dimer assembles to place an α4 clockwise from the β2 in the pentamer (Fig. 3). In this case, the two subunits in the dimer generate an internal α4/β2 interface that forms a canonical ACh-binding site, with the positive side contributed by α4 and the negative side by β2. This binding site is located within the dimer, and the fifth, free subunit occupies the non-binding position in the pentamer and contributes to β2/X and X/α4 interfaces (where X indicates the surface contributed by the free subunit). In contrast, the α4-β2 dimer forms one canonical ACh-binding site between the α4 subunit of one dimer and the β2 of the adjacent dimer and the second
canonical site is formed between a dimer and the free subunit (either $\alpha_4/X$ or $X/\beta_2$; see Fig. 3).

Role of the membrane spanning region

An initial thought was that inhibition by physostigmine might reflect an increased sensitivity to open-channel block due to the differences in sequence of the channel-lining regions contributed by the second transmembrane domains of the $\alpha_4$ and $\beta_2$ subunits. Accordingly we constructed chimeras in which the regions forming the major portion of the ion channel (the 1st to 3rd transmembrane regions, TM1 to TM3) were exchanged between the subunits. When the chimeras were expressed with the $\beta_2$-$\alpha_4$ dimeric construct, activation by ACh showed the EC$_{50}$ values expected for the number of $\beta_2$ subunit extracellular domains present in the receptor (Table 2). Critically, inhibition by physostigmine did not depend on the number of copies of the $\beta_2$ TM1-TM3 region, but rather on the number of copies of the $\beta_2$ extracellular domain. A second set of chimeric subunits was made in which the region following TM3 comprising the main cytoplasmic loop and TM4 domain was swapped. Expression of these constructs indicated that this region did not contribute to the differential effects of physostigmine (Table 2). That is, none of the transmembrane regions appeared to be critical for the difference in responses to physostigmine. We conclude from these results that key determinants underlying the difference in response for the two stoichiometric forms of the receptors are located in the N-terminal, extracellular domain of the subunits.

This finding is in agreement with the weak concentration- and voltage-dependencies seen (Fig. 2) and indicates that the inhibition we observe is unlikely to result from open-channel block. We note that voltage-dependent open channel block has been reported for inhibition of both muscle and neuronal nicotinic receptors (Wachtel, 1993; Zwart et al., 2000; Militante et al., 2008). In general these experiments have used higher concentrations of ACh to activate and higher concentrations of physostigmine to inhibit, and so a contribution from open-channel block would be enhanced. We also note that Smulders et al. (2005) concluded that for inhibition by
physostigmine of receptors formed from α4 and β2 subunits injected at a 1:1 ratio “a mechanism more complex than simple channel block appears to be required.” It is likely that the inhibition we observe at lower concentrations reflects an additional action of physostigmine on the receptor form containing 2 copies of α4 and 3 of β2, while the inhibition seen at higher concentrations of physostigmine and ACh may well result from open-channel block.

The E loop in the N-terminal extracellular domain plays a key role

Since the difference in response to physostigmine appeared to lie in the amino-terminal extracellular domain we constructed a series of chimeric subunits in which regions were exchanged (summarized in Fig. 3). Chimeric subunits were made by transferring the loops that form the canonical ACh-binding sites between the α4 and β2 subunits. In a canonical site the α4 subunit contributes loops to the positive side of the interface (the A, B and C loops) while the β2 subunit contributes loops to the negative side (D, E and F) (see Fig. 3). The chimeric subunit is named for the subunit contributing the major portion, followed by the transferred region in parentheses (e.g. β2(E) indicates a β2 subunit containing the E loop from α4).

We first transferred loops from the α4 subunit to the β2 subunit, in an effort to either remove inhibition or confer potentiation. These constructs were initially tested with the β2-α4 dimer, in which the free subunit will occupy a position that does not form a canonical ACh binding site. The chimeric construct will provide the minus side of the interface to a β2/β2 interface, and the positive side to a β2/α4 interface (see Fig. 3). None of loops contributing to the positive side (the A, B and C loops) had any effect on inhibition by physostigmine (Table 3). A construct in which all 3 loops were transferred also had no effect on inhibition. We also tested the β2(ABC) chimera with the α4-β2 dimer, and again saw no effect on physostigmine action (Table 3). These results indicated that the β2 regions homologous to the regions contributed by α4 to the positive side of the ACh binding site were not involved in determining the actions of physostigmine, whether located at a β*/β interface (when expressed with the α4-β2 dimer) or a
β*/α interface (with the β2-α4 dimer). (Here, β* indicates that the chimera affected the “plus” side of an interface contributed by a β subunit.)

We then constructed chimeras in which loops contributed by β2 to the negative side were swapped (the D, E and F loops). Transferring the D and F loops from α4 to β2 did not affect any properties examined (Table 3). When the α4 E loop was transferred, however, the average maximal response was increased significantly and inhibition by physostigmine was reduced, albeit insignificantly (Table 3, Fig 1C, E). The β2(E) construct was also tested with the α4-β2 dimer, and again inhibition by physostigmine was reduced (Table 3, Fig 1D, F). Again, the effect of the chimera was the same whether the affected interface was α/*β or β/*β.

We did not test chimeras made in the α4 subunit by transferring the β2 A, B or C loops. However, since there appeared to be an effect when the α4 E loop was transferred into the β2 subunit, we examined the consequences of moving the β2 E loop to the α4 subunit. This transfer had dramatic effects on receptor function, converting potentiation by physostigmine to inhibition and reducing the EC50 for ACh (Table 3, Fig 1G, H). Indistinguishable effects occurred whether the chimera was expressed with the α4-β2 (α/*α) or β2-α4 (β/*α) dimer (Table 3, Fig 1I, J).

Finally, we constructed 3 pentameric concatemers: β2-α4-β2-α4-α4(E), β2-α4-β2-α4-β2(E), and β2-α4-β2(E)-α4-β2. Previous work (Carbone et al., 2009; Mazzaferro et al., 2011) has found that pentameric concatemers assemble with subunits in clockwise order (Fig. 3), so the first would contain a β/*α interface with a transposed E loop, the second an altered β/*β interface and the third an altered α/*β interface. Only the third would have affected a canonical ACh-binding site. The results are summarized in Table 3, and agree with the results obtained with dimers. Altering the α4 E loop at a β/*α interface resulted in reduced potentiation and a significant reduction in the EC50 for ACh. Altering the β2 E loop at a β/*β interface resulted in a small potentiation. Finally, altering the β2 E loop at a canonical ACh-binding interface (α/*β) removed block.
Effects of E loop transfer on actions of other potentiating agents.

We performed initial studies of 3 additional potentiating agents: galantamine, atropine and zinc ions. Galantamine was chosen because it photolabels the same residues in the Torpedo nicotinic receptor as physostigmine (Hamouda et al., 2013). Atropine has been reported to be a strong potentiator of α4β2 receptors, but the effects of stoichiometry have not been examined (Smulders et al., 2005). Finally, Zn²⁺ potentiates the form containing 3 copies of α4 and inhibits the form containing 2 copies, but the residues involved do not lie in the E loop (Hsiao et al., 2006; Moroni et al., 2008). We tested selected concentrations of the drugs on receptors formed from free subunits at different ratios of injected cRNA and on chimeric α4(E) and β2(E) subunits expressed with the β2-α4 dimeric construct. The results are summarized in Table 4.

We tested 0.5 µM galantamine, a concentration that has been reported to produce maximal potentiation for human α4β2 receptors expressed in HEK cells (Samochocki et al., 2003). It produced minimal potentiation or inhibition in our experiments, as was reported in a previous study of human α4β2 receptors expressed in Xenopus oocytes (Smulders et al., 2005). We then used 10 µM galantamine, which produces inhibition of responses for receptors expressed in oocytes (Smulders et al., 2005). At this concentration galantamine strongly inhibited receptors containing 3 copies of α4 and less strongly inhibited receptors containing only 2 copies (Table 4; the difference in inhibition has a p value of P < 0.0001; one way ANOVA on the 4 constructs with Bonferroni’s correction). Indeed, inhibition by galantamine of responses from receptors containing only 2 copies of α4 was less than inhibition by physostigmine (0.69 response ratio, Table 1). Replacing the E loop of the α4 subunit reduced inhibition (comparison to free subunits at 8:1 ratio P < 0.0001), while replacing the E loop of the β2 subunit increased inhibition (P = 0.001). These results show a similar pattern to the results obtained with physostigmine, although in the case of galantamine inhibition occurred rather than potentiation.
This suggests that the inhibition produced by galantamine differs from that of physostigmine and might reflect an inverse agonist action at the site mediating potentiation by physostigmine.

Atropine (100 µM) strongly potentiated receptors containing 3 copies of the α4 subunit, and strongly inhibited receptors containing 2 copies (Table 4). Replacement of the E loop of the α4 subunit converted potentiation to inhibition, while replacement of the E loop in the β2 subunit significantly reduced inhibition (compared to the 1:8 ratio injection; P = 0.02) but did not confer potentiation. These results are quite similar to those obtained with physostigmine.

Zinc ion (100 µM) was chosen as a negative control potentiator, as it is a stoichiometry-dependent modulator while the residues identified as critical for its actions do not lie in the E loop (Moroni et al., 2008). We confirmed the stoichiometry-dependence of the actions of Zn²⁺ for receptors assembled from free subunits (Table 4). Expression of the α4(E) chimera with the β2-α4 chimera somewhat reduces potentiation compared to free subunits (P = 0.02) but not when compared to the level when wild-type α4 was expressed with β2-α4 (P = 1.0). However, transfer of the α4 E loop to β2 conferred potentiation (Table 4). This is surprising, as the transferred E loop should contribute to the (-) side of a β2/*β2 interface (Figure 2). Potentiation by Zn²⁺ is the result of an interaction of zinc at the α4/α4 interface (specifically between α4(H165) in the F loop on the (-) side and α4(E194) on the (+) side in the C loop) (Moroni et al., 2008). Accordingly, it would be expected that potentiation would not be affected, as we observed. However, inhibition reflects interaction of zinc at the β2/α4 interface (between α4(H165) on the (-) side and β2(D193) on the (+) side), which should not have been altered when the β2(E) chimera is expressed.

To examine the actions of Zn²⁺ further we expressed the chimeric subunits with the α4-β2 dimer, to place the transferred E loop at an α4/*X interface. Responses of receptors containing either the α4(E) or β2(E) chimera were potentiated by 100 µM Zn²⁺ (Table 4). Indeed, when the β2(E) chimera was expressed with this dimer the level of potentiation was the largest we observed (Table 4), and the amplitude of the potentiated response actually exceeded the
maximal response of the oocyte to 1 mM ACh alone (average relative potentiated response was 1.24 times the maximal response to ACh alone, P < 0.001).

Overall, the actions of transferring the E loop on potentiation by atropine were very similar to effects on physostigmine. Galantamine, in contrast, did not potentiate either form of the receptor when expressed in oocytes. However, the inhibition produced by galantamine at 10 µM differed from the inhibition by physostigmine: galantamine inhibition was strongest on receptors containing 3 copies of α4. The effects of transferring the E loop on inhibition by galantamine are consistent with the idea that galantamine acts as an inverse agonist at the same site at which physostigmine acts as an agonist and results in potentiation. In contrast, the unexpected effects on modulation by Zn²⁺ ions followed a different pattern, in that the transfer into the β2 subunit changed inhibition to potentiation while the reciprocal transfer had no effect. The consequences of the transfer were similar for Zn²⁺ ions and physostigmine, in that it did not matter whether the affected interface formed a canonical binding site or occurred elsewhere in the pentamer.
Discussion

Our initial observation was that the effect of physostigmine on currents elicited by ACh differed between the two stoichiometric forms of the nicotinic α4β2 receptor: physostigmine potentiated responses from receptors containing 3 copies of α4, and inhibited receptors containing 3 copies of β2.

The inhibition by physostigmine we studied is unlikely to reflect open-channel block, based on 3 lines of evidence: low dependence on the concentration of physostigmine, weak voltage-dependence and lack of effect when the membrane-spanning regions were swapped between the α4 and β2 subunits. However it is likely that this inhibition reflects an additional interaction between physostigmine and the α4β2 receptor, and physostigmine can also act as an open channel blocker.

Our results indicate that the E loop of the α4 and β2 subunits is a key determinant of the actions of physostigmine. Although transferring the E loop from α4 to β2 did not confer potentiation, inhibition was reduced. In contrast, transferring the E loop from β2 to α4 resulted in strong block of the response to ACh. Our data do not distinguish whether the potentiation and inhibition we have studied result from interaction of physostigmine with a single site (i.e. as agonist and inverse agonist) or at separate sites. Because potentiation, inhibition or both could have been affected it is not clear whether the E loop has predominant effects on one or the other process.

A role for loops in the ACh-binding site suggests that physostigmine might bind to the canonical ACh-binding site, as proposed earlier (Zwart et al., 2000; Smulders et al., 2005). This is unlikely, however, since the E loop of the α4 subunit does not contribute to the canonical site. Alternatively, recent studies have indicated that the pharmacology of nicotinic receptors is significantly influenced by sites located at intersubunit interfaces in addition to the canonical ACh-binding site found at the α/β interface. These include a site located at an α/α interface (Harpsoe et al., 2011; Mazzaferro et al., 2011; Lucero et al., 2016) and sites at β/α interfaces.
(Moroni et al., 2008; Seo et al., 2009). We examined the role that interface location plays by expressing chimeric subunits with concatemers of subunits and by incorporating chimeric subunits into pentameric concatemers. We found no indication that the position of the transferred E loop is critical in determining the actions of physostigmine, suggesting that interactions of physostigmine are not restricted to either canonical or non-canonical binding sites.

At this point we need to consider whether the overall data support the idea that physostigmine binds to the receptor in a site including the E loop. The results of photolabeling studies of the *Torpedo* nicotinic receptor strongly support the idea that binding does occur there. Other results indicate that the E loop plays a more general role in determining the properties of the receptor.

Turning first to the photolabeling studies, Hamouda et al. (2013) used the intrinsic photosensitivity of physostigmine to covalently label aromatic residues in the *Torpedo* electric organ nicotinic receptor (related to the muscle nicotinic receptor). They found that several residues in the canonical ACh-binding site were labeled in the absence of a nicotinic agonist (carbamylcholine) or a competitive snake neurotoxin, whereas this labeling was blocked in the presence of carbamylcholine. However they found other residues that were labeled even in the presence of agonist. None of this second group were located in the α1 subunit, but several were found in or near the E loop of the γ subunit (in this receptor the γ subunit contributes the (-) side to a canonical ACh-binding interface). These residues are shown in Fig. 4. Galantamine also photolabeled the same residues in the presence of agonist. These results strongly support the idea that physostigmine and galantamine directly interact with nicotinic receptors, in part with residues in the E loop. However a significant caveat is that physostigmine is only a weak agonist on muscle nicotinic receptors (Militante et al., 2008 Shaw et al., 1985; Cooper et al., 1996), although one study reports that it can potentiate the response of skeletal muscle receptors to low concentration of ACh (Svobodova et al., 2006).
On the other hand, the E loop plays a major role in determining the properties of the α4β2 receptor. We found that incorporating the α4(E) subunit had a significant effect on activation by ACh irrespective of the predicted interface at which it was placed. A previous study had mutated 3 residues in the α4 E loop and found a similar shift in the EC50 for ACh (Harpsoe et al., 2011). In that study receptors were expressed from free subunits at various ratios, and it was concluded that the mutations affected binding of ACh to the non-canonical binding site at the α4/α4 interface. Recently, Lucero et al. (2016) extended these results using pentameric concatemers of subunits containing α4 or β2 subunits with the same 3 mutations in the E loop of either the α4 or β2 subunit. Lucero et al. found that incorporating a single copy of the mutated α4 subunit greatly decreased the EC50 for ACh irrespective of whether the altered E loop contributed to a β/*α or an α/*α interface. Our results agree well with both of these studies, and confirm that a shift in the EC50 for ACh can occur even when the altered E loop is present at a single β2/*α4 interface.

In terms of unexpected effects of swapping regions, the most surprising of our findings is that transferring the α4 E loop to β2 conferred potentiation by zinc ions on the 3 β2 stoichiometric form of the receptor. Based on the location of the residues implicated in potentiation and inhibition (see Fig. 4) this would definitely not have been expected. It is also surprising because inhibition is proposed to occur at the β2/α4 interface and potentiation at the α4/α4 interface (Moroni et al., 2008), neither of which would be predicted to be altered by a change in the (-) side of the β2 subunit. In an earlier study we found that transfer of a larger region, including the E loop, between the α2 and α4 subunits altered the efficacy of 17β estradiol at potentiation (Jin and Steinbach, 2015). In that work we proposed that the basis for the alteration was a change in the transduction process for potentiation by estradiol. These findings suggest that the E loop is a critical determinant of potentiation by drugs believed to bind elsewhere in the receptor, in addition to having significant actions on activation of the receptor by agonists. Accordingly, these considerations support the idea that the effects of swapping the
E loop on the actions of physostigmine might well result from changes in either activation by ACh or transduction of the potentiating effect following physostigmine binding to a different region of the receptor.

One significant issue in interpreting our results is that the effects of transferring E loops are independent of the interface affected. A possible interpretation is that the structure of binding sites may be influenced by regions at other interfaces. It has been proposed that interaction of a conotoxin with the canonical site on nicotinic α3β2 receptors depends on whether there is an α3/α3 or β2/β2 interface present in the receptor (Grishin et al., 2010). Furthermore, the apparent affinity for competitive antagonists at the canonical sites of α4β2 receptors is influenced by the presence of an α4/α4 or a β2/β2 interface in the receptor (Moroni et al., 2006). These studies of the actions of antagonists avoid the significant problem of interpreting effects on activation by agonists, that necessarily are confounded by the problem of separating effects on affinity (potency) and transduction (efficacy) (Colquhoun, 1998). Most recently, Lucero et al (2016) propose that there is a strong interaction between adjacent subunits so that the structure at one interface (influenced by the structure of the E loop) can alter activation mediated by binding of ACh to neighboring subunits. The authors did not specify whether the interaction alters binding or the coupling of binding to channel gating.

Perhaps the simplest summary of our results is that they confirm the physiological relevance of the photolabeling studies, and support the idea that physostigmine associates with the receptor at a site or sites including the E loop. This may also be true for galantamine and atropine. However the lack of dependence on a specific interface and the presence of multiple effects as a result of swapping the E loop both indicate the existence of greater complexity. The facts that there are significant changes in activation by ACh and in the pharmacology of unrelated modulatory drugs clearly indicate that many properties of the receptor have been altered, and it remains possible that physostigmine may associate with other (or additional) regions of the receptor to have its effects. The precise mechanisms mediating the actions of
physostigmine require further study. There are similarities to the role the E loop plays in activation of the receptor by ACh, suggesting that the structure-function relationship for the nicotinic α4β2 receptor involves extensive interactions of regions involving the entire pentamer.
Acknowledgments

We thank John Bracamontes for helpful comments and criticism and Daniel Shin for performing some of the oocyte recordings. JHS was the Russell and Mary Shelden Professor of Anesthesiology.

Authorship contributions

Participated in research design: Jin, Akk and Steinbach
Conducted experiments: Jin, McCollum and Germann
Contributed new reagents or analytic tools: Jin
Performed data analysis: Jin, Akk and Steinbach
Wrote or contributed to the writing of the manuscript: Jin, Akk and Steinbach
References


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Jin X, Bermudez I, Steinbach JH (2014) The nicotinic alpha5 subunit can replace either an acetylcholine-binding or nonbinding subunit in the alpha4beta2* neuronal nicotinic receptor. Mol Pharmacol 85:11-17.


Footnotes

This work was supported by the National Institutes of Health National Institute of Neurological Diseases and Stroke [Grant NS22356].

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Figure Legends

Figure 1. Effect of transplanting the E loop between the α4 and β2 subunits.
Traces are shown from oocytes when ACh was initially applied alone and then the perfusion was switched to ACh + physostigmine (ACh alone application indicated by the lower line above the trace, and the time of the application of both ACh + physostigmine shown by the upper line). The constructs injected are shown above the trace; the horizontal bar shows 20 sec for all traces while the vertical bar shows the current calibration for each trace. The ACh concentration was adjusted to result in a response of less than 20% of the maximal response for that oocyte. Concentrations used were: α4β2 8:1 1 µM ACh; α4β2 1:8 0.3 µM; α4-β2 & β2 0.3 µM; β2-α4 & β2 0.3 µM; α4-β2 & β2(E) 0.3 µM; β2-α4 & β2(E) 0.1 µM; α4-β2 & α4 1 µM; β2-α4 & α4 1 µM; α4-β2 & α4(E) 0.3 µM; β2-α4 & α4(E) 0.3 µM. 15 µM physostigmine was used for all traces.

Figure 2. Concentration and voltage dependence of physostigmine actions.
Panel A shows the effect of different concentrations of physostigmine on responses from oocytes injected with free α4 and β2 subunits at the indicated ratios (mean ± SEM). Data are from 6 to 40 oocytes, except for the point at 10 µM physostigmine on α4&β2 1:8, which is a single experiment. The symbols indicate the p value that the value differs from 1 (no effect) by chance (one sample t-test; ns: P > 0.05, **: P < 0.01; *** P < 0.001). Panel B shows the inhibition of responses from oocytes injected with α4&β2 1:8 (0.3 µM ACh, 15 µM physostigmine) at different voltages. Symbols show mean ± SEM for 4 oocytes tested at both -50 and -100 mV (“cells at 2 voltages”) and for 10 eggs tested at -50 mV (“mean” including the 4 tested at both voltages). The heavy line shows the average relative response for 4 oocytes subjected to a voltage ramp (see Methods) while the dashed lines show the standard error.

Figure 3. Cartoons of receptor structure.
Panel A shows the arrangement of subunits in the receptors formed following injections of various combinations of subunits. The diagrams are of the receptor viewed from the extracellular side. The locations of canonical ACh-binding sites (α4/β2 interface) are shown by the stars. The contributions of loops to a canonical ACh-binding site are also shown. Panel B shows a linear diagram of a generic nicotinic subunit with the relative positions of the binding site loops (A through F) and the transmembrane domains (TM1 through TM4) shown. Extracellular portions are indicated by white shading, intracellular by gray shading and transmembrane regions by black. Below the diagram the residues transferred in the chimeric constructs are shown, together with the initial residue number in the mature subunit.

Figure 4. Location of residues photolabeled by physostigmine or implicated in the actions of Zn2+.

Panel A shows sections of aligned sequence for segments of the extracellular domains of the human α4 and β2 subunits and the Torpedo α1, γ and δ subunits. The letters on a blue background above the sequences indicate the locations of chimera segments at the (+) side of an interface (loops A & C) while those on orange those on the (-) side (E & F). Residues are color coded to indicate that they were labelled by physostigmine only in the absence of carbamylcholine (green, in α1 subunit), labelled by physostigmine in the presence of carbamylcholine (red, in γ and δ subunits). Note that no label was reported from the β1 subunit, and it was not possible to sequence the E loop of the δ subunit. Yellow backgrounds indicate residues implicated in the actions of Zn2+: in α4 H(165) participates in potentiation at the α4/α4 interface and inhibition at the β2/α4 interface, while α4(E194) participates in potentiation and β2(D193) in inhibition. The 3 residues in the E loops pf α4 and β2 highlighted in gray are the residues switched in the studies by Harpsoe et al. (2011) referred to in the Discussion. Panel B shows a homology model of the extracellular domains of 2 α4 subunits, based on the GluCl structure (3RIF; Hibbs and Gouaux, 2011) to provide an idea of the positions of the residues.
highlighted in Panel A. The view is from the outside of the receptor looking into the binding site for ACh, and the extracellular portion is at the top. The subunit contributing the (+) side is shown as a cyan ribbon. The C loop on the (+) side is colored blue, and the residue in α4 contributing to the Zn²⁺ potentiating site is shown as yellow spheres. The residues in α1 that were photolabeled by physostigmine are not shown. The subunit contributing the (-) side is shown as a light gray ribbon and the E loop is colored orange. The histidine contributing to both the Zn²⁺ sites is shown as yellow spheres. The residues homologous to the residues photolabeled by physostigmine and for which carbamylcholine did not protect against labeling are shown as red spheres. Finally the residues in the E loop interchanged by Harpsoe et al. (2011) are shown as black spheres. One residue was both interchanged and photolabeled; it is colored purple. Note that the residues contributing to the Zn²⁺ sites are relatively far from the E loop, while several residues photolabeled by physostigmine cluster near the E loop and to residues shown to be important in determining the properties of the receptor.
### Table 1

<table>
<thead>
<tr>
<th>Subunits</th>
<th>Response to 1 mM ACh (nA)</th>
<th>ACh EC$_{50}$ (µM)</th>
<th>Response to physostigmine</th>
</tr>
</thead>
<tbody>
<tr>
<td>α&amp;β 8:1</td>
<td>-13042 ± 1303 (51) [-]</td>
<td>132 ± 10 (33) [-]</td>
<td>1.42 ± 0.04 (38) [-, &lt;0.001]</td>
</tr>
<tr>
<td>α-β&amp;α</td>
<td>-7238 ± 1179 (68)</td>
<td>78 ± 5 (47) [&lt;0.001]</td>
<td>1.51 ± 0.08 (25) [0.3, &lt;0.001]</td>
</tr>
<tr>
<td>β-α&amp;α</td>
<td>-14421 ± 1081 (94) [0.6]</td>
<td>121 ± 10 (59) [0.6]</td>
<td>1.38 ± 0.03 (51) [0.7, &lt;0.001]</td>
</tr>
<tr>
<td>α&amp;β 1:8</td>
<td>-1029 ± 193 (47) [-]</td>
<td>4.5 ± 0.6 (20) [-]</td>
<td>0.69 ± 0.03 (15) [, &lt;0.001]</td>
</tr>
<tr>
<td>α-β&amp;β</td>
<td>-341 ± 98 (32) [0.01]</td>
<td>2.9 ± 0.5 (14) [0.3]</td>
<td>0.78 ± 0.07 (10) [0.6, 0.01]</td>
</tr>
<tr>
<td>β-α&amp;β</td>
<td>-1007 ± 116 (91) [1.0]</td>
<td>3.7 ± 0.5 (57) [0.5]</td>
<td>0.72 ± 0.05 (28) [0.9, &lt;0.001]</td>
</tr>
<tr>
<td>α-β</td>
<td>-761 ± 136 (45)</td>
<td>90 ± 11 (30)</td>
<td>1.02 ± 0.05 (19) [, 0.7]</td>
</tr>
<tr>
<td>β-α</td>
<td>-4837 ± 738 (88)</td>
<td>105 ± 12 (55)</td>
<td>1.00 ± 0.07 (23) [, 1.0]</td>
</tr>
</tbody>
</table>

Table 1. The effect of physostigmine depends on the subunit stoichiometry. The first column gives the subunit combination injected, for examples α&β 8:1 indicates that free subunits were injected at a ratio of 8:1:α4:β2, while α-β&α indicates that the α4-β2 dimer was injected with free α4 subunit (1:1 ratio). The second and third columns give mean ± SE (number of cells) for the response to 1 mM ACh and for the concentration of ACh producing a half-maximal response (EC$_{50}$). The number in square brackets gives the significance of the difference in value to that of
the first entry in the set (one way ANOVA with Dunnett’s correction), and values are in bold type when the p value is less than 0.01. The final column gives the ratio of the response to a low concentration of ACh in the presence of physostigmine to the response of the same cell to ACh in the absence of physostigmine. The numbers in square brackets give, first, the significance of the difference to the first entry in the set and, second, the difference of the potentiation to a value of 1 (that is, no effect; one-sample t-test). Data for dimers injected in the absence of a free subunit are given in the last two rows, for comparison. Note that responses can be seen when dimers alone are injected, but the pharmacology of the responses differs from that seen when a free subunit is also injected demonstrating the presence of the free subunit in the pentamers (see also Jin and Steinbach, 2011).
Table 2

<table>
<thead>
<tr>
<th>Subunits</th>
<th>Response to 1 mM ACh (nA)</th>
<th>ACh EC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>Response to physostigmine</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-α&amp;α</td>
<td>14,421 ± 1,081 (94) [-]</td>
<td>121 ± 10 (59) [-]</td>
<td>1.38 ± 0.03 (51) [-, &lt;0.001]</td>
</tr>
<tr>
<td>β-αα(TM1 to TM3)</td>
<td>15,034 ± 3,105 (12) [0.98]</td>
<td>316 ± 61 (8) [&lt;0.001]</td>
<td>1.28 ± 0.06 (11) [0.19, 0.001]</td>
</tr>
<tr>
<td>β-αα(TM3 to C)</td>
<td>27,249 ± 3,339 (17) [&lt;0.001]</td>
<td>82 ± 24 (10) [0.37]</td>
<td>1.19 ± 0.04 (7) [0.03, 0.005]</td>
</tr>
<tr>
<td>β-α&amp;β</td>
<td>1,007 ± 116 (91) [-]</td>
<td>4 ± 0 (57) [-]</td>
<td>0.72 ± 0.05 (28) [-, &lt;0.001]</td>
</tr>
<tr>
<td>β-αβ(TM1 to TM3)</td>
<td>959 ± 162 (28) [0.97]</td>
<td>2 ± 0 (25) [0.14]</td>
<td>0.76 ± 0.05 (14) [0.82, 0.001]</td>
</tr>
<tr>
<td>β-αβ(TM3 to C)</td>
<td>1,801 ± 543 (6) [0.14]</td>
<td>5 ± 1 (6) [0.74]</td>
<td>0.63 ± 0.06 (3) [0.80, 0.03]</td>
</tr>
</tbody>
</table>

Table 2. The effect of physostigmine does not depend on the transmembrane domain. Data are presented as in Table 1 (mean ± SE (number of cells) [p values]). Chimeric subunits are named as subunit contributing the amino terminus followed by the region swapped between subunits (in parentheses), so α(M1-M3) indicates an α4 subunit containing β2 sequence for the TM1-TM3 domains. The joining points for the chimeras were: start of TM1 α4 V(208)IRR β2 V(204)IRR, end of TM3 α4N(344)VHH β2 (N(340)VHH.
Table 3.

<table>
<thead>
<tr>
<th>Subunits</th>
<th>Response to 1 mM ACh (nA)</th>
<th>ACh EC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>Response to physostigmine</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-α&amp;β</td>
<td>-1007 ± 116 (91) [-]</td>
<td>3.7 ± 0.5 (57) [-]</td>
<td>0.72 ± 0.05 (28) [-, &lt;0.001]</td>
</tr>
<tr>
<td>β-α&amp;β(ABC)</td>
<td>-511 ± 120 (4) [1.0]</td>
<td>6.1 ± 1.1 (4) [1.0]</td>
<td>0.52 ± 0.04 (4) [0.3, 0.001]</td>
</tr>
<tr>
<td>β-α&amp;β(A)</td>
<td>-479 ± 66 (5) [1.0]</td>
<td>8.0 ± 3.2 (5) [0.7]</td>
<td>0.71 ± 0.07 (3) [1.0, 0.06]</td>
</tr>
<tr>
<td>β-α&amp;β(B)</td>
<td>-323 ± 121 (13) [0.8]</td>
<td>12.6 ± 2.5 (3) [&lt;0.001]</td>
<td>0.71 ± 0.05 (6) [1.0, 0.001]</td>
</tr>
<tr>
<td>β-α&amp;β(C)</td>
<td>-317 ± 88 (6) [1.0]</td>
<td>9.3 ± 1.6 (6) [0.3]</td>
<td>0.69 ± 0.05 (5) [1.0, 0.004]</td>
</tr>
<tr>
<td>β-α&amp;β(D)</td>
<td>-207 ± 36 (4) [1.0]</td>
<td>3.6 ± 0.6 (4) [1.0]</td>
<td>0.66 ± 0.04 (4) [1.0, 0.003]</td>
</tr>
<tr>
<td>β-α&amp;β(E)</td>
<td>-8437 ± 1662 (14)</td>
<td>3.0 ± 0.3 (12) [1.0]</td>
<td>0.90 ± 0.04 (4) [0.4, 0.1]</td>
</tr>
<tr>
<td>β-α&amp;β(F)</td>
<td>-244 ± 77 (4) [1.0]</td>
<td>3.2 ± 0.6 (4) [1.0]</td>
<td>0.69 ± 0.01 (3) [1.0, 0.002]</td>
</tr>
<tr>
<td>α-β&amp;β</td>
<td>-341 ± 98 (32) [-]</td>
<td>2.9 ± 0.5 (14) [-]</td>
<td>0.78 ± 0.07 (10) [-, 0.01]</td>
</tr>
<tr>
<td>α-β&amp;β(ABC)</td>
<td>-68 ± 27 (4) [0.6]</td>
<td>5.7 ± 0.7 (2) [0.02]</td>
<td>0.63 ± 0.00 (2) [0.7, &lt;0.001]</td>
</tr>
<tr>
<td>α-β&amp;β(E)</td>
<td>-236 ± 51 (4) [1.0]</td>
<td>7.6 ± 1.4 (4) [0.8]</td>
<td>1.00 ± 0.07 (4) [0.2, 1.0]</td>
</tr>
<tr>
<td>β-α&amp;α</td>
<td>-14421 ± 1081 (94) [-]</td>
<td>121 ± 10 (59) [-]</td>
<td>1.38 ± 0.03 (51) [-, &lt;0.001]</td>
</tr>
<tr>
<td>β-α&amp;α(E)</td>
<td>-24237 ± 2810 (24)</td>
<td>3.7 ± 0.4 (14) [&lt;0.001]</td>
<td>0.53 ± 0.02 (11) [&lt;0.001, &lt;0.001]</td>
</tr>
<tr>
<td>α-β&amp;α</td>
<td>-7238 ± 1179 (68) [-]</td>
<td>78 ± 5 (47) [-]</td>
<td>1.51 ± 0.08 (25) [-, &lt;0.001]</td>
</tr>
<tr>
<td>α-β&amp;α(E)</td>
<td>-387 ± 105 (6) [0.09]</td>
<td>3.1 ± 0.2 (5) [&lt;0.001]</td>
<td>0.59 ± 0.03 (4) [&lt;0.001, 0.001]</td>
</tr>
</tbody>
</table>
Table 3. The E loop is important in determining the effect of physostigmine. Data are presented as in Tables 1 and 2. The significance of differences in relative response was determined as for Tables 1 and 2, except when two conditions were compared (e.g. α4-β2 expressed with α4 or α4(E)) in which case a two-tailed t-test with unequal variances was used. The locations of the chimeras are shown in Figure 2.

<table>
<thead>
<tr>
<th>Chimeras</th>
<th>Relative Response</th>
<th>p-value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-α-β-α-α</td>
<td>-2020 ± 265 (25) [-]</td>
<td>153 ± 12 (9) [-]</td>
<td>1.43 ± 0.23 (8) [-, 0.1]</td>
</tr>
<tr>
<td>β-α-β-α-α(E)</td>
<td>-1674 ± 507 (9) [0.5]</td>
<td>4.6 ± 0.6 (7) [&lt;0.001]</td>
<td>1.08 ± 0.18 (8) [0.2, 0.7]</td>
</tr>
<tr>
<td>β-α-β-α-β</td>
<td>-110 ± 23 (20) [-]</td>
<td>3.2 ± 0.8 (16) [-]</td>
<td>0.54 ± 0.04 (6) [-, &lt;0.001]</td>
</tr>
<tr>
<td>β-α-β-α-β(E)</td>
<td>-265 ± 70 (16) [0.9]</td>
<td>7.8 ± 1.3 (11) [0.002]</td>
<td>1.20 ± 0.18 (12) [0.03, 0.3]</td>
</tr>
<tr>
<td>β-α-β(E)-α-β</td>
<td>-2592 ± 677 (12) [&lt;0.001]</td>
<td>4.6 ± 0.4 (7) [0.6]</td>
<td>1.01 ± 0.19 (6) [0.2, 1.0]</td>
</tr>
</tbody>
</table>
Table 4

<table>
<thead>
<tr>
<th>Subunits</th>
<th>Potentiator</th>
<th>Relative response</th>
<th>P to 1</th>
<th>P to pair</th>
<th>P to free</th>
</tr>
</thead>
<tbody>
<tr>
<td>α4&amp;β2 8:1</td>
<td>GAL 0.5</td>
<td>0.94 ± 0.03 (7)</td>
<td>0.09</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>β-α&amp;α(E)</td>
<td>GAL 0.5</td>
<td>0.95 ± 0.02 (6)</td>
<td>0.08</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>α4&amp;β2 1:8</td>
<td>GAL 0.5</td>
<td>1.01 ± 0.04 (9)</td>
<td>0.85</td>
<td>-</td>
<td>-</td>
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<tr>
<td>β-α&amp;β(E)</td>
<td>GAL 0.5</td>
<td>0.91 ± 0.03 (6)</td>
<td>0.03</td>
<td>0.24</td>
<td>-</td>
</tr>
<tr>
<td>α4&amp;β2 8:1</td>
<td>GAL 10</td>
<td>0.52 ± 0.02 (6)</td>
<td>&lt;0.001</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>β-α&amp;α(E)</td>
<td>GAL 10</td>
<td>0.88 ± 0.03 (6)</td>
<td>0.01</td>
<td>&lt;0.001</td>
<td>-</td>
</tr>
<tr>
<td>α4&amp;β2 1:8</td>
<td>GAL 10</td>
<td>0.90 ± 0.02 (8)</td>
<td>0.004</td>
<td>-</td>
<td>-</td>
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<tr>
<td>β-α&amp;β(E)</td>
<td>GAL 10</td>
<td>0.68 ± 0.06 (5)</td>
<td>0.005</td>
<td>0.001</td>
<td>-</td>
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<tr>
<td>α4&amp;β2 8:1</td>
<td>ATR 100</td>
<td>1.51 ± 0.05 (8)</td>
<td>&lt;0.001</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>β-α&amp;α(E)</td>
<td>ATR 100</td>
<td>0.29 ± 0.03 (7)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>-</td>
</tr>
<tr>
<td>α4&amp;β2 1:8</td>
<td>ATR 100</td>
<td>0.35 ± 0.06 (7)</td>
<td>&lt;0.001</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>β-α&amp;β(E)</td>
<td>ATR 100</td>
<td>0.55 ± 0.04 (8)</td>
<td>&lt;0.001</td>
<td>0.002</td>
<td>-</td>
</tr>
<tr>
<td>α4&amp;β2 8:1</td>
<td>ZN 100</td>
<td>1.97 ± 0.05 (11)</td>
<td>&lt;0.001</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>α-β&amp;α</td>
<td>ZN 100</td>
<td>2.98 ± 0.12 (11)</td>
<td>&lt;0.001</td>
<td>-</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>α-β&amp;α(E)</td>
<td>ZN 100</td>
<td>2.85 ± 0.17 (6)</td>
<td>&lt;0.001</td>
<td>1.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>β-α&amp;α</td>
<td>ZN 100</td>
<td>1.38 ± 0.05 (4)</td>
<td>0.005</td>
<td>-</td>
<td>0.01</td>
</tr>
<tr>
<td>β-α&amp;α(E)</td>
<td>ZN 100</td>
<td>1.50 ± 0.07 (7)</td>
<td>&lt;0.001</td>
<td>1.0</td>
<td>0.02</td>
</tr>
<tr>
<td>α4&amp;β2 1:8</td>
<td>ZN 100</td>
<td>0.21 ± 0.02 (6)</td>
<td>&lt;0.001</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>α-β&amp;β(E)</td>
<td>ZN 100</td>
<td>11.17 ± 0.84 (6)</td>
<td>&lt;0.001</td>
<td>-</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>β-α&amp;β</td>
<td>ZN 100</td>
<td>0.37 ± 0.03 (7)</td>
<td>&lt;0.001</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td>β-α&amp;β(E)</td>
<td>ZN 100</td>
<td>3.59 ± 0.41 (7)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Table 4. Effects of transferring the E loop on actions of other potentiators. Three additional potentiating drugs were tested: galantamine at 0.5 µM (GAL 0.5) and 10 µM (GAL 10), atropine at 100 µM (ATR 100) and Zn\textsuperscript{2+} at 100 µM (ZN 100). Three p values are given: P to 1 indicates the p value for the difference to 1 (no effect) (1 sample t-test), P to pair indicates that the relative response differs from the value immediately above (e.g. α4&β2 1:8 to β-α&β(E)) (one way ANOVA on all conditions in the group with Bonferroni’s post-hoc correction) and P to free gives the p value for the difference from the value at the top of the group (one way ANOVA on all conditions in the group with Bonferroni’s post-hoc correction).
Figure 1
Figure 2
Figure 3
Figure 4