

Effects of substitution of putative transmembrane segments on nicotinic acetylcholine receptor function

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Mutants of the *Torpedo* nicotinic acetylcholine receptor in which each of the putative transmembrane segments of the α -subunit is replaced by the hydrophobic transmembrane segment of the vesicular stomatitis virus glycoprotein or of the human interleukin-2 receptor have been produced in *Xenopus* oocytes by cDNA manipulations. Functional analysis of these mutants shows that the hydrophobic segment M4 can be replaced by foreign transmembrane sequences without loss of channel activity. It is also suggested that the hydrophobic segments M1, M2 and M3 and the amphipathic segment MA are important for efficient expression of the acetylcholine receptor on the cell surface and that the specific amino acid sequence of segment M2 may be involved in channel activity.

Nicotinic acetylcholine receptor; Transmembrane segment; Site-directed mutagenesis; cDNA expression; Channel activity; α -Bungarotoxin binding

1. INTRODUCTION

The nicotinic acetylcholine receptor from *Torpedo* electroplax is composed of four kinds of subunits assembled in a molar stoichiometry of $\alpha_2\beta\gamma\delta$ [1]. The primary structures of the four subunits of the *Torpedo californica* AChR, deduced from the cDNA sequences [2–5], exhibit conspicuous amino acid sequence homology. This observation, as well as electron image analysis [6], supports the notion that the AChR subunits are oriented in a pseudosymmetric fashion across the

membrane, thus forming the ionic channel. The carboxy-terminal half of each subunit contains four strongly hydrophobic segments (M1–M4) [2–5], which are considered to traverse the membrane. An amphipathic segment (MA) [7,8] and two additional segments [9] have been proposed as potential membrane-spanning regions.

The combination of cDNA expression and site-directed mutagenesis provides a powerful tool for studying the structure-function relationship of the AChR [10–16]. The results of deletion mapping suggest that segments M1, M2, M3, M4 and MA are involved directly or indirectly in the formation of the ionic channel [11,17]. These segments may constitute part of the channel wall or may alternatively be implicated in the correct folding, assembly or transport of the subunits. The present investigation deals with the question as to whether the putative transmembrane segments of the AChR α -subunit can be functionally replaced by hydrophobic transmembrane segments from other proteins.

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Abbreviations: ACh, acetylcholine; AChR, acetylcholine receptor; α -BTX, α -bungarotoxin; VSV, vesicular stomatitis virus

2. MATERIALS AND METHODS

cDNAs encoding *T. californica* AChR α -subunit mutants with substitutions were constructed using synthetic oligodeoxyribonucleotides prepared with an automatic DNA synthesizer (Applied Biosystems) and restriction endonuclease-cleaved DNA fragments from the plasmids pSP α , pSPE α , pSP α N Δ and pSP α C Δ [11] and from pSP α 4 and pSP α 5 (see below). Some silent mutations were introduced by the strategies used (see below). The double-stranded oligonucleotides GP-1 and GP-2 were synthesized to encode the transmembrane segment of the VSV glycoprotein [18] (nucleotide residues 1416–1475 encoding amino acid residues 463–482), and the double-stranded oligonucleotides IR-1, IR-2 and IR-3 to encode the transmembrane segment of the human interleukin-2 receptor [19,20] (nucleotide residues 895–951 encoding amino acid residues 220–238; for the residue numbers, see [20]). These synthetic oligonucleotides differed from the original cDNAs as follows: GP-1, TCA for nucleotides 1416–1418, G for 1421, 1451 and 1460, C for 1424, 1448, 1449 and 1463, AAGC for 1427–1430 and A for 1469; GP-2, identical with GP-1 except A for 1421 and 1475 and T for 1473; IR-1, G for 897 and 933 C for 900 and 945, and A for 903; IR-2, G for 897 and 933, C for 900 and A for 903; IR-3, G for 897 and 933 and C for 900. The plasmids constructed differed from the parental plasmids (given in parentheses) as follows (nucleotide numbers refer to those of the *T. californica* AChR α -subunit cDNA [2]); pSP α 4 (pSP α [11]), C for nucleotides 1113, 1122, 1128, 1143 and 1146, G for 1149, T for 1155, GAGCTC for 1173–1178 and the junctional sequence between the 3'-end of the α -subunit cDNA and the *Eco*RI site of the vector (referred to hereafter as the junctional sequence) taken from pSPE α [11]; pSP α 5 (pSP α 4), C for 1209; pSP α -M1-GP (pSP α), GP-2 for 631–711 and the junctional sequence from pSPE α ; pSP α M2-GP (pSP α), GP-1 for 727–783, C for 717, 789 and 801, T for 792 and G for 795, 798 and 804; pSP α M3-GP (pSP α), GP-1 for 829–894, G for 810, 825, 930 and 936, A for 813, 816 and 903, C for 822 and 939, GTCG for 909–912 and T for 927; pSP α M4-GP (pSP α 5), GP-1 for 1225–1281, C for 1215 and T for 1224; pSP α MA-GP (pSP α 4), GP-1 for 1114–1164 and A for 1170; pSP α M1-IR

(pSP α), IR-1 for 631–711, C for 627 and the junctional sequence from pSPE α ; pSP α M2-IR (pSP α), IR-2 for 727–783, C for 717 and 801, G for 720, 795, 798 and 804 and the junctional sequence from pSPE α ; pSP α M3-IR (pSP α), IR-1 for 829–894, G for 810, 825, 930 and 936, A for 813, 816 and 903, C for 822 and 939, GTCG for 909–912 and T for 927; pSP α M4-IR (pSP α 5), IR-3 for 1225–1281; pSP α MA-IR (pSP α 5), IR-2 for 1114–1164. The plasmids pSP α M4-17, pSP α -M4-15, pSP α M4-14, pSP α M4-11 and pSP α M4-9, which carried cDNAs encoding α -subunit mutants with deletions, were constructed as in [11]. All the constructs were confirmed by sequence analysis [21] of the region encompassing the mutation. The construction of the plasmids pSP α Δ 224–237, pSP α Δ 249–257, pSP α Δ 279–284, pSP α Δ 355–389, pSP α Δ 366–389, pSP α Δ 371–377, pSP α Δ 376–383, pSP α Δ 376–389, pSP α Δ 382–389 and pSP α Δ 409–420 has been described [11].

mRNAs encoding wild-type and mutant AChR subunits were synthesized in vitro as in [11,15], using *Eco*RI-cleaved plasmids, except that the plasmid pSP γ was cleaved with *Sma*I. *Xenopus laevis* oocytes were injected with the wild-type or a mutant α -subunit-specific mRNA, combined with the wild-type β -, γ - and δ -subunit-specific mRNAs (final concentrations, 100, 50, 50 and 50 ng/ μ l, respectively; average volume injected per oocyte, about 40 nl). The oocytes were incubated [16] for 2 days before being tested. The procedures for preparation of cell extracts [16], assay of 125 I- α -BTX binding activity in the cell extract [11] and on the cell surface [13], labelling with L-[35 S]methionine, immunoprecipitation and SDS-polyacrylamide gel electrophoresis of AChR subunits [11] and measurement of ACh-activated whole cell currents [16] have been described.

3. RESULTS

We constructed cDNAs encoding *T. californica* AChR α -subunit mutants in which each of the hydrophobic segments M1–M4 or the amphipathic segment MA was replaced by the transmembrane segment of the VSV glycoprotein [18] or of the human interleukin-2 receptor [19,20]. The α -subunit mutants containing the 20-amino-acid transmembrane segment of the VSV glycoprotein

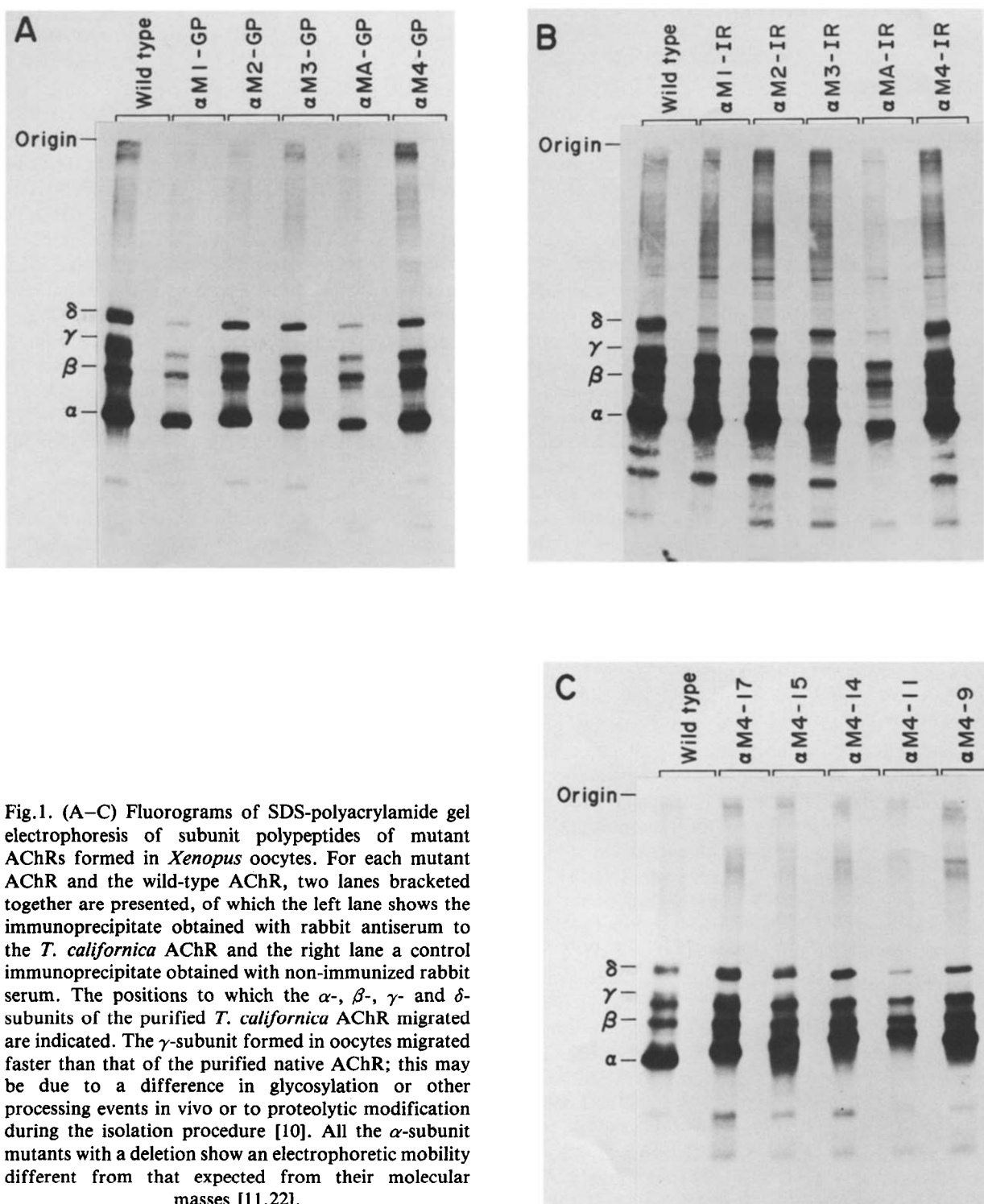


Table 1
Functional properties of AChR mutants with altered α -subunit

α -Subunit	^{125}I - α -BTX binding (fmol per oocyte)		Whole-cell current (nA)
	Surface	Extract	
Wild type	7.4 $\pm$ 4.1 (30)	43.6 $\pm$ 14.3 (19)	189 $\pm$ 169 (228/230)
α M1-GP	ND (9)	3.2 $\pm$ 2.2 (6)	ND (0/60)
α M2-GP	ND (9)	34.8 $\pm$ 16.6 (6)	ND (0/60)
α M3-GP	ND (9)	16.5 $\pm$ 5.2 (7)	ND (0/60)
α MA-GP	ND (9)	8.2 $\pm$ 4.8 (7)	ND (0/60)
α M4-GP	0.1 $\pm$ 0.1 (9)	34.4 $\pm$ 10.3 (7)	62 $\pm$ 107 (52/60)
α M1-IR	ND (8)	0.02 $\pm$ 0.02 (5)	ND (0/40)
α M2-IR	0.6 $\pm$ 0.5 (9)	44.5 $\pm$ 20.8 (5)	ND (0/40)
α M3-IR	ND (8)	26.4 $\pm$ 6.3 (5)	ND (0/40)
α MA-IR	ND (8)	4.1 $\pm$ 2.5 (5)	ND (0/40)
α M4-IR	3.4 $\pm$ 2.3 (10)	43.9 $\pm$ 10.2 (6)	2080 $\pm$ 2480 (50/50)
α M4-17	2.4 $\pm$ 1.5 (3)	23.3 $\pm$ 6.6 (3)	24 $\pm$ 23 (27/30)
α M4-15	3.4 $\pm$ 1.6 (3)	29.1 $\pm$ 12.7 (3)	1490 $\pm$ 1120 (30/30)
α M4-14	ND (3)	19.6 $\pm$ 6.6 (3)	0.1 $\pm$ 0.7 (1/30) ^a
α M4-11	ND (3)	5.1 $\pm$ 1.8 (3)	ND (0/30)
α M4-9	ND (3)	12.6 $\pm$ 5.4 (3)	ND (0/30)

^a 11 $\pm$ 15 nA (19/30) in response to 10 μ M ACh

Data are given as means $\pm$ SD. Numbers in parentheses indicate the number of experiments for ^{125}I - α -BTX binding activity or the number of responsive oocytes/number of oocytes tested for ACh response. Whole-cell currents represent membrane currents activated by bath application of 1 μ M ACh at -70 mV membrane potential. Our detectable limits were ~ 0.1 fmol per oocyte for ^{125}I - α -BTX binding activity on the cell surface, ~ 0.01 fmol per oocyte for ^{125}I - α -BTX binding activity in the cell extract and ~ 3 nA for ACh response. The means for ACh-activated currents were calculated by taking the value for unresponsive oocytes as zero. ND, not detectable. When the concentration of ^{125}I - α -BTX used for the assay was reduced to 0.2 nM, the ^{125}I - α -BTX binding activities in the cell extract of the mutant AChRs (except for the AChR with α M1-IR) as well as of the wild-type AChR were 38–55% of the activities measured with 2 nM ^{125}I - α -BTX, suggesting that the apparent binding affinity for ^{125}I - α -BTX was not essentially affected by the mutations. The extent of inhibition of ^{125}I - α -BTX binding by 0.5 mM carbamylcholine was 42–57% for the mutant AChRs (except for the AChR with α M1-IR) as well as for the wild-type AChR, suggesting that the apparent binding affinity for the agonist was not substantially affected by the mutations

(SSIASFFFIIGLIIGLFLVL) in place of segment M1 (PLYFVVNVIIPELLFSFLTGLVFYLP-T), M2 (MTLSISVLLSLTVFLLIV), M3 (YMLFTMIFVISSIIITVVVINT), M4 (ILLCVFMLICII-GTVSVFA) or MA (VKSAIEGVKYIAEHMKS) are designated as α M1-GP, α M2-GP, α M3-GP, α M4-GP and α MA-GP, and the corresponding mutants containing the 19-amino-acid transmembrane segment of the human interleukin-2 receptor

(VAVAGCVFLLISVLLLSGL) as α M1-IR, α M2-IR, α M3-IR, α M4-IR and α MA-IR, respectively. Each of the mutant α -subunit-specific mRNAs synthesized with the respective cDNA templates, combined with the wild-type β -, γ - and δ -subunit-specific mRNAs, was injected into *Xenopus* oocytes. The amounts of individual AChR subunits formed in the oocytes were estimated by labelling the polypeptides with [^{35}S]methionine,

followed by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. Densitometric scanning of the resulting fluorograms (fig.1A and B) showed that, for all the mutant AChRs with a substitution in the α -subunit, there were sufficient concentrations of the four subunit polypeptides (at least 10% of the wild type) to allow the assay of functional parameters for the AChR.

As indicated in table 1, oocytes implanted with most of the mutant AChRs with a substitution in the α -subunit exhibited ^{125}I - α -BTX binding activities in the cell extract comparable to the wild-type activity, whereas the mutant AChR with $\alpha\text{M1-IR}$ showed a markedly reduced activity, which was much lower than expected from the concentrations of the subunit polypeptides found. The relatively low α -BTX binding activities of some other mutants (for example, the AChR with $\alpha\text{M1-GP}$ or $\alpha\text{MA-IR}$) can roughly be accounted for by the observed decreases in the amounts of the subunit polypeptides (see [16]). Table 1 also shows that oocytes implanted with the mutant AChR containing $\alpha\text{M2-IR}$, $\alpha\text{M4-IR}$ or $\alpha\text{M4-GP}$ exhibited ^{125}I - α -BTX binding activities on the cell surface, which were diminished to various extents, however, as compared with the wild-type activity. In contrast, no α -BTX binding activity was detectable on the surface of oocytes implanted with the other mutant AChRs carrying a substitution in the α -subunit, despite the presence of significant α -BTX binding activities in the cell extract (except for the AChR with $\alpha\text{M1-IR}$).

The ACh responses of oocytes implanted with the mutant AChRs with a substitution in the α -subunit were measured under voltage clamp at -70 mV membrane potential, using bath application of ACh (table 1). Oocytes implanted with the mutant AChR containing $\alpha\text{M4-IR}$ or $\alpha\text{M4-GP}$ responded to $1\text{ }\mu\text{M}$ ACh, the currents being much larger than expected from the α -BTX binding activities found on the cell surface. In contrast, no detectable response ($< \sim 3$ nA) was observed for oocytes implanted with the other mutant AChRs carrying a substitution in the α -subunit even after application of 3 (or 10) μM and $100\text{ }\mu\text{M}$ ACh. In view of the significant α -BTX binding activity found on the surface of oocytes implanted with the mutant AChR containing $\alpha\text{M2-IR}$ (0.6 fmol per oocyte), a similar amount of the wild-type AChR was expressed on the surface of oocytes (0.8 fmol

per oocyte) by injecting 10-fold smaller amounts of the four subunit-specific mRNAs. These oocytes responded clearly to ACh, the currents activated by 1 , 10 and $100\text{ }\mu\text{M}$ ACh being 29 , 910 and 4150 nA, respectively (means of 20 oocytes, six of which failed to respond to $1\text{ }\mu\text{M}$ ACh).

We next examined the effect of shortening of segment M4 of the α -subunit on AChR function. By introducing internal deletions into the cDNA, segment M4 of the α -subunit, composed of 19 amino acid residues (see above), was shortened by 2, 4, 5, 8 and 10 amino acids to generate the mutants $\alpha\text{M4-17}$ (in which CII was changed to A), $\alpha\text{M4-15}$ (MLICII to IA), $\alpha\text{M4-14}$ (MLICII to A), $\alpha\text{M4-11}$ (CVFMLICII to A) and $\alpha\text{M4-9}$ (LLCVFMLICII to A), respectively. These deletions did not strongly affect either the amounts of the subunit polypeptides found or the α -BTX binding activity in the cell extract (fig.1C and table 1). As shown in table 1, the decrease in the length of segment M4 to 17 ($\alpha\text{M4-17}$) or 15 amino acids ($\alpha\text{M4-15}$) did not substantially prevent the expression of functional AChR on the cell surface; the current measured for the AChR with $\alpha\text{M4-15}$ was much larger than expected from the α -BTX binding activity on the cell surface. The shortening of segment M4 to 11 ($\alpha\text{M4-11}$) or 9 amino acids ($\alpha\text{M4-9}$) abolished, however, the α -BTX binding activity on the cell surface as well as the response to ACh (1 , 10 and $100\text{ }\mu\text{M}$); similarly, neither α -BTX binding activity on the cell surface nor ACh response was detected for oocytes implanted with the AChR containing another α -subunit mutant with segment M4 of 9 amino acids in length ($\alpha\Delta 409-420$ [11]; ILLCVFMLICII to EL), despite the significant α -BTX binding activity found in the cell extract [11]. Oocytes implanted with the AChR containing the α -subunit mutant with segment M4 of 14 amino acids in length ($\alpha\text{M4-14}$) showed no detectable α -BTX binding activity on the cell surface, but responded weakly to ACh.

Oocytes implanted with the AChRs containing α -subunit mutants with a deletion in segment M1, M2, M3 or MA [11] were also tested for α -BTX binding activity on the cell surface and for response to bath-applied ACh. An internal deletion in segment M1, M2 or M3 of the α -subunit ($\alpha\Delta 224-237$, $\alpha\Delta 249-257$ and $\alpha\Delta 279-284$ [11], respectively) abolished the α -BTX binding activity on the cell surface as well as the response to ACh

(1, 10 and 100 μ M), despite the fact that significant α -BTX binding activities were found in cell extracts from oocytes implanted with the AChRs containing these α -subunit mutants except for $\alpha\Delta 224$ –237 [11]. On the other hand, oocytes implanted with the AChRs containing α -subunit mutants with a deletion of part or all of segment MA ($\alpha\Delta 355$ –389, $\alpha\Delta 366$ –389, $\alpha\Delta 371$ –377, $\alpha\Delta 376$ –383, $\alpha\Delta 376$ –389 and $\alpha\Delta 382$ –389 [11]) exhibited low α -BTX binding activities on the cell surface (0.1–0.5 fmol per oocyte, mean of 3–4 experiments for each mutant AChR); the α -BTX binding activities in the cell extract were comparable to the wild-type activity [11]. These oocytes showed virtually no response to 1 μ M ACh, but most or all (68–100%) of the oocytes implanted with each of these mutant AChRs responded to 10 μ M ACh, the average currents (from 20–30 oocytes each) ranging from 14 to 131 nA. Using ionophoretic application of ACh, we previously observed that all or some of oocytes implanted with the mutant AChR containing $\alpha\Delta 355$ –389, $\alpha\Delta 366$ –389 or $\alpha\Delta 371$ –377 responded to ACh, whereas oocytes implanted with the mutant AChR containing $\alpha\Delta 376$ –383, $\alpha\Delta 376$ –389 or $\alpha\Delta 382$ –389 showed no detectable response [11]. This difference can be accounted for principally by the use of bath application of ACh in the present study. The absence of detectable ACh response in oocytes implanted with the AChRs containing the α -subunit mutants with a deletion in segment M1, M2, M3 or M4 (segment M4 of 9 or 11 amino acids in length) confirms our previous results [11].

4. DISCUSSION

The present investigation shows that the hydrophobic segment M4 of the AChR α -subunit can be replaced by foreign hydrophobic transmembrane sequences without loss of channel activity. This finding suggests that the sequence requirement for segment M4 is not strict, provided that its hydrophobic character is retained. An appropriate length of segment M4 (14 or more amino acids) is required, however, for the expression of functional AChR on the cell surface. These results are consistent with the view that segment M4 traverses or is embedded in the membrane. The higher channel activities observed for the mutant AChRs containing α M4-IR, α M4-GP or α M4-15 with a substitu-

tion or a deletion in segment M4 may reflect a higher open probability of the channel or also a larger elementary current; the apparent binding affinity for carbamylcholine seems not to be substantially affected by these mutations (see the legend to table 1).

Replacement of the hydrophobic segment M1, M2 or M3 of the α -subunit by foreign hydrophobic transmembrane sequences, as well as an internal deletion in these segments, abolishes the α -BTX binding activity on the cell surface and the ACh response, except that oocytes implanted with the mutant AChR containing α M2-IR with a substitution in segment M2 exhibit a reduced but significant α -BTX binding activity on the cell surface. This observation suggests that these clustered hydrophobic segments, which presumably represent transmembrane α -helices, are important for efficient expression of the AChR in the plasma membrane, possibly through their involvement in the correct folding, assembly or transport of the AChR subunits. It is also suggested that the specific amino acid sequence of segment M2 may be involved in channel activity, although the possibility that not all AChR subunits are expressed on the cell surface cannot be excluded. In accord with this notion is our study on the single-channel conductances of mutant AChRs with chimaeric δ -subunits, which suggests that a region comprising segment M2 and the adjacent bend portion between segments M2 and M3 is involved in determining the rate of ion transport through the channel [15]. It has also been reported that segment M2 contributes to the binding site of noncompetitive antagonists [23,24].

Our results further show that the amphipathic segment MA of the α -subunit cannot be functionally replaced by foreign hydrophobic transmembrane sequences. On the other hand, deletion of part or all of segment MA of the α -subunit strongly diminishes but does not abolish the ACh response and the α -BTX binding activity on the cell surface. This suggests that the amphipathic segment MA is also important for efficient expression of the AChR in the plasma membrane.

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