Determination of the residues in the extracellular domain of the nicotinic α subunit required for the actions of physostigmine on neuronal nicotinic receptors

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List of nonstandard abbreviations.
ACh: acetylcholine chloride, 2-acetoxy-N,N,N-trimethylethanaminium chloride
Abstract

Physostigmine can potentiate and inhibit neuronal nicotinic receptors, in addition to inhibiting the activity of acetylcholinesterase. We found that receptors containing 3 copies of the α2 subunit are inhibited by low concentrations of physostigmine in contrast to receptors containing 3 copies of the α4 subunit that are potentiated. We exploited this observation to determine regions required for the actions of physostigmine. Chimeric constructs of the α2 and α4 subunits located two regions in the extracellular amino-terminal domain of the subunit: the E loop (a loop of the transmitter-binding domain) and a region closer to the amino-terminus that collectively could completely determine the different effects of physostigmine. Point mutations then identified a single residue, α2(I92) versus α4(R92) that when combined with transfer of the E loop could convert the inhibition seen with α2 subunits to potentiation, and the potentiation seen with α4 subunits to inhibition. In addition, other point mutations could affect the extent of potentiation or inhibition, indicating that a more extensive set of interactions in the amino-terminal domain plays some role in the actions of physostigmine.
Introduction

Physostigmine has been used in clinical practice to raise ACh levels in brain because of its ability to inhibit the enzyme acetylcholinesterase but also 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). In addition to acting as an agonist and potentiator physostigmine also can inhibit nicotinic receptors (Zwart et al., 2000; Smulders et al., 2005). There has been recent interest in the ability of physostigmine and other drugs to act as “positive allosteric modulators” for neuronal nicotinic receptors, with possible therapeutic uses in, for example, treatment of dementias including Alzheimer’s disease (Triggle et al., 1998; Maelicke and Albuquerque, 2000).

The site at which physostigmine acts on neuronal nicotinic receptors is not known, although it has been proposed that it may interact with the ACh-binding site (Smulders et al., 2005; however see Militante et al., 2008). The ACh-binding site is in the extracellular domain of the receptor, located at the interface between the α and β subunits (Figure 1). The site comprises regions from the α subunit (the so-called A, B and C loops) and the β subunit (D, E and F loops). We recently found in studies involving the α4 and β2 subunits that the E loop of the ACh-binding site is a key determinant of the actions of physostigmine (Jin et al., 2017). However finer mapping of the critical residues was difficult because of the sequence divergence between the α4 and β2 subunits. In the course of other studies we found that physostigmine did not potentiate receptors comprising α2 and β2 receptors, and pursued this preliminary observation in the present work. The α4 and α2 subunits are highly homologous in terms of amino acid sequence (Jin and Steinbach, 2015) and occupy the same position in the assembled pentameric receptor so that it was possible to examine the structural differences between the α2 and α4 subunits that underlay this pharmacological difference. We utilized chimeric constructs between the α2 and α4 subunits to first determine that the basis for the different responses to physostigmine lay in the extracellular domain of the subunit. A series of finer chimeras identified two regions of the extracellular domain that were required for both inhibition and potentiation by physostigmine. The first is
the E loop that was necessary for both actions but, by itself, could not confer full potentiation or inhibition. The second is a region containing the D loop and stretching to the A loop (the D-A region). These two regions could quantitatively account for the differences in the actions of physostigmine on the α2 and α4 subunits. Within the D-A region a single residue, R92 in the α4 subunit and I92 in the α2 subunit, could largely duplicate the effects of exchanging the entire D-A region in association with the E loop. However, other mutations in the D-A region also affected physostigmine actions, indicating that other interactions were important.
Materials and Methods

The methods used were generally as described earlier (Jin and Steinbach, 2015; Jin et al., 2017).

Generation and expression of constructs

Constructs of the coding region were kindly provided by Dr. Jon Lindstrom (University of Pennsylvania) for the human α4 and β2 subunits and by Dr. Jim Boulter (UCLA) for the mouse α2 and β4 subunits. The mouse subunits were supplied in pSGEM, all other subunits were in the pcDNA3 vector (Invitrogen, Carlsbad CA). Chimeric subunits were constructed by overlap extension and smaller mutations were constructed using QuikChange (Stratagene, La Jolla CA), and the complete coding region was sequenced to determine that only the desired changes had been made. Concatemers of the human α4 and β2 subunits were described earlier (Jin and Steinbach, 2011).

cRNA was synthesized using the mMessage mMachine T7 kit (Ambion, Austin TX) and the concentration of RNA was estimated from the OD260 value. Subunits were injected at a ratio of 20:1 (α:β) for combinations of an α subunit with β4 to ensure a subunit ratio of 3α:2β, unless otherwise noted in Results. We tested responses of receptors containing either the human or mouse form of the β4 subunit and found no differences (data not shown) and so the data are pooled in the Results. Oocytes were injected with 12 to 15 ng of cRNA in a volume of 18 to 23 nL. Oocytes were maintained at 18°C for 2 to 4 days before physiological study.

Xenopus oocytes were prepared in Dr. C. Zorumski’s laboratory (Washington University, St. Louis MO). The protocols for animal use have been approved by the Washington University Animal Studies Committee. Animals are purchased from approved suppliers and are cared for in a Washington University Animal Care Facility. This facility is operated under the supervision of the Washington University Division of Comparative Medicine. This is an approved animal care facility providing housing and veterinary care for animals. Frogs were anaesthetized with 0.1% tricaine (3-aminobenzoic acid ethyl ester) during a partial ovariectomy. Eggs are harvested twice per frog. Frogs are killed under
tricaine anesthesia by rapid decapitation. This is an approved method of euthanasia.

**Recording and data analysis**

Standard methods were used for two-electrode voltage clamp of *Xenopus* oocytes (Jin and Steinbach, 2015; Jin et al., 2017). Records were digitized at 50 Hz with a Digidata 1200 interface; (Molecular Devices, Sunnyvale, CA), stored using pClamp 8.0 (Molecular Devices) and analyzed with Clampfit (Molecular Devices). Oocyte recordings were performed in a RC-1Z chamber (Warner Instruments) that was continuously perfused with saline. Drug applications were made using a manually controlled perfusion system. The bath 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.

The Hill equation was used to characterize the concentration-response relationship for activation by ACh ($Y_{[ACh]} = Y_{max} ([ACh] / ([ACh] + (EC_{50})^{nHill})$, where $Y$ is the response to a concentration of ACh, $EC_{50}$ is the concentration producing half-maximal activation, $Y_{max}$ is the maximal response, and $nHill$ is the Hill coefficient. Concentration-response data for each cell were normalized to the response to 1 mM ACh for that cell, fit with the Hill equation, then overall mean values were calculated for oocytes injected with that set of constructs.

To test the effect of physostigmine a low concentration of ACh was chosen for each subunit combination, that was able to evoke less than 20% of the maximal current. A test consisted of first applying the low concentration of ACh alone. After a stable response was reached the application was switched to ACh plus physostigmine (10 or 15 µM physostigmine). The response was terminated by application of bath solution. The relative response in the presence of physostigmine to that in the absence of physostigmine was then calculated. Applications were separated by 3 to 4 minutes to allow full washout.

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

Multiple regression analysis of the chimeric constructs was conducted using the sources of the
sequence in the swapped regions as the independent variables, assigning the number 0 to a region containing sequence from the α2 subunit and 1 to a region containing sequence from the α4 sequence.

The published structure of the nicotinic receptor containing 2 copies of the α4 subunit and 3 copies of β2 (5kxi.PDB; Morales-Perez et al., 2016) was viewed and figures were made using Chimera 1.11 (http://www.rbvi.ucsf.edu/chimera).

**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. Working solutions were prepared on the day of experiments.
Results

**Neuronal nicotinic receptors containing the α2 subunit are inhibited by physostigmine**

Our initial observation was that receptors containing the α2 subunit were inhibited by physostigmine when either 2 or 3 copies of α2 were present (Table 1, Figure 2). This contrasts to receptors containing the α4 subunit that were potentiated by physostigmine when 3 copies of α4 were present and inhibited when only 2 copies were present (Table 1). These results were obtained when α subunits were expressed with either the β2 or β4 subunit. The α2 subunit expressed poorly with the β2 subunit (Table 1) so all subsequent work was performed using the β4 subunit. In the rest of the experiments α constructs were expressed with β4 at a 20:1 α:β ratio, so that receptors containing 3 copies of α4 showed potentiation by physostigmine.

**The presence of a single α2 subunit in the receptor affects potentiation**

We also expressed α2 with two concatemeric constructs of the α4 and β2 subunit (Jin and Steinbach, 2011), to assess whether the position of the α2 subunit in the pentameric receptor affected responses to physostigmine (see Figure 1). These receptors both had the stoichiometry α4β2α2, but when expressed with the α4-β2 concatemer the α2 subunit occupied a position between α4 and β2 (when viewed from the extracellular medium, the subunit order is counterclockwise α4-α2-β2) while with the β2-α4 concatemer the order was β2-α2-α4. Drug binding sites are formed at subunit interfaces such that one subunit contributes the “+” side while the counterclockwise subunit contributes the “−” side. A canonical ACh binding site is formed when the order is α-β, while additional drug binding sites have been identified at β-α and α-α interfaces (Seo et al., 2009; Harpsoe et al., 2011; Mazzaferro et al., 2011; Lucero et al., 2016). Accordingly, with the α4-β2 concatemer the α2 subunit participated in a canonical (α2-β2) interface and a non-canonical interface (α4-α2) whereas with the β2-α4 concatemer it participated in two non-canonical interfaces (β2-α2 and α2-α4).

The effect of physostigmine did not differ depending on which concatemer the α2 subunit was
expressed with (Table 2; \( P = 0.16; \) two-tailed t-test with unequal variances), indicating that it did not matter which interfaces the \( \alpha_2 \) subunit participated in. In both cases the receptors were potentiated by physostigmine while the potentiation was less than when the \( \alpha_4 \) subunit was expressed with either concatemer and greater than when \( \beta_2 \) was (Table 2).

**The difference in effect does not depend on sequence differences in the transmembrane domains**

We first asked whether differences in the transmembrane domains were important. The \( \alpha_2 \) and \( \alpha_4 \) subunits are identical over the first 3 transmembrane domains (TM1 to TM3), so we transferred the region containing TM4 and the short C-terminal tail to produce 2 chimeric constructs: \( \alpha_4(TM4-C) \) and \( \alpha_2(TM4-C) \), where the construct is identified as the parent subunit (region transferred into it). Swapping the TM4 regions had no effect on the actions of physostigmine. For the \( \alpha_4 \) subunit physostigmine potentiated both: \( \alpha_4 1.51 \pm 0.04 \) (29) and \( \alpha_4(TM4-C) 1.50 \pm 0.02 \) (6) \( P = 0.85 \) (data given as mean \( \pm \) SE (number of oocytes tested), P value that the two values are the same assessed using a 2-tailed t-test with unequal variance). For the \( \alpha_2 \) subunit physostigmine inhibited both: \( \alpha_2 0.81 \pm 0.03 \) (14) and \( \alpha_2(TM4-C) 0.83 \pm 0.03 \) (6) \( P = 0.58 \).

Some of the constructs we made were also used in a previous study of potentiation by 17\( \beta \)-estradiol (Jin and Steinbach, 2015), and examined the effects of the presence of either a tryptophan (\( \alpha_4 \): PPWLAGMI) or phenylalanine (\( \alpha_2 \): PPFLAGMI) in the carboxyl terminal tail immediately following TM4. The lack of effect of swapping the TM4 to C region indicates that the W to F difference is not important for the actions of physostigmine. We also verified a lack of difference for subunits in which both the W and F forms were tested: \( \alpha_4 1.51 \pm 0.04 \) (29) vs. \( \alpha_4(F) 1.51 \pm 0.10 \) (2) \( P = 0.96 \) and \( \alpha_2 0.81 \pm 0.03 \) (14) vs. \( \alpha_2(W) 0.73 \pm 0.06 \) (9) \( P = 0.22 \). Accordingly we pooled results for N-terminal constructs made with either W or F present in the C-terminal tail.

These results indicated that the difference in the responses to physostigmine was determined by residues in the amino-terminal extracellular region, as we had previously found in the case of the \( \alpha_4 \)
and β2 subunits (Jin et al., 2017).

**Substituting regions of the α2 N-terminal domain with homologous regions from the α4 subunit can remove inhibition and confer potentiation**

The α2 and α4 subunits are quite similar in sequence in the amino-terminal extracellular region (Figure 3). Therefore it seemed likely that we could make relatively large exchanges of sequence and still obtain constructs that would produce functional receptors. We constructed a set of overlapping chimeras that covered the extracellular domain. The chimeras are identified in terms of the domains contributing to a canonical binding site for ACh: the A, B and C loops contributed by an α subunit on the “+” side of the interface and the D, E and F loops contributed by a β subunit on the “-“ side. For example, a chimera based on the α2 subunit but containing sequence from the α4 subunit covering the region from the amino terminus to the A loop is named α2(N-A). The chimeras made are diagrammed in Figure 3.

We first examined the α2 subunit and sought to confer potentiation. The results are shown in Figure 4 and presented in Table 3. When the extreme amino-terminal region from the α4 subunit (N-terminal to the A loop) was swapped into the α2 subunit (α2(N-A)) inhibition was reduced but potentiation was not conferred. When the remaining portion of the extracellular domain was swapped (α2(A-TM1)) inhibition was also reduced but potentiation was not conferred (Table 3). However, chimeras with the joining point further from the amino terminus did not affect inhibition (α2(B-TM1) and α2(C)). These results suggested that the region conferring inhibition was relatively widely distributed, but located between the amino terminus and the B loop.

Our previous study of the α4 and β2 subunits had indicated that the E loop is a key determinant of the actions of physostigmine (Jin et al., 2017), so we examined the consequences of swapping this domain. When the E loop from α4 was inserted into α2 block was fully removed, but again potentiation was not conferred. This suggested that the E loop was the basis for the effects of the α2(A-TM1) chimera. However, the results had indicated that a region further to the N-terminus was also important
in determining the effects of physostigmine. When we combined transfer of the D-A region with the E loop strong potentiation was conferred (α2(D-A + E), Table 3, Figure 4). We also made additional chimeras incorporating regions of the α4 subunit between the E loop and the start of the transmembrane domains without significant differences to the chimera involving the E loop alone (α2(E+B-TM1) and α2(E+C)), or the D to A+E chimera (α2(D-A+E+C) (Table 3, Figure 4).

The E loop and D-A region contain all the determinants necessary to confer full potentiation

The physostigmine effect on receptors containing the α2(D-A + E) chimera was comparable to that for α4 (1.42 ± 0.05 vs 1.51 ± 0.03, P = 0.14 that the effects are the same by two-tailed t-test), indicating that these regions contained all the determinants necessary to confer full potentiation.

Linear multiple regression of the physostigmine relative response on the origin of the regions transferred into the α2 subunit (see Methods) indicated that the largest effects were due to the presence of regions from α4 in the D-A region (P = 2 × 10⁻¹⁹ that the correlation arose by chance) and the E loop region (P = 5 × 10⁻¹⁹). In both cases the presence of α4 sequence increased potentiation. Other regions did not show strong effects (P > 0.005).

The effects of mutations on the background of chimeric α2 subunits

We then refined the residues required by making point mutations in the D-A region on the background of α2 chimeric receptors. We performed the mutations symmetrically, seeking residues in the D-A region for which mutations increased the potentiation of the α2(E) chimera to that of the α2(D-A+E) and, conversely decreased the potentiation of the α2(D-A+E) to that of the α2(E) chimera. We selected 4 pairs of mutations to make, one in the D loop (α4(V61) vs α2(L61)), one immediately following the D loop (α4(H66) vs α2(N66)), a group of 3 adjacent residues in the middle of the D-A region (α4(D76,Y77,E78) vs α2(E76,F77,G78)) and one near the A loop (α4(R92) vs α2(I92)). These were chosen because the residues differ between α2 and α4 but are the same for α2 and β2 (L61, E76, F77), or because there is a major difference in side chain properties (H to N, R to I). On the background
of α2(E) the residue was changed from the α2 to the α4 residue (e.g. L61 to V61), while on the background of α2(D-A+E) it was changed from α4 to α2 (V61 to L61).

The results are shown in Table 4 and Figure 5. There was one pair of mutations that showed the predicted pattern: α2(E+I92R) showed potentiation and α2(D-A+E+R92I) showed a loss of potentiation. The physostigmine effect on α2(E) was increased by the mutation I92R to 1.19 ± 0.05, as compared to 0.98 ± 0.01 for α2(E) (P = 0.01 that the physostigmine effects are equivalent based on a one-way ANOVA with Bonferroni correction for all the constructs shown in Table 4). However, the physostigmine effect was less than that for α2(D-A + E) (1.42; P = 0.01). The converse mutation, α2(D-A +E + R92I) reduced the physostigmine effect to 0.99 ± 0.01, which did not differ from that for α2(E) (P = 1) and was significantly less that α2(D-A + E) (P < 0.001).

However, the other pairs showed discordant results. On the background of α2(E) both the N66H and EFG to DYE mutations significantly increased inhibition by physostigmine, while on the α2(D-A+E) background neither the H66N nor the DYE to EFG mutations had significant effects on potentiation. In contrast, the L61V mutation had no effect on the α2(E) background, while V61L significantly reduced potentiation on the α2(A-D+E) background. This pattern of results is difficult to interpret. Overall it appears that there are several regions in the D-A region that interact to influence the binding of physostigmine and/or the transduction of binding into functional effect.

The effects of mutations in the E loop of the α2 subunit

We also mutated two residues in the E loop of wild type α2 subunits: α2(H122Q) and α2(V124T). The mutations change two of the residues in the E loop that previous work has shown are critical in determining the difference in EC$_{50}$ for ACh between receptors containing 3 copies of α4 and 3 copies of β2 (Harpsoe et al., 2011; Lucero et al., 2016). The third residue studied by these authors (α2(H114)) is identical between α2 and α4. The double mutant α2(Q122, T124) was inhibited by physostigmine (ratio 0.92 ± 0.01, N= 5). Inhibition was reduced compared to α2 wild-type subunits (0.81 ± 0.03, P = 0.001 from a t-test with unequal variance) but larger than for the α2(E) chimera (0.98 ±
The double mutant had an EC\textsubscript{50} for ACh of 242 ± 9 µM (5 cells), compared to 91 µM for α2 wild-type (P = 0.01) and 209 µM for α2(E) (P = 0.2), supporting the role of these residues in determining the EC\textsubscript{50}.

When we made the α2(I92R) mutation on the background of α2(Q122, T124) the physostigmine response was increased to 1.04 ± 0.01 (N = 5), to a level greater than for the double mutant alone (0.92 ± 0.01; P = 5 × 10\textsuperscript{-5}) but still less than that of the α2(E+I92R) construct (1.19 ± 0.05, P = 0.02). These observations suggest that these residues in the E loop contribute to determining the effects of physostigmine, but are not the sole determinants in the E loop.

**Substituting regions of the α4 N-terminal domain with homologous regions from the α2 subunit can remove potentiation and confer block**

We also constructed 4 constructs based on the α4 subunit. Transfer of both the D-A and E regions fully removed potentiation and conferred block (Table 5, Figure 4), whereas the separate transfer of the regions produced partial effects. The physostigmine effect on receptors containing the α4(D-A + E) chimera (0.73 ± 0.02) was comparable to that for receptors containing α2 (0.78 ± 0.03, P = 0.02 that the effects are the same by two-tailed t-test). These consequences are the inverse of the effect of the transfers into the α2 subunit, and support the idea that these regions are the full basis for the differences in the actions of physostigmine.

**Receptor activation by ACh**

Many of the constructs based on the α2 subunit showed EC\textsubscript{50} values for ACh that differed significantly from wild-type (see Tables 3 & 4), with values spread over almost a 10-fold range for the 17 constructs tested. However there was not a strong relationship between change in activation by ACh and the effect of physostigmine. There was a relatively weak rank correlation between the physostigmine effect and EC\textsubscript{50} (P = 0.03) indicating that a larger physostigmine ratio and higher EC\textsubscript{50} values tend to be associated. It seems likely that the correlation between the magnitude of the
physostigmine effect and the EC<sub>50</sub> reflects the strong tendency of α4 sequence in the E loop to increase both parameters. There was no significant rank correlation between the maximal current and the physostigmine effect ratio for these constructs (P = 0.4; data not shown).

Multiple linear regression (as described for the physostigmine effect) for the 11 chimeras shown in Table 3 showed no significant effect of the origin of the D-A region on the EC<sub>50</sub> value (P = 0.3), while both the E loop and C regions showed strong effects (P = 8×10<sup>-25</sup> and 2×10<sup>-19</sup>, respectively). When α4 sequence was in the E loop the EC<sub>50</sub> value was larger, while when it was in the C region it was lower. The effects of sequence on activation differed from those on physostigmine, in that no dependence on the D-A region was present. Furthermore, the C region strongly affected ACh EC<sub>50</sub> but not physostigmine effect.

These results indicate that there is no strong relationship between the effects of the various molecular manipulations on activation by ACh and modulation by physostigmine. Although regions of the α subunit that contribute to the ACh-binding sites do contain residues that affect modulation by physostigmine, the importance of the regions is not identical for effects on physostigmine and ACh.
Discussion

Our initial observation was that the effect of physostigmine differed between neuronal nicotinic receptors containing 3 copies of the α2 subunit versus 3 copies of the α4 subunit. We found that we could fully convert the responses by interchanging regions in the amino-terminal extracellular regions of the subunits.

The inhibition of responses seen with the α2 subunit was not influenced by the residues in the transmembrane region. The α2 and α4 subunits are identical in the TM1-TM3 regions, and swapping the TM4 regions did not affect the responses to physostigmine. The various chimeric constructs also indicate that inhibition is fully accounted for by the effects of residues in the amino-terminal extracellular domain. Our previous study of the actions of physostigmine on receptors containing α4 and β2 subunits obtained similar results (Jin et al., 2017). In that work the inhibition seen with low concentrations of ACh and physostigmine for receptors containing 2 copies of α4 and 3 of β2 was unlikely to result from open channel block. We did not examine the properties of inhibition in the present study, but it seems highly likely that the inhibition seen with receptors containing α2 subunits also does not result from open channel block.

Swapping the E loops between α2 and α4 subunits to produce the α4(E) and α2(E) chimeras resulted in receptors that showed weak inhibition in both cases. This result makes it more likely that interchange of the E loop affected both potentiation and inhibition, as an effect on only one action would not result in ablation of both potentiation and inhibition. Further, it indicates that the E loop is necessary but not sufficient for either action of physostigmine. To fully interconvert the inhibition found with the α2 subunit and the potentiation found with the α4 subunit, we had to exchange both the D-A region and the E loop.

We identified one residue in the D-A region that interacted with the E loop in a fashion that we had expected based on the interactions between the full D-A region and the E loop. If a residue in the D-A region underlay the difference in the actions of physostigmine on the α2(E) and α2(D-A+E) constructs then in the simplest case swapping the residue would swap the effects of physostigmine.
The residue at position 92 (α2(I92) vs. α4(R92)) essentially meets this criterion. These results clearly implicate this residue as a critical element in determining the effects of physostigmine in concert with the E loop. However, other mutations in the D-A region resulted in significant changes in physostigmine effect that were not reciprocal between the background chimeras. This implies that additional interactions are important although the rules appear obscure. For example, on the background of α2(E) mutating the residues E76, F77 and G78 of the α2 sequence to DYE of the α4 sequence actually significantly increased inhibition by physostigmine. If a simple mapping of α4 sequence to increased potentiation (or decreased inhibition) existed this result is completely unexpected. It could be that some residues in the D-A region more strongly influence processes resulting in inhibition and others potentiation.

Three residues in the E loop have been identified as critical in determining the pharmacological differences between stoichiometric forms of the α4β2 receptor: α4(H114), α4(Q122) and α4(T124) (Harpsoe et al., 2011; Lucero et al., 2016). H114 is present in both α2 and α4, while the other two residues differ: α2(H122) and α2(V124). We made the double mutation to the wild-type α2 subunit (α2(Q122, T124)) and found that physostigmine still inhibited the response to ACh although to a lesser extent than for α2. The addition of the α2(I92R) mutation on the background of α2(Q122, T124) conferred a small level of potentiation, although less than that seen with the α2(E + I92R) construct. These observations suggest that the E loop has residues affecting the actions of physostigmine in addition to those that affect activation by ACh.

In the structure of the α4 subunit (Morales-Perez et al., 2016) the D-A region curves around the E loop (Figure 6B, C), suggesting that extensive physical interactions could take place. The α4(R92) residue lies behind the E loop and is oriented to approach the conserved tryptophan at position 123. The residues in or near the D loop (α4(V61) and α4(H66)) are near the carboxy-terminal end of the E loop, although not oriented directly towards the E loop. However, the sidechains of the triplet starting at D76 are oriented towards the initial α helix, far from the E loop itself. None of these residues is positioned in such a way to indicate that they participate in forming a binding site also involving the E...
loop, suggesting that they either participate in a transduction process or alter the conformation of the receptor to facilitate interaction of physostigmine with the receptor. The residues are also located in positions that are unlikely to associate with an adjacent subunit, suggesting that all the interactions take place within a single subunit.

There was no indication that the loops contributed by the α subunit to the “+” side of an interface played a role in determining the effects of physostigmine. The A and B loops are identical for the α2 and α4 subunits (Figure 3) and swapping the region containing the C loop had no effect (Table 2).

The E loop of the α subunit does not participate in a canonical ACh-binding site as it lies on the “-” or complementary side of the interface. This side is contributed by a β or other non-α subunit in a canonical binding site. A surprising result from previous studies of α4 and β2 subunits (Jin et al., 2017) was that swapping the E loop from β2 to α4 resulted in physostigmine becoming an inhibitor regardless of whether the chimeric α4 subunit was placed counterclockwise from an α4 subunit so that the chimera provided the “-” side to an α4-α4 interface or with a β2 subunit to contribute to a β2-α4 interface. This appears to be the case for the α2 subunit as well. We expressed free α2 subunits with concatemers of the α4 and β2 subunits constructed so that the free α2 subunit would contribute the “-” side to either an α4-α2 interface or a β2-α2 interface (see Figure 1) and obtained similar low levels of potentiation by physostigmine.

Although the nature of the amino acids in the E loop affects both the actions of physostigmine and activation by ACh there does not seem to be a mechanistic connection between the two effects. There was no or a weak relationship between in terms of the effects of the various constructs on physostigmine effects and activation parameters, and the regions of the α subunit implicated in mediating effects were not identical.

Our experiments focused on the properties of the α subunit, but inspection of Table 1 suggests that the nature of the β subunit has some effect on the response to physostigmine: in every expression context the β4 subunit resulted in larger potentiation or less inhibition than the β2 subunit.

The present results agree with our previous studies of physostigmine effects on receptors
containing α4 and β2 subunits (Jin et al., 2017), and confirm the role of the E loop. In combination the D-A region and the E loop are able to fully explain the differences in physostigmine effect between the α2 and α4 subunits. Studies of the related nicotinic receptor from Torpedo electroplax have shown that physostigmine photolabels residues near the E loop (Hamouda et al., 2013), consistent with the idea that physostigmine binds at or near the E loop. The additional residues we have identified in the D-A region appear likely to be involved in transduction of binding into either potentiation or inhibition.
Acknowledgments

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Authorship contributions

Participated in research design: Jin, Akk and Steinbach

Conducted experiments: Jin, Germann and Shin

Contributed new reagents or analytic tools: Jin, Germann

Performed data analysis: Jin, Germann, Shin, Akk and Steinbach

Wrote or contributed to the writing of the manuscript: Jin, Akk and Steinbach
References


Footnotes

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Legends for Figures

Figure 1. Summary of subunit structure and arrangement in the pentameric receptor.

The figure shows cartoon representations of the structure of neuronal nicotinic receptors. Panel A shows main structural regions of the primary sequence for both α and β subunits. Each subunit has a similar overall structure (Panel A lower) with a relatively large N-terminal extracellular domain that contains the loops of the ACh-binding site. This is followed by three transmembrane (TM) domains; the channel is lined by the TM2 domains from each subunit. There is then a variable length intracellular domain followed by a fourth transmembrane domain and a short C-terminal extracellular domain. Panel B shows the arrangement of subunits in the pentameric receptor viewed from the extracellular side (left); a canonical ACh-binding site (indicated by a star) is formed at an interface between an α subunit (“+” surface) counterclockwise to a β subunit (“−” surface). The α subunit contributes the A, B and C loops (right) while the β subunit contributes the D, E and F loops. Panel C shows the location of the α2 subunit when expressed with the α4-β2 and β2-α4 dimeric constructs. Note that the nature of the interfaces formed by the α2 subunit depends on the particular constructs used.

Figure 2. Effect of transplanting the D-A and E loop regions between the α4 and α2 subunits.

Current traces are shown for responses when ACh was initially applied alone and then the perfusion was switched to ACh + physostigmine (ACh alone application indicated by the lower bar above the trace, and the time of the application of both ACh and physostigmine shown by the upper bar). The constructs injected are shown above the trace (20:1 ratio for α:β subunits); 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. The physostigmine concentration was 10 µM, The ACh concentrations used were: α4β4 10 µM; α2β4 20 µM; α4(D-A+E)β4 3 µM and α2(D-A+E)β4 10 µM.
Figure 3. The extracellular domains of α2 and α4 subunits.

Sequences of the human α4 (Accession number NP_000735.1) and mouse α2 (NP_659052.1) subunits are shown for the amino-terminal extracellular region. In the line below the sequences (“Differ”) a “+” indicates that the residues differ at that position in the two subunits, while the approximate locations of the loops in the canonical ACh-binding site are shown above the sequences (“Loops”). Loops A, B and C are contributed by the subunit at the “+” side of the interface, while loops D, E and F are contributed by the subunit on the “-” side. The regions transferred in the various chimeras are indicated by the lines above the loops, and identified at the left (e.g. “A-TM1” starts at the end of the A loop and extends to the start of the first transmembrane domain). Finally, the residues mutated in the D-A region are indicated by solid arrows below the sequences in the line marked “Mutations” and the residues in the E loop by hollow arrows.

Figure 4. Effects of N-terminal region chimeras on actions of physostigmine.

The left-hand panel shows a cartoon representation of the structure of the N-terminal region of the various chimeras tested. Regions derived from the α2 subunit are shown in red, while regions from α4 are shown in blue. Regions that are identical between the two subunits are in gray. The chimeras are identified in the left hand boxes; those based on the α2 subunit are shown in the bottom portion, while those based on α4 are shown in the upper portion. The chimera joining points are shown in Figure 3. The mean ± SE physostigmine effect is shown in the bar to the right, while the asterisks give the P value that the effect is identical to the effect for the wild type subunit (one-way ANOVA with Dunnett’s correction; *** P< 0.001, ** P<0.01). The solid vertical line shows an effect ratio of 1 (no effect) while the dashed lines show the mean effects for wild-type α2 and α4 subunits. Data values are given in Tables 3 and 5. All α subunits were expressed with β4 at a 20:1 ratio.

Figure 5. Effects of mutations in the D-A region.

The figure is similar to Figure 4. In the left panel a cartoon of the region from the D loop to the end of
the E loop is shown, with regions derived from the α2 subunit in red and regions from α4 in blue. The A loop (gray) is identical in these subunits, while the region between the A and E loops (white) was not mutated. The mutations were made on the background of the α2(E) chimera (upper 4 lines, hatched bars) or the α2(D-A+E) chimera (lower 4 lines, solid black bars), while the middle 4 lines show results for relevant chimeras and wild-type α2 subunits. The approximate location of the residues mutated is shown by the colored bars shown in the cartoons (left panel). On the background of the α2(E) chimera the mutation changed a residue found in the α2 sequence to that found in α4 and so is shown as red and vice versa. The dashed lines show the mean effects for the background chimeras, and the asterisks indicate the P value that the effect is identical to that for the background chimera (one-way ANOVA with Dunnett’s correction; *** P < 0.001, ** P < 0.01). For locations of chimera joining points and mutations see Figure 3. Data shown are mean ± SE; data values given in Table 4.

Figure 6. Location of mutated residues in the N-terminal domain of the α4 subunit.

The figure shows views of the extracellular N-terminal domain of the nicotinic α4β2 receptors (Morales-Perez et al., 2016) to indicate the position of the residues mutated. The color coding is kept constant for all panels. Panels A and B provide overall orientation: Panel A shows a view from the extracellular fluid looking down at the receptor. The α4 subunits are shown as blue ribbons, while β2 are shown in green. The yellow arrows show the locations of canonical ACh-binding sites (α4 counterclockwise, contributing the “+” side). The red bracket indicates the β2-α4 interface shown in the remaining panels. Panel B shows a side view of a β2-α4 pair from the outside of the receptor with the extracellular fluid at the top. The approximate position of the cell membrane is shown by the black bracket on the right. The E loop of the α4 subunit is shown in orange, and the D-A region in cyan. Panel C shows the region indicated by the red box in Panel B, enlarged and with the β2 subunit removed to reveal the α4 subunit. The view is from the outside of the receptor, looking into the cavity that binds drugs. The face of the “−” side of the binding pocket is shown. The peptide chain of the α4 subunit is shown as a blue ribbon, with the E loop in orange and the D-A region in cyan. The mutated residues are shown in stick format: V61 and H66
residues in green to the right of the E loop, D76, Y77 and E78 residues in black at the top of the panel and R92 in red behind the E loop. Panels D through F show enlarged views of mutated residues. Panel D shows the V61 and H66 residues (green stick format) seen from a perspective similar to panel A. Although these residues are close to the E loop they do not appear to be in a position to directly interact nor do they extend into the drug-binding pocket. Panel E shows the D76, Y77 and E78 residues (black stick format), seen from a perspective further into the extracellular milieu. These residues appear to be close to the initial α helix of the subunit and far from the E loop. Panel F shows the R92 residue in red seen from a perspective approximately 180° from that in A, R92 is behind the E loop, and appears to approach the W123 sidechain in the E loop (shown in orange stick format).
Table 1

<table>
<thead>
<tr>
<th>Subunits</th>
<th>Maximal response</th>
<th>ACh EC\textsubscript{50}</th>
<th>Physostigmine effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nA)</td>
<td>µM</td>
<td>P in pair</td>
</tr>
<tr>
<td>α4&amp;β2 8:1</td>
<td>-13042 ± 1303 (51)</td>
<td>132 ± 10 (33)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>α4&amp;β2 1:8</td>
<td>-1029 ± 193 (47)</td>
<td>5 ± 1 (20)</td>
<td>1.42 ± 0.04 (38)</td>
</tr>
<tr>
<td>α2&amp;β2 8:1</td>
<td>-130 ± 21 (26)</td>
<td>217 ± 42 (15)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>α2&amp;β2 1:8</td>
<td>-190 ± 35 (59)</td>
<td>4 ± 0 (36)</td>
<td>0.78 ± 0.04 (13)</td>
</tr>
<tr>
<td>α4&amp;β4 10:1</td>
<td>-10607 ± 473 (118)</td>
<td>31 ± 2 (21)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>α4&amp;β4 1:10</td>
<td>-14898 ± 1221 (27)</td>
<td>14 ± 2 (21)</td>
<td>1.10 ± 0.05 (27)</td>
</tr>
<tr>
<td>α2&amp;β4 20:1</td>
<td>-929 ± 102 (85)</td>
<td>91 ± 6 (28)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>α2&amp;β4 1:8</td>
<td>-11509 ± 1635 (22)</td>
<td>22 ± 3 (16)</td>
<td>0.90 ± 0.07 (9)</td>
</tr>
</tbody>
</table>

Table 1. Physostigmine does not potentiate responses of receptors containing the α2 subunit. The first column gives the subunit combination injected and the ratio of α to β subunits. The column headed "Maximal response" gives mean ± SEM (number of cells) for the peak amplitude in response to 1 mM ACh. The column headed "ACh EC\textsubscript{50}" gives for the concentration of ACh producing a half-maximal response (EC\textsubscript{50}). The column headed "Physostigmine effect" gives the ratio of the response to a low concentration of ACh in the presence of Physo to the response of the same cell to ACh in the absence of Physo. The column headed P in pair gives the P value that the parameter (EC\textsubscript{50} or effect ratio) is the same for the injections at different ratios (two-tailed t-test with unequal variances).
Table 2

<table>
<thead>
<tr>
<th>Subunits</th>
<th>ACh EC$_{50}$ µM</th>
<th>P to α2 ratio</th>
<th>P to α2</th>
</tr>
</thead>
<tbody>
<tr>
<td>α4-β2&amp;α2</td>
<td>294 ± 22 (5)</td>
<td>1.20 ± 0.05 (5)</td>
<td></td>
</tr>
<tr>
<td>α4-β2&amp;α4</td>
<td>78 ± 5 (47)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>α4-β2&amp;β2</td>
<td>2.9 ± 0.5 (14)</td>
<td>&lt;0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>β2-α4&amp;α2</td>
<td>96 ± 6 (8)</td>
<td>1.12 ± 0.01 (10)</td>
<td></td>
</tr>
<tr>
<td>β2-α4&amp;α4</td>
<td>121 ± 10 (59)</td>
<td>0.04</td>
<td>1.38 ± 0.03 (51)</td>
</tr>
<tr>
<td>β2-α4&amp;β2</td>
<td>3.7 ± 0.5 (57)</td>
<td>&lt;0.001</td>
<td>0.72 ± 0.05 (28)</td>
</tr>
</tbody>
</table>

Table 2. The response to physostigmine is less when the α2 subunit replaces a single α4 subunit. The first column gives the subunit combination injected. The concatemer construct α4-β2 has the α4 subunit at the amino-terminal end joined to the β2 subunit by a linker, while the β2-α4 concatemer has the β2 subunit at the amino-terminal end. The concatemers assemble in a clockwise fashion (N-terminal to C-terminal) as viewed from the extracellular space (Zhou et al., 2003; Jin and Steinbach, 2011). The column headed P to α2 gives the P value that the parameter (EC$_{50}$ or effect ratio) is the same for the concatemer expressed with the studied subunit as with the α2 subunit (two-tailed t-test with unequal variances). Date are mean ± SEM (number of cells).
Table 3

<table>
<thead>
<tr>
<th>α subunit</th>
<th>ACh EC$_{50}$</th>
<th>Physostigmine effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µM</td>
<td>P ratio</td>
</tr>
<tr>
<td>α2</td>
<td>93 ± 4 (38)</td>
<td>X 0.78 ± 0.03 (23)</td>
</tr>
<tr>
<td>α2(D-A)</td>
<td>52 ± 6 (10)</td>
<td>0.3 0.79 ± 0.03 (10)</td>
</tr>
<tr>
<td>α2(C)</td>
<td>37 ± 2 (9)</td>
<td>0.03 0.80 ± 0.02 (14)</td>
</tr>
<tr>
<td>α2(B-TM1)</td>
<td>36 ± 2 (9)</td>
<td>0.02 0.86 ± 0.02 (20)</td>
</tr>
<tr>
<td>α2(N-A)</td>
<td>96 ± 3 (7)</td>
<td>1 0.93 ± 0.01 (14)</td>
</tr>
<tr>
<td>α2(A-TM1)</td>
<td>211 ± 32 (7)</td>
<td>&lt;0.001 0.95 ± 0.02 (20)</td>
</tr>
<tr>
<td>α2(E)</td>
<td>209 ± 16 (3)</td>
<td>&lt;0.001 0.98 ± 0.01 (12)</td>
</tr>
<tr>
<td>α2(E + C)</td>
<td>99 ± 5 (7)</td>
<td>0.9 1.01 ± 0.03 (30)</td>
</tr>
<tr>
<td>α2(E + B-TM1)</td>
<td>159 ± 6 (5)</td>
<td>0.006 1.05 ± 0.02 (31)</td>
</tr>
<tr>
<td>α2(D-A + E + C)</td>
<td>93 ± 14 (13)</td>
<td>0.9 1.31 ± 0.03 (20)</td>
</tr>
<tr>
<td>α2(D-A + E)</td>
<td>257 ± 12 (23)</td>
<td>&lt;0.001 1.42 ± 0.05 (10)</td>
</tr>
</tbody>
</table>

Table 3. The D-A region and the E loop are important in determining the effect of physostigmine. Chimeric subunits are named as subunit contributing the amino terminus followed by the region swapped between subunits (in parentheses), so α2(D-A) indicates an α2 subunit containing α4 sequence for the D-A region. The joining points for the chimeras are shown in Figure 3. The data are arranged in terms of increasing physostigmine effect. The columns headed P gives the P value that the parameter value is the same as that for the control for that group, indicated by X (one way ANOVA with Dunnett’s correction). All α subunits were injected with β4 at a ratio of 20:1. Data are mean ± SEM (number of cells).
Table 4

<table>
<thead>
<tr>
<th>α2 Background</th>
<th>Mutation</th>
<th>ACh EC_{50}</th>
<th>Physostigmine effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>μM</td>
<td>P</td>
</tr>
<tr>
<td>α2(E)</td>
<td>L61V</td>
<td>ND</td>
<td>1.00 ± 0.01 (5)</td>
</tr>
<tr>
<td>α2(E)</td>
<td>N66H</td>
<td>ND</td>
<td>0.67 ± 0.04 (5)</td>
</tr>
<tr>
<td>α2(E)</td>
<td>EFG→DYE</td>
<td>231 ± 4 (2)</td>
<td>0.68 ± 0.05 (5)</td>
</tr>
<tr>
<td>α2(E)</td>
<td>I92R</td>
<td>235 ± 8 (5)</td>
<td>1.19 ± 0.05 (7)</td>
</tr>
<tr>
<td>α2(D-A + E)</td>
<td></td>
<td>257 ± 12 (23)</td>
<td>X</td>
</tr>
<tr>
<td>α2(D-A + E)</td>
<td>V61L</td>
<td>ND</td>
<td>1.13 ± 0.03 (5)</td>
</tr>
<tr>
<td>α2(D-A + E)</td>
<td>H66N</td>
<td>121 ± 50 (3)</td>
<td>1.32 ± 0.11 (5)</td>
</tr>
<tr>
<td>α2(D-A + E)</td>
<td>DYE→EFG</td>
<td>92 ± 13 (7)</td>
<td>1.38 ± 0.04 (5)</td>
</tr>
<tr>
<td>α2(D-A + E)</td>
<td>R92I</td>
<td>184 ± 10 (5)</td>
<td>0.99 ± 0.01 (6)</td>
</tr>
</tbody>
</table>

Table 4. The effect of mutations in the D-A region differs depending on the background. The first two columns give the background chimera (α2(E) or α2(D-A + E)) and the mutation made on that background. The data are arranged in order of the location of the mutation, with the site closest to the amino-terminus given first. The column headed P gives the P value that the parameter value is the same as that for the control for that group, indicated by X (one way ANOVA with Dunnett’s correction). The locations of mutations and the joining points for the chimeras are shown in Figure 3. Data are presented as in Table 3. ND: not determined.
Table 5

<table>
<thead>
<tr>
<th>α subunit</th>
<th>ACh EC&lt;sub&gt;50&lt;/sub&gt; µM</th>
<th>P value</th>
<th>Physostigmine effect</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>α4(D-A + E)</td>
<td>16 ± 2 (6)</td>
<td>0.005</td>
<td>0.73 ± 0.02 (13)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>α4(E)</td>
<td>7 ± 0 (4)</td>
<td>&lt;0.001</td>
<td>0.96 ± 0.01 (12)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>α4(N-A)</td>
<td>ND</td>
<td>ND</td>
<td>1.08 ± 0.01 (13)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>α4(D-A)</td>
<td>74 ± 6 (8)</td>
<td>&lt;0.001</td>
<td>1.27 ± 0.02 (24)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>α4</td>
<td>31 ± 2 (24)</td>
<td>X</td>
<td>1.51 ± 0.03 (31)</td>
<td>X</td>
</tr>
</tbody>
</table>

Table 5. Reciprocal exchanges in the α4 subunit have inverse effects. The data are arranged in terms of increasing physostigmine effect. The column headed P gives the P value that the parameter value is the same as that for the control for that group, indicated by X (one way ANOVA with Dunnett’s correction). All α subunits were injected with β4 at a ratio of 20:1. Data are mean ± SEM (number of cells).
Figures

Figure 1

A

B

C

\( \alpha_4 - \beta_2 \) dimer

\( \beta_2 - \alpha_4 \) dimer
Figure 2
Figure 3

[Diagram showing molecular sequences and mutations]

- D-A
- N-A
- Loops
  - α2
  - α4
- Differences
  - Mutations

- E
  - A-TM1
  - D-A
  - N-A
  - Loops
    - α2
    - α4
  - Differences
    - Mutations

- C
  - B-TM1
  - A-TM1
  - Loops
    - α2
    - α4
  - Differences
    - Mutations
Figure 5
Figure 6