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A ring of uncharged polar amino acids as a component of channel constriction in the nicotinic acetylcholine receptor

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The channel pore of the nicotinic acetylcholine receptor (AChR) has been investigated by analysing single-channel conductances of systematically mutated *Torpedo* receptors expressed in *Xenopus* oocytes. The mutations mainly alter the size and polarity of uncharged polar amino acid residues of the acetylcholine receptor subunits positioned between the cytoplasmic ring and the extracellular ring. From the results obtained, we conclude that a ring of uncharged polar residues comprising threonine 244 of the α-subunit (αT244), βS250, γT253 and δS258 (referred to as the central ring) and the anionic intermediate ring, which are adjacent to each other in the assumed α-helical configuration of the M2-containing transmembrane segment, together form a narrow channel constriction of short length, located close to the cytoplasmic side of the membrane. Our results also suggest that individual subunits, particularly the γ-subunit, are asymmetrically positioned at the channel constriction.

Nicotinic acetylcholine receptor; Ionic channel; Channel constriction; Site-directed mutagenesis; cDNA expression; Single-channel recording

1. INTRODUCTION

The nicotinic acetylcholine receptor (AChR) is a pentameric transmembrane protein composed of 4 kinds of homologous subunits which are assembled in the molar stoichiometry $\alpha_2\beta\gamma\delta$ and arranged pseudosymmetrically around a central cation-conducting channel (see [1] for review). It is generally assumed that the hydrophobic segments M1–M4 of the 5 subunits of the AChR form membrane-spanning α-helices and that polar side chains of some of the transmembrane segments line the channel pore [2]. By analysing single-channel current properties of site-specific mutants of the AChR, we have previously identified 3 clusters of negatively charged and glutamine residues as major determinants of the rate of ion transport through the AChR channel [3]. These amino acid clusters, neighbouring the M2 segments of the constituent subunits of the AChR, are suggested to form three (cytoplasmic, intermediate and extracellular) anionic rings (see Fig. 1A). Mutations in the intermediate ring exert much stronger effects on single-channel conductance [3] as well as on ion selectivity [4] than do mutations in the cytoplasmic and extracellular rings, which suggests that the residues forming the intermediate ring come into close contact with permeating cations.

The present investigation deals with further characterization of the ion-conducting pore of the AChR channel. We systematically mutated uncharged polar amino acid residues of the *Torpedo* AChR subunits positioned between the cytoplasmic ring and the extracellular ring to alter the size and polarity of their side chains. Some other mutations were also introduced into the γ-subunit to alter its unique sequence features. Mutant AChR channels were expressed in *Xenopus* oocytes by microinjection of mRNAs derived from specifically mutated cDNAs and were analysed for single-channel conductance. The results obtained indicate that a cluster of uncharged polar residues, which probably forms a ring-like structure adjacent to the intermediate ring in the assumed α-helical configuration of the M2-containing transmembrane segment, is critical for determining the rate of ion transport. The present investigation, in conjunction with our previous studies, suggests that this uncharged polar ring (referred to as the central ring) and the anionic intermediate ring together form a narrow channel constriction which is confined to a small region close to the cytoplasmic side of the membrane. Our results also suggest that individual subunits, particularly the γ-subunit, are asymmetrically arranged at the channel constriction. Functional implications of the proposed molecular architecture of the AChR channel are discussed.

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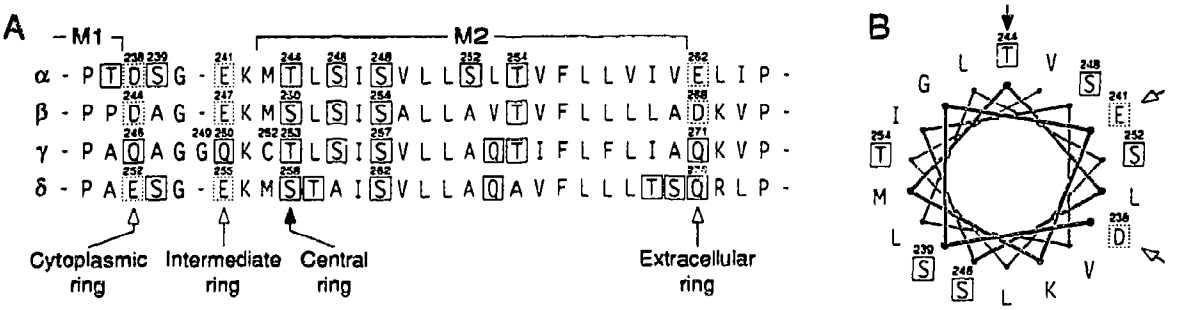

Fig. 1. The channel-forming region of the *T. californica* AChR subunits subjected to mutations. (A) The relevant amino acid sequences (one-letter code) of the α-, β-, γ- and δ-subunits [2,9,10] are aligned [2,15], and the positions of the M1 and M2 segments [2] are shown. The positions of the cytoplasmic, intermediate and extracellular anionic rings [3] are indicated by open arrows, and the position of the uncharged polar residues that have been suggested to take part in forming a channel constriction is indicated by a filled arrow; this cluster of the uncharged polar residues is referred to as the central ring. The numbers [2,9,10] of the amino acid residues subjected to mutations and of the residues composing the 3 anionic rings are given. Uncharged polar residues are boxed with solid lines, and negatively charged residues with broken lines. (B) Schematic representation of part of the channel-forming region of the α-subunit (residues αD238– αV255) in the assumed α-helical configuration, viewed from the amino-terminal (cytoplasmic) side. Circles indicate the positions of the α-carbons of the amino acid residues shown; arrows, numbers and boxes are as in (A).

2. MATERIALS AND METHODS

2.1. *Construction of mutant AChR subunit cDNAs*

Recombinant plasmids carrying the bacteriophage SP6 promoter [5] linked to cDNAs encoding the wild-type and mutant subunits of the *Torpedo californica* AChR were used for invitro synthesis of specific mRNAs. The plasmids that carry cDNAs encoding the wild-type α-(pSPα), β- (pSPβ), γ- (pSPγ) and δ-subunits (pSPδ) have been described previously [6]. The plasmids pSPα6 [3] and pSPα9 (see below) carry cDNAs encoding the wild-type α-subunit into which silent mutations have been introduced to generate additional restriction endonuclease sites. The plasmids carrying cDNAs encoding mutants of the α-, β-, γ- and δ-subunits were constructed [7,8], using synthetic oligodeoxyribonucleotides prepared with an automatic DNA synthesizer (Applied Biosystems). The plasmids prepared differ from the parental plasmids (given in parentheses) as follows (the substituted nucleotides with residue numbers [2,9,10] are given unless otherwise specified, and the plasmids carrying mutated cDNAs are named after the mutant specification): pSPα9 (pSPα): T 648; C 651, 690, 691; AGC 676-678; A 687. pSPαS239N (pSPα6): AAT 715–717. pSPαS239V (pSPα6): G 715; T 716. pSPαS239Y (pSPα6): A 716; T 717. pSPαT244A (pSPα9): C 717, 748, 750; G 720, 730, 732, 762; ATCG 735–738; A 741, 744, 747; T 751; AAGCT 753–757. pSPαT244G (pSPα9): G 730, 731. pSPαT244N (pSPα6): A 731. pSPαT244S (pSPα6): G 731. pSPαT244V (pSPα9): G 730; T 731. pSPαT244Y (pSPα6): T 730; A 731. pSPαS246A (pSPα9): C 717, 748, 750; G 720, 732, 762; AGCG 735–738; A 741, 744, 747; T 751; AAGCT 753–757. pSPαS246N (pSPα9): A 737. pSPαS246V (pSPα9): G 736; T 737. pSPαS246Y (pSPα9): T 736; A 737. pSPαS248N (pSPα9): A 742, 743. pSPαS248V (pSPα9): G 742; T 743. pSPαS248Y (pSPα9): A 743. pSPαS252N (pSPα6): A 754, 755. pSPαS252V (pSPα6): G 754; T 755. pSPαS252Y (pSPα6): A 755. pSPαT254N (pSPα6): A 761. pSPαT254V (pSPα6): G 760; T 761. pSPαT254Y (pSPα6): T 760; A 761. pSPβS250A (pSPβ): G 748; C 749. pSPβS250G (pSPβ): G 748. pSPβS250N (pSPβ): A 749. pSPβS250T (pSPβ): C 749. pSPβS250V (pSPβ): G 748; T 749. pSPβS250Y (pSPβ): T 748; A 749. pSPβS254N (pSPβ): AAC 760–762. pSPβS254V (pSPβ): G 760; T 761. pSPβS254Y (pSPβ): A 761; C 762. pSPγG249Δ (pSPγ): deletion (Δ) of three of the consecutive G residues 742–746. pSPγG249Δ·γQ250K (pSPγG249Δ): A 748. pSPγG249Δ·γT253N (pSPγG249Δ): A 758. pSPγG249Δ·γT253V (pSPγG249Δ): G 757; T 758. pSPγG249Δ·γT253Y (pSPγG249Δ): T 757; A 758. pSPγC252A (pSPγ): G 754; C 755. pSPγC252M (pSPγ): ATG 754–756. pSPγC252S (pSPγ): C 755. pSPγC252T (pSPγ): A 754; C 755. pSPγT253A (pSPγ): G 757. pSPγT253G (pSPγ): G 757, 758. pSPγT253N (pSPγ): A 758. pSPγT253S (pSPγ): G 758. pSPγT253V (pSPγ): G 757; T 758. pSPγT253Y (pSPγ): T 757; A 758. pSPγS257N (pSPγ): A 769, 770. pSPγS257V (pSPγ): G 769; T 770. pSPγS257Y (pSPγ): A 770. pSPδS258A (pSPδ): G 772; C 773. pSPδS258G (pSPδ): G 772. pSPδS258N (pSPδ): A 773. pSPδS258T (pSPδ): C 773. pSPδS258V (pSPδ): G 772; T 773. pSPδS258Y (pSPδ): T 772; A 773. pSPδS262N (pSPδ): A 784, 785. pSPδS262V (pSPδ): G 784; T 785. pSPδS262Y (pSPδ): A 785. mRNAs specific for the wild-type and mutant AChR subunits were synthesized in vitro [5], using *Eco*RI-cleaved plasmids carrying the respective cDNAs as templates, except that the plasmid pSPγ and its mutants were cleaved with *Sma*I; pSPα was used for the synthesis of mRNA specific for the wild-type α-subunit. Transcription was primed [11] with the cap dinucleotide G(5′)ppp(5′)G (0.5 mM).

2.2. *Electrophysiological measurements*

Xenopus laevis oocytes were injected with the 4 kinds of wild-type or mutant AChR-subunit-specific mRNAs; molar ratio of the α-, β-, γ- and δ-subunit-specific mRNAs, 2:1:1:1, total mRNA concentration, 0.1 μg/μl; average volume injected per oocyte, ~50 nl. The oocytes were incubated at 19°C for 3–5 days in modified Barth's medium [12] containing 0.1 mg/ml of cefmenoxime. Single-channel current measurements were made at 12 ± 1°C in inside-out membrane patches isolated from the oocytes as described previously [3]. The bath solution contained 100 mM KCl, 10 mM EGTA and 10 mM HEPES (pH adjusted to 7.2 with KOH). The pipette solution was the same as the bath solution, except that acetylcholine was added in a final concentration of 0.5–4 μM. Single-channel current-voltage relations were obtained as described previously [3,13].

3. RESULTS

3.1. *Mutations of αT244 and equivalent residues*

Threonine 244 of the α-subunit (αT244) and the uncharged polar residues at the equivalent positions of the other subunits (βS250, γT253 and δS258) are predicted to reside at 0.8 α-helix turn from the residues forming the intermediate ring, facing the channel pore (Fig. 1A and B). Mutations were systematically introduced into these uncharged polar residues to alter the size and polarity of their side chains. The resulting mutant channels were measured for acetylcholine-activated currents in a nominally divalent-cation-free, symmetrical K^+-

rich solution, using inside-out membrane patches. Fig. 2 shows single-channel current-voltage relations of the wild-type channel and some of the mutant channels. Most of the mutations used in the present investigation effect little or no change in rectification in comparison with the wild-type channel, although slight inward rectification is caused by some of the mutations (for example, the mutation δS258V); mutations are denoted by amino acid residues (in one-letter code) of the wild-type and the mutant preceding and following the number of the altered residue, respectively.

When single-channel conductance (for inward and outward current) is plotted against the volume of the substituted side chain, two classes of effect become evident for the mutations of the residues αT244 (Fig. 3A), βS250 (Fig. 3B) and δS258 (Fig. 3D), one resulting from hydrophobic substitutions and the other resulting from polar substitutions. In each case, an approximately inverse relationship is found between channel conductance and size of the substituted side chain. When the substituted side chains compared are similar in size, stronger reductions in conductance are observed for hydrophobic substitutions than for polar substitutions. Remarkably, substitution of glycine for the residue αT244 and for the residue βS250, which removes the wild-type polar side chain, yields a conductanct even higher than that of the wild-type channel; when both αT244 and βS250 are replaced by glycine, the effects of the 2 mutations are additive (Fig. 2). The extent of reduction in conductance also varies depending on the subunit into which the mutations are introduced. Stronger effects are generally observed for the mutations of the residue δS258, particularly when the subunit stoichiometry of $\alpha_2\beta\gamma\delta$ is taken into consideration. In contrast, the mutations of the residue γT253 (Fig. 3C) cause much smaller changes in conductance than do the mutations of the equivalent residues of the 3 other subunits. These results, taken together, indicate that both the size and polarity of the residues αT244, βS250 and δS258 are critical for determining the rate of ion transport, suggesting that their side chains take part in forming a narrow channel constriction. They also suggest that individual residues at this position, particularly γT253, are not symmetrically arranged.

3.2. *Mutations of αS248 and equivalent residues*

Serine 248 of the α-subunit (αS248) and the serine residues at the equivalent positions of the other subunits (βS254, γS257 and δS262) are assumed to lie at 1.9 α-helix turns from the residues forming the intermediate ring, facing the channel pore (Fig. 1A and B). The mutations of these residues cause much smaller decreases in conductance than do the analogous mutations of the residues αT244, βS250 or δS258, although some correlation is still observed between the decrease in conductance and the size and polarity of the substituted side chain (Fig. 4A–D). These results suggest that the channel pore is larger in cross section at the position of αS248 and the equivalent residues.

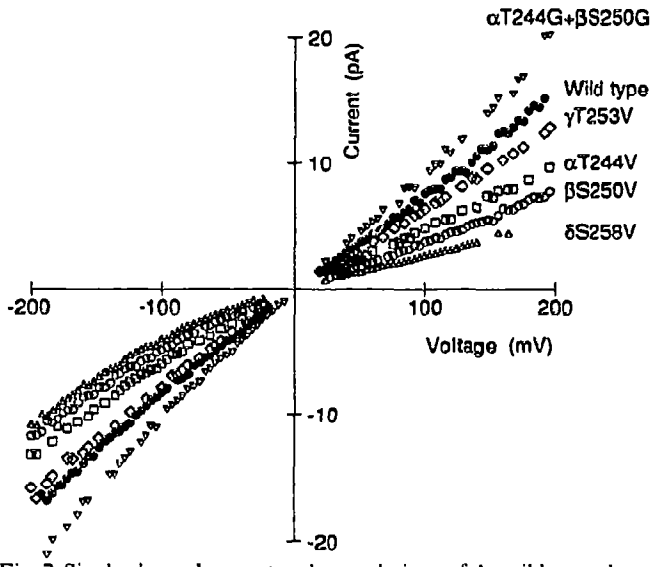

Fig. 2. Single-channel current–voltage relations of the wild-type channel (●) and the channels carrying the mutations αT224V (□), βS250V (○), γT253V (◇), δS258V (△) or both αT244G and βS250G (▽). Each symbol represents a mean of at least 4 amplitude measurments within 4-mV intervals.

3.3. *Mutations of other residues*

Mutations were also introduced into the remaining uncharged polar residues of the α-subunit positioned between the cytoplasmic ring and the extracellular ring (αS239, αS246, αS252 and αT254) (Fig. 1A). The residue αS252 is assumed to reside at 3.1 α-helix turns from the residue αE241 of the intermediate ring, facing the channel pore, whereas the residues αS239, αS246 and αT254 are predicted to lie on the opposite side of the assumed α-helix of the M2-containing transmembrane segment, not facing the channel pore (Fig. 1B). The mutations of these four residues of the α-subunit exert much weaker effects on conductance than do the analogous mutations of the residue αT244, except that the mutation αS252N reduces conductance more than the mutation αT244N (Fig. 5A–D). The effects on conductance of the mutations of the residue αS252, however, do not correlate with the size and polarity of the substituted side chains. These results suggest that the channel pore is wider at the position of αS252.

3.4. *Deletion of glycine of the γ-subunit preceding the intermediate ring*

Asymmetrical behaviour of the γ-subunit is observed not only when the residue γT253 is mutated but also when the residue γQ250 in the intermediate ring is mutated [3]. The mutation γQ250K reduces conductance more than expected from the effect of decreasing the net negative charge of the equivalent residues of the α-, β-and δ-subunits (the mutations αE241Q, βE247Q and δE255Q). Furthermore, the mutation γQ250K does not

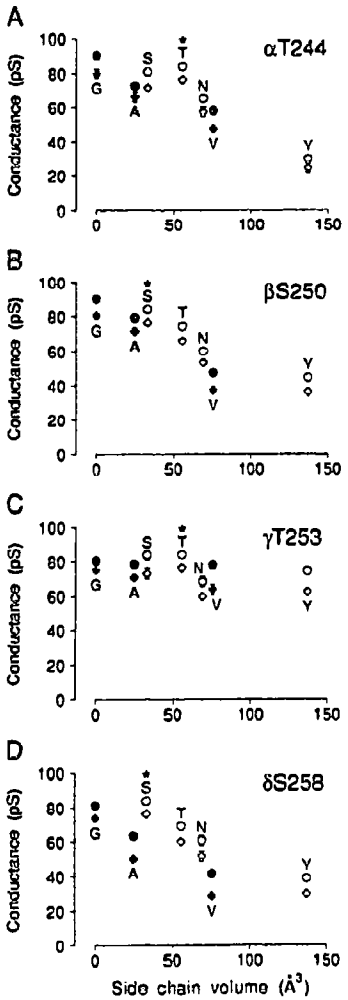

Fig. 3. Effects on conductance of altering the size and polarity of the side chains of the residues αT244 (A), βS250 (B), γT253 (C) and δS258 (D). Single-channel chord conductances at membrane potentials of −100 mV (●, ○) and +100 mV (♦, ◇) are plotted as a function of the side chain volume of the wild-type and substituted residues; (●, ♦) residues with hydrophobic side chains (glycine, alanine and valine); (○, ◇) residues with polar side chains (serine, threonine, asparagine and tyrosine); the wild-type residues are marked with asterisks. The side chain volumes used [38] are differences with respect to glycine. Each symbol represents a mean from 3–8 experiments. Error bars are ± SD and indicated only when larger than the symbol size.

evoke inward rectification which results from the mutations αE241Q, βE247Q and δE255Q. To test the possibility that the asymmetrical behaviour of the γ-subunit is attributable to this subunit containing an extra glycine residue, which immediately precedes the intermediate ring (Fig. 1A), we examined effects of deleting this additional glycine (Table I). When this glycine is deleted from the γ-subunit, the reduction in conductance caused by the mutation γQ250K becomes smaller such that it is comparable to the reduction expected from the effect of decreasing the net negative charge of the residues αE241, βE247 and δE255 [3]. Furthermore, this deletion significantly increases the conductances of the wild-type channel and the channels carrying mutations of the residue γT253 (γT253V and γ253Y). On the other hand, the absence of inward rectification observed for the channel carrying the mutation γQ250K is retained and the effect on conductance of mutating the residue γT253 remains small. Thus part of the asymmetrical behaviour of the γ-subunit is eliminated by deleting the extra glycine residue preceding the intermediate ring. The insertion of this glycine presumably causes a localized distortion of the γ-subunit, thus altering the position of the residue γQ250 relative to the other residues in the intermediate ring, so that the pore size becomes smaller at this constricted site. This view is supported by the observation that the mutation γQ250N, which is expected to enlarge the channel pore at this site, significantly increases conductance [4].

3.5. *Mutations of cysteine 252 unique to the γ-subunit*

Another unique feature of the γ-subunit sequence is the occurrence in the M2 segment of cysteine 252 (γC252) located 2 residues from the residue γQ250 of the intermediate ring, whereas the equivalent position of the other subunits is occupied by methionine (Fig. 1A); the δ-subunit of mammalian muscle AChRs has threonine at this position [14–16]. The residue γC252 is predicted to be located away from the channel pore on the assumed α-helix of the M2-containing transmembrane segment (see Fig. 1B for the residue αM243 at the equivalent position). Mutating this cysteine to methionine or to threonine, serine and alanine exerts only marginal effects on conductance for inward and outward current, the conductances of the mutant channels being 94–97% of the wild-type values (data not shown).

4. DISCUSSION

The results presented suggest that the uncharged polar residues αT244, βS250 and δS258, which are adjacent to the residues of the intermediate anionic ring in the assumed α-helical configuration of the M2-containing transmembrane segment, take part in constituting a narrow constriction of the AChR channel, coming into close contact with permeating cations. These uncharged polar residues, together with the residue γT253, probably from a ring-like structure (referred to as the central ring). Our results also suggest that the channel pore is larger in cross section at the position of the serine residues αS248, βS254, γS257 and δS262, which are assumed to reside about two α-helix turns apart from the residues of the intermediate ring, as well as at the position of the serine residue αS252, which is assumed to lie about three α-helix turns apart from the residue αE241 of the intermediate ring. Our previous studies have suggested that the intermediate ring contributes to forming a narrow constriction of the channel, whereas the extracellular ring and the cytoplasmic ring, located near the external and internal mouths, respectively, surround wider portions of the channel pore [3,4]. All these findings lead to the conclusion that the uncharged polar

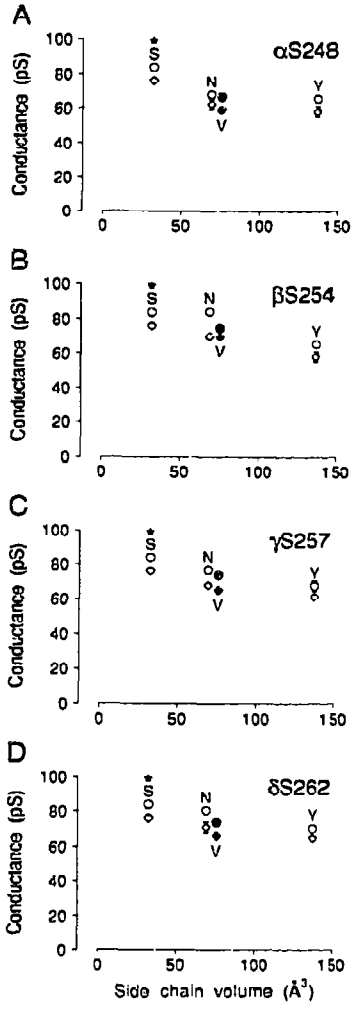

Fig. 4. Effects on conductance of altering the size and polarity of the side chains of the residues αS248 (A), βS254 (B), γS257 (C) and δS262 (D). Single-channel chord conductances at membrane potentials of −100 mV (●, ○) and +100 mV (♦, ◇) are plotted as in Fig. 3. Each symbol represents a mean from 3–6 experiments with one exception indicated below. Error bars are as in Fig. 3. The conductances of the channel carrying the mutation αS248V were obtained from a single current–voltage relation derived from 3 experiments because openings of this channel were short and infrequently well resolved.

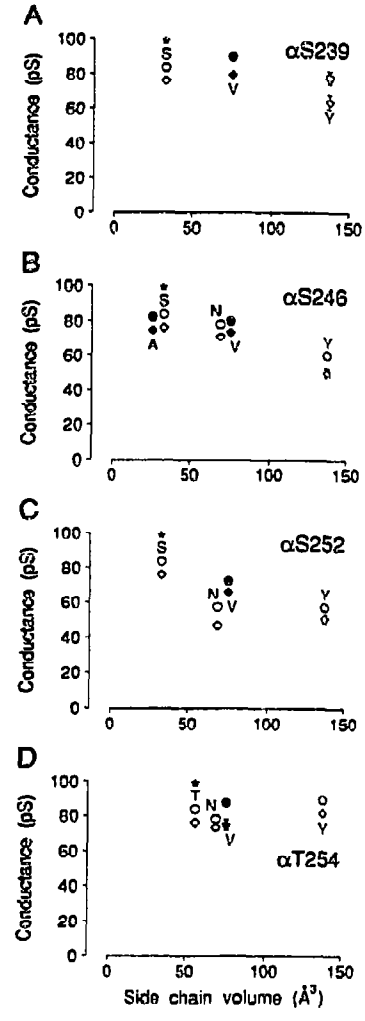

Fig. 5. Effects on conductance of altering the size and polarity of the side chains of the residues αS239 (A), αS246 (B), αS252 (C) and αT254 (D). Single-channel chord conductances at membrane potentials of −100 mV (●, ○) and +100 mV (♦, ◇) are plotted as in Fig. 3. Each symbol represents a mean from 3–7 experiments. Error bars are as in Fig. 3. The conductance of the channel carrying the mutation αS239N could not reliably be determined because of insufficient functional expression.

residues αT244, βS250 and δS258 in the central ring and the glutamic acid and glutamine residues in the intermediate ring together form a narrow channel constriction, whereas the channel pore is larger in cross section on both sides of this constriction. Because the uncharged polar residues of the central ring and the anionic and polar residues of the intermediate ring are adjacent to each other on the assumed α-helices of the M2-containing transmembrane segments of the individual subunits, the channel constriction is suggested to be confined to a small region comprising these 2 rings, which is close to the cytoplasmic side of the membrane. A simplistic estimate of its length would be one of the α-helix (5.4 Å), which agrees with the value obtained from streaming potential measurements (3–6 Å) [17]. In accord with this conclusion is also the recent report that the threonine residue of the rat AChR α-subunit equivalent to αT244 of the *Torpedo* AChR contributes to forming a channel constriction [18].

The wider portion of the channel pore on the external side of the short constriction has been shown to interact with bulky organic compounds. Mutating residues of the mouse AChR corresponding to αS248 and αS252 and the equivalent residues of the *Torpedo* AChR affects interactions with the open-channel blocker QX-222, but hardly reduces conductance with one exception [19,20]. Mutating residues corresponding to αT244 and the equivalent residues exerts little effect on binding of

QX-222 and causes only slight decreases in conductance [20]; the failure to observe stronger effects on conductance may be accounted for by the use of mutant AChRs in which replacement of amino acid residues (threonine by alanine, serine by alanine and glycine by serine) decreases (or increases) both the size and the polarity of their side chains. It has also been reported that αS248 and the equivalent serine residues of the other subunits and some other residues assumed to lie on the adjacent α-helical turns are labelled by noncompetitive antagonists, such as [^{3}H]chlorpromazine [21–24] and [^{3}H]triphenylmethylphosphonium [25,26]. These findings are in line with the view that the pore size at the position of the central ring is too small to be accessible to these bulky organic compounds. Away from the narrowest portion, other transmembrane segments may also participate in forming the channel lining, because five α-helices can encircle a pore of limited cross section. This view is consistent with the observation that another noncompetitive antagonist, [^{3}H]quinacrine azide, labels the α-subunit of the AChR in the open state within the cyanogen bromide fragment comprising the residues αQ208–αM243 [27], which includes the M1 segment. It is also possible that this antagonist interacts with the carboxy-terminal portion (αD238–αM243) of the cyanogen bromide fragment, which corresponds to part of the channel-forming transmembrane segment (Fig. 1A).

The variable extents of change in conductance resulting from the mutations of the residues αT244, βS250, γT253 and δS258 in the central ring suggest that the individual subunits do not equally participate in forming the channel constriction. Particularly, the mutations of γT253 cause only weak effects on conductance. This, together with the aberrant effects of the mutation γQ250K in the intermediate ring [3], suggests that the γ-subunit is asymmetrically positioned at the channel constriction. The asymmetric behaviour of the γ-subunit seems to be attributable partly to the presence of an additional glycine residue immediately preceding the intermediate ring, a unique structural feature of the γ-subunit. There are several lines of evidence that point to unique functional roles of the γ-subunit in controlling the conductance and gating of the AChR channel. The γ-subunit of the embryonic form of skeletal muscle AChR is replaced by the ε-subunit [28], the adult counterpart of the γ-subunit, during muscle development [29]. This switch from the γ- to the ε-subunit increases the conductance and shortens the average duration of elementary currents of the AChR channel. The higher channel conductance of the adult form of AChR can be ascribed to the fact that the mammalian ε-subunit, which is equivalent to the *Torpedo* γ-subunit with respect to the amino acid residues in the 3 anionic rings, is more negative in net charge in the extracellular and cytoplasmic rings than is the mammalian γ-subunit [3]. A recent observation suggests that the M2 segment is also an important determinant of the difference in gating behaviour between the two forms of AChR [30]. It has also been shown that the bovine muscle AChR channel devoid of both the γ- and ε-subunits exhibits frequent spontaneous openings in the absence of agonist [31]. Because in this defective receptor the position normally occupied by the γ-subunit (or the ε-subunit) is presumably filled by one of the remaining subunits, the aberrant gating behaviour of this channel suggests that the γ-subunit (or the ε-subunit) is important in stabilizing the closed state of the unliganded AChR channel. A recent report also suggests that the γ-subunit contributes significantly to the control of channel closure [32]. Additionally, the residue γT253, unlike the residues of the other subunits at the equivalent position, has been shown to incorporate [^{3}H]chlorpromazine [24].

Our results also show that the mutations of the residue δS258 generally cause stronger effects on conductance than do the mutations of the equivalent residues of the other subunits. This may indicate that the posi-

Table I

Effects of deleting an extra glycine of the γ-subunit preceding the intermediate ring on the conductance of wild-type and mutant channels

	Without deletion				With deletion			
Mutation of γQ250 or γT253	−100 mV (pS)	(%)	+100 mV (pS)	(%)	−100 mV (pS)	(%)	+100 mV (pS)	(%)
None	84.0 ± 1.7	(100)	76.3 ± 2.0	(100)	95.9 ± 1.6	(100)	86.5 ± 2.7	(100)
γQ250K	17.7 ± 2.4	(21)	18.6 ± 1.0	(24)	46.1 ± 2.0	(48)	44.9 ± 1.7	(52)
γT253N	68.4 ± 2.9	(81)	59.8 ± 1.6	(78)	79.5 ± 0.5	(83)	70.4 ± 0.5	(81)
γT253V	77.8 ± 2.4	(93)	63.7 ± 2.8	(83)	85.5 ± 1.8	(89)	70.2 ± 2.7	(81)
γT253Y	74.5 ± 1.4	(89)	62.4 ± 2.3	(82)	84.5 ± 2.9	(88)	73.7 ± 2.2	(85)

Single-channel chord conductances at −100 mV and +100 mV membrane potentials of the wild-type channel and of the channels that carry the indicated mutations with or without deletion of an extra glycine of the γ-subunit immediately preceding the intermediate ring are given as means ± SD from 3–8 experiments; the means are also given in percentages. The data for the wild-type channel and the channels carrying the mutations of the residue γT253 without deletion of the extra glycine are the same as given in Fig. 3. The data for the channel carrying the mutation γQ250K without deletion of the extra glycine are the same as described previously [3].

tion of the δ-subunit also deviates somewhat from symmetry at the central ring. It is known that replacing the δ-subunit of the *Torpedo* AChR by the bovine δ-subunit decreases channel conductance (measured in a low-divalent-cation solution) and increases the average duration of elementary currents, making the resulting hybrid channel similar to the bovine channel [13,33]. The region responsible for this difference in conductance has been localized to the M2 segments and its vicinity [13]. Because this hybrid channel shows a voltage sensitivity of the average current duration similar to that of the bovine channel, the δ-subunit has been suggested to contribute to the channel-closing step [33].

The unequal participation of the γ- and δ-subunits in forming the channel constriction may have relevance to the agonist-induced structural transition of the AChR, demonstrated by cryoelectron microscopy of flattened vesicular crystals [34]. Exposure of the AChR to carbamylcholine elicits a tangential displacement of the δ-subunit and a radial displacement of the γ-subunit, whereas no gross alteration in the positioning of the α-and β-subunits is observable. Also from an evolutionary point of view, the γ-subunit (or the ε-subunit) and the δ-subunit are the most closely related among the AChR subunits, exhibiting the highest degree of amino acid sequence identity [35]. Thus the asymmetric behaviour of the γ- and δ-subunits may be important for AChR function.

The proposed architecture of the channel constriction of the AChR allows inferences about the mechanism of permeation. Because of the short length of the channel constriction, a large and steep drop of the membrane potential should occur there to generate a strong driving force for permeating cations. Furthermore, the negatively charged residues in the intermediate ring would attract cations by long- range electrostatic interactions from either the external or the internal mouth of the channel into the channel constriction, where cations would gain free energy from the coordinating negatively charged residues, thus being partially dehydrated [36]. Concurrently, as suggested by the observed effect on conductance of altering the polarity of the side chain, the uncharged polar residues in the central ring would contribute to dehydration of permeating cations by replacing some of the water molecules of their hydration shell [37]. Such a cooperation of the negatively charged and uncharged polar residues at the channel constriction seems to ensure the cation selectivity of the AChR channel without hampering the high rate of ion transport.

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