Volume 320, number 3, 261–266 FEBS 12299 April 1993

Design, synthesis and functional characterization of a pentameric channel protein that mimics the presumed pore structure of the nicotinic cholinergic receptor

Myrta Oblatt Montal[a], Takeo Iwamoto[b], John M. Tomich[b] and Mauricio Montal[a]

[a]*Department of Biology, University of California San Diego, La Jolla, CA 92093-0319, USA* and [b]*Department of Biochemistry and Biotechnology Core Facility, Kansas State University, Manhattan, KS 66506-3702, USA*

Received 23 February 1993

Nicotinic cholinergic receptors are membrane proteins composed of five subunits organized around a central aqueous pore. A pentameric channel protein, $T_5M2\delta$, that emulates the presumed pore-forming structure of this receptor was generated by assembling five helix-forming peptide modules at the lysine ε-amino groups of the 11-residue template [K*AK*KK*PGK*EK*G], where * indicates attachment sites. Helical modules represent the sequence of the M2 segment of the *Torpedo californica* acetylcholine receptor (AChR) δ subunit; M2 segments are considered involved in pore-lining. Purified $T_5M2\delta$ migrates in SDS-PAGE with an apparent M_r ~14,000, concordant with a protein of 126 residues. $T_5M2\delta$ forms cation-selective channels when reconstituted in planar lipid bilayers. The single channel conductance in symmetric 0.5 M KCl is 40 pS. This value approximates the 45 pS single channel conductance characteristic of authentic purified *Torpedo* AChR, recorded under otherwise identical conditions. These results, together with conformational energy calculations, support the notion that a bundle of five amphipathic α-helices is a plausible structural motif underlying the inner bundle that forms the pore of the pentameric AChR channel.

Neurotransmitter receptor; Ionic channel; Protein design; Lipid bilayer; Signal transduction

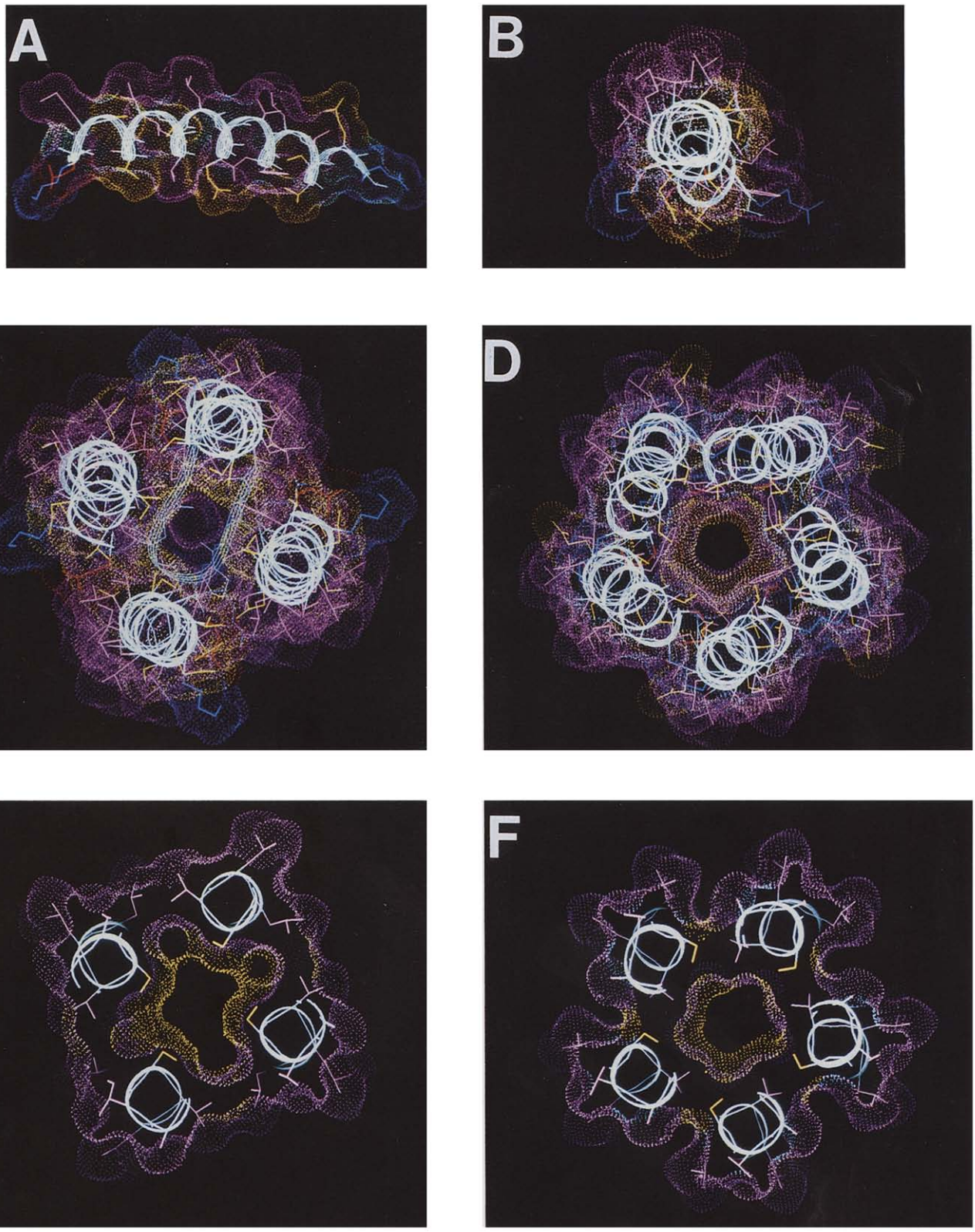
1. INTRODUCTION

The acetylcholine receptor (AChR) from *Torpedo californica* is a pentameric glycoprotein (M_r ~250 kDa) with a stoichiometry of $\alpha_2\beta\gamma\delta$, assembled around a central ion conducting pore [1–7]. The AChR channel is cation selective, with an effective pore diameter of ~7 A; [8,9]. Analysis of the primary structure predicts the occurrence of four α-helical transmembrane segments, M1– M4, in each subunit [1–3,6]. M2 is amphipathic, highly conserved among all members of the superfamily of ligand-gated channels, and is thought to be involved in lining the pore [1–3,6,10–24]. A bundle of five M2 α-helices contributed by each of the subunits that cluster together to form a hydrophilic channel has been considered as a plausible structural motif for the pore-forming element of the AChR [1,2,6,10–24].

Molecular modeling results [10,11,15,17] show that the ion-conducting pore may be mimicked by a parallel homo-oligomer of five amphipathic α-helices arranged such that polar residues line a central hydrophilic pathway and apolar residues face the hydrophobic bilayer interior [11]. We have demonstrated that a four-helix bundle protein designated as $T_4M2\delta$, in which four identical helical modules with the sequence of $M2\delta$ were covalently attached to a multifunctional carrier (template) [25,26], forms ionic channels in lipid bilayers. The $T_4M2\delta$ channel is cation-selective and blocked by mM concentrations of the local anesthetic channel blocker QX-22, properties that are characteristic of the AChR channel [25]. However, the single channel conductance (γ) of $T_4M2\delta$ (γ=20 pS), presumably expressing the activity of a conductive tetramer, differs from that of the authentic pentameric AChR channel (γ=45 pS) [25]. Here, we describe the total chemical synthesis of a five-helix bundle protein $T_5M2\delta$. The purified protein exhibits in SDS-polyacrylamide gels an apparent M_r ~14,000, consistent with a 126-residue protein. The designed molecule forms cation-selective channels after reconstitution in lipid bilayers with a single channel conductance similar to that of the authentic *Torpedo* AChR channel. Results demonstrate that a five helix bundle with the sequence of M2 is sufficient to form the conduction pathway of the authentic AChR and suggest that this motif may represent the inner bundle that lines the pore of the authentic pentameric channel with one M2 segment contributed by each of the five component subunits.

Correspondence address M. Montal, Department of Biology, University of California San Diego, La Jolla, CA 92093-0319, USA. Fax: (1) (619) 534-0173.

Abbreviations. AChR, acetylcholine receptor; γ, single channel conductance; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; τ_c, closed times; τ_o, open times


A
B
C
D
E
F

2. MATERIALS AND METHODS

2.1. *Conformational energy computations*

Molecular modeling, including energy minimization and molecular dynamics simulations, were conducted on a Silicon Graphics IRIS 4D/21 GTX workstation using the INSIGHT and DISCOVER molecular modeling packages of Biosym (San Diego, CA) [11,25,27]. Low-energy arrangements of α-helices and helical bundles were calculated with semi-empirical potential energy functions and optimization routines, and further refined. Constraints were used for symmetry and to maintain regular helical backbone dihedral angles of $\phi=-45$ and $\varphi=-60$ [11].

2.2. *Protein synthesis, purification and characterization*

The designed five-helix bundle protein was synthesized by solid-phase methods using an Applied Biosystems Model 431 peptide synthesizer (ABI, Foster City, CA), according to general principles previously described for the synthesis of four-helix bundle proteins [25–29]. The multifunctional carrier template has the sequence: [K*AK*KK*PGK*EK*G], where * indicates attachment sites for peptide modules at the ε-amino groups of lysine. M2δ modules have the sequence: EKMSTAISVLLAQAVFLLLTSQR [6,10,11,25]. Underlined residues are considered to line the pore [11]. Multiple couplings (3–5) were performed to boost coupling efficiencies to >99% per site for the pentameric unit [25,26]. Cleaved proteins were subjected to multiple purification steps by reverse-phase HPLC, and characterized by amino acid analysis, microsequencing and capillary zone electrophoresis [25,26]. SDS-PAGE analysis was according to Laemmli [30] on 10–20% polyacrylamide gradient Tricine gels (Novex, San Diego, CA). Molecular weight was estimated using CNBr-treated myoglobin molecular weight markers [25,26].

2.3. *Single channel recordings in lipid bilayers*

Lipid bilayers were assembled by apposition of two monolayers, initially formed at the air-water interface, at the tip of patch pipettes [31]. Purified protein was incorporated into lipid bilayers by extraction with lipid, POPE/POPC [1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3- phosphocholine (POPC) (Avanti Biochemicals, Alabaster, AL)], 4:1, in hexane (5 mg/ml), as described [25,26]. The aqueous subphase was composed of 0.5 M KCl, 1 mM $CaCl_2$ and 10 mM HEPES (pH 7.4). Acquisition and analysis of single channel currents were as described [31–35]. Records were filtered with an 8-pole Bessel filter (Frequency Devices, Haverhill, MA) and digitized at 0.1 ms per point using an Axon TL-1 interface (Axon Instruments, Burlingame, CA) connected to an Everex Step 386 computer (Everex, Fremont, CA). pClamp 5.5 (Axon Instruments) was used for data processing. Conductance and lifetime values were calculated from continuous recordings lasting 30 s or longer and with ≥300 openings; openings with $\tau_o \leq 0.3$ ms were ignored. Values are reported as mean ± S.E.M., *n*=number of experiments. Bilayer experiments were performed at 24 ± 2°C.

3. RESULTS AND DISCUSSION

3.1. *Bundles of amphipathic α-helices as a structural motif for an ionic pore*

Conformational energy calculations suggest that a homopentameric bundle of α-helices is a plausible model for the pore-structure of the cholinergic receptor. Fig. 1 illustrates optimized helical structures for the M2δ monomer (A, side view; B, end view), the homotetramer $T_4M2\delta$ (C, end view) and the homopentamer $T_5M2\delta$ (D, end view). The template portion of the bundles may be discerned in Fig. 1C as a β-hairpin at the C-terminal of the helical units [25,27]. The N-terminus is in front and corresponds to the untethered end of helical modules. Glu-1, which corresponds to E255 in the protein sequence, is postulated to face the intracellular end of the receptor channel which is considered to be negatively charged [1,2,6,11,18,23]. Attached α-helical modules are parallel. Most of the hydrophobic and hydrophilic residues occur on opposite faces of the helices (A–D). The lumen of the pore in both tetrameric and pentameric bundles is lined with polar/neutral residues. Ser-4 and Ser-8, which correspond to S258 and S262 in the actual protein sequence, face the aqueous pore. These residues are considered to be exposed to the pore lumen and to participate in ionic conduction through the pore [1,2,11,14,17,20–24]. Fig. 1E and F

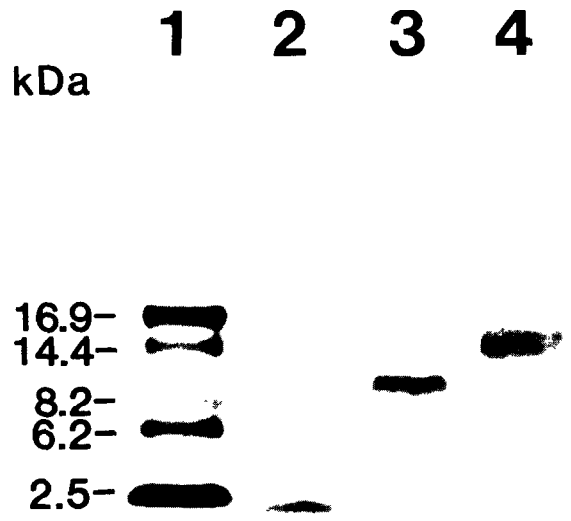


Fig. 2. SDS-PAGE analysis of M2δ channel-forming peptides and oligomeric proteins. HPLC-purified synthetic M2δ peptide, tetrameric $T_4M2\delta$ and pentameric $T_5M2\delta$ were subjected to gel electrophoresis on 10–20% polyacrylamide gels. Protein bands were visualized with silver stain. Lanes: 1, molecular weight markers; 2, monomeric M2δ peptide, 3, tetrameric $T_4M2\delta$ protein, 4, pentameric $T_5M2\delta$ protein. Apparent M_r are 2,000, 11,000 and 14,000, respectively.

←

Fig. 1. A bundle of five amphipathic α-helices is a plausible structure for the pentameric channel protein $T_5M2\delta$. Side view (A) and end view (B) of an energy-optimized molecular model of the helix with the sequence of M2δ. The N-terminus is on the left (A) and at the top (B). The van der Waals envelope (dotted surface) shows the solvent-accessible surface with ribbon representation of the α-carbon backbone. (C and D) End views of the parallel tetramer $T_4M2\delta$ (C) and pentamer $T_5M2\delta$ (D) with the N-terminus in front. The central pore is lined by polar/neutral residues (yellow) and the location of lipophilic residues (purple) is at the exterior of the bundle. The length of the bundle is sufficient to span the bilayer. (E and F) End views of a cross-section at the level of S8 showing approximately two turns of helix, for $T_4M2\delta$ (E) and $T_5M2\delta$ (F). This region corresponds to the section of the pore that features the ring of serines thought to be involved in ionic conduction, with pore dimensions of 4 A; by 5 A; for the tetramer (E) and a pore diameter of 7 A; for the pentamer (F). Color code: light blue, α-carbon backbone; red, acidic, yellow, polar-neutral; white, proline and glycine; and purple, lipophilic residues.

show transverse sections across the bundles depicting approximately one turn of the helix at the location of exposed serines; the pore cross-section, measured at the boundaries of the solvent accesible surface (dotted surface) is, for the tetramer, irregular, with dimensions of 4×5 A;, and for the pentamer ~7 A;. The narrowest section of the pore (tetramer, 4 A;, and pentamer, 4.5 A;) is formed by a ring of phenylalanines at position 16, corresponding to F270 in the protein sequence [6,11]. Thus, tetrameric and pentameric bundles of M2 helices satisfy structural and energetic requirements for the inner bundle that forms the pore of the nicotinic AChR channel [11,14,15], and the pentamer fulfills the constraints imposed by the pentameric subunit structure.

3.2. *Chemical characterization of $T_5M2\delta$*

We have previously described the strategy and procedures to synthesize a homotetrameric protein $T_4M2\delta$ by the covalent attachment of four $M2\delta$ modules to a carrier molecule [25,26]. Similar protocols were applied for the synthesis, purification and characterization of $T_5M2\delta$. The salient novelty is the modification of the carrier template to generate five attachment sites. $T_5M2\delta$ was synthesized by a two-step procedure: the

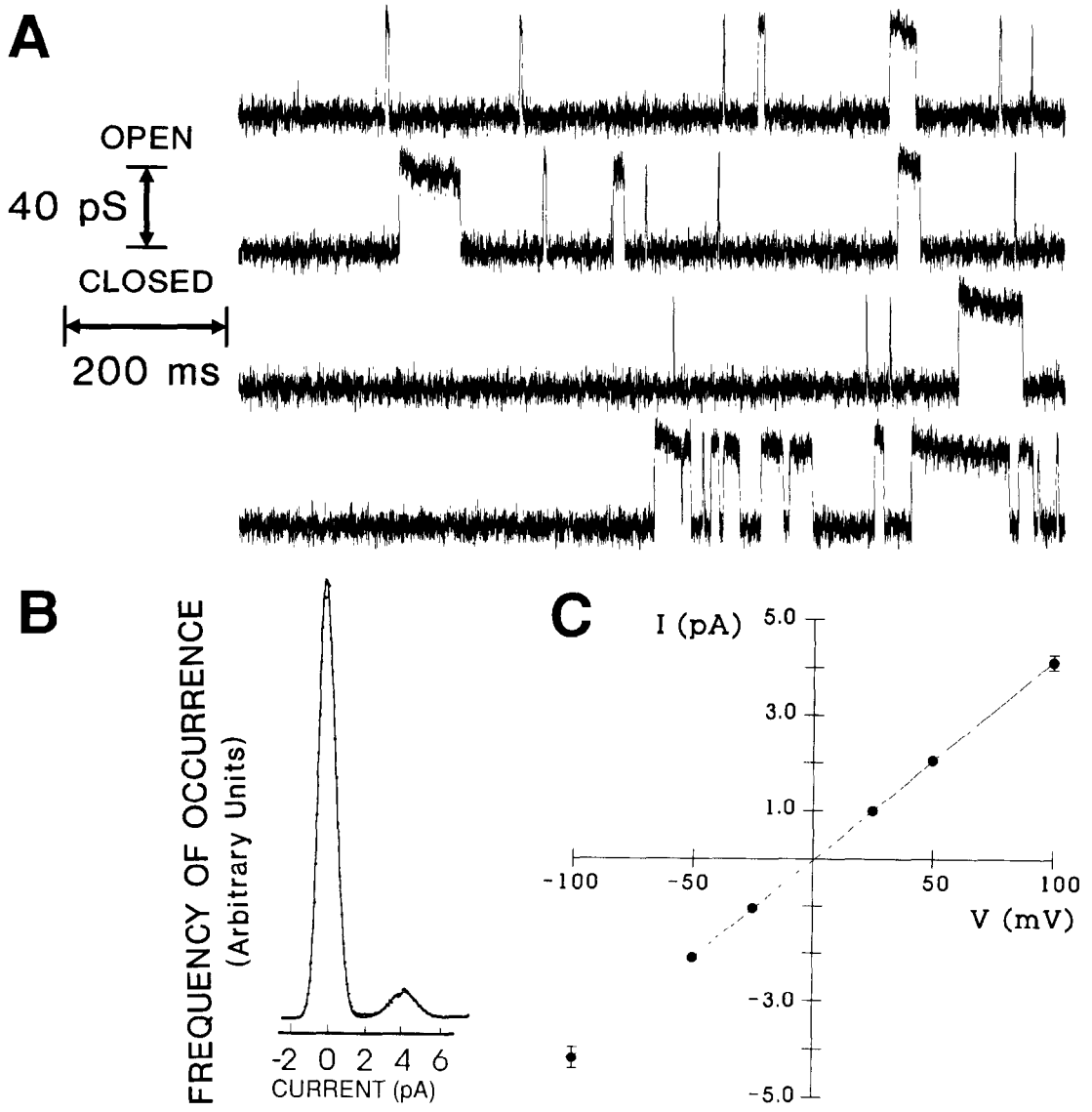


Fig. 3. Single channel currents from POPE/POPC bilayers containing the synthetic pentameric protein $T_5M2\delta$. Currents were recorded at V=100 mV in symmetric 0.5 M KCl, 1 mM $CaCl_2$, 10 mM HEPES, pH 7.4. (A) Segments of a continuous record displayed at a time resolution sufficient to illustrate the occurence of discrete openings of homogeneous current amplitudes that exhibit short and long residence times in the open and closed states. Records filtered at 2 kHz. (B) Current histogram generated from segments of records lasting several minutes Data points were fitted by the sum of two Gaussian distributions (smooth curve). The prominent fitted distribution centered at zero current corresponds to the channel closed state and that centered at 4 pA to the open state. The single channel current, calculated as the difference between the peaks associated with the closed and open states, is 4 pA; the corresponding value for single channel conductance is 40 pS. (C) Current–voltage (I-V) characteristics of single channel events of $T_5M2\delta$ in symmetric 0 5 M KCl The single channel I-V is ohmic with a slope conductance of 40 pS. Currents reported as mean values ± S.D ; n=5.

carrier molecule was synthesized using orthogonal lysine side-chain protection followed by the simultaneous assembly of M2δ peptide modules [25,26]. Amino acid composition, sequence, and purity of the protein were confirmed by amino acid analysis (hydrolysis with 6 M HCl at 165°C), microsequencing (automated Edman degradation), and analytical HPLC. T_5M2δ migrates in SDS-polyacrylamide gels as a single band with M_r ~14,000, as illustrated in Fig. 2 (lane 4). This molecular weight is consistent with a protein of 126 residues. In contrast, the tetrameric protein T_4M2δ (lane 3) appears as a band with M_r ~11,000, compatible with a protein of 101 residues [25,26], whereas the monomer form of M2δ (lane 2) migrates with M_r ~2,000 [10,25,26].

3.3. *Channel formation by $T_5M2\delta$*

T_5M2δ forms channels in lipid bilayers, as shown in Fig. 3. Conductance events are homogeneous in amplitude (Fig. 3A) with single channel conductance $\gamma=40 \pm 3$ pS ($n=8$) in symmetric 0.5 M KCl (Fig. 3B). Channel openings with $\gamma=80 \pm 5$ pS were detected at significantly lower frequency of occurrence (<10%). The channel is ohmic (Fig. 3C) and cation selective; the cation transference number, determined from reversal potential measurements under single KCl concentration gradients, is 0.90 ± 0.05 ($n=3$). Channel openings tend to occur in bursts of openings followed by very long salient periods with little or no activity. The probability of the channel being open, P_o, calculated from the area under the fitted Gaussian curve (Fig. 3B), is 0.078 ± 0.013 ($n=3$). Analysis of channel lifetimes in the open and closed states during the periods of activity shows that channel open (τ_o) and closed (τ_c) times are well fitted by the sum of two exponentials, $\tau_{o1}=1.5 \pm 0.2$ ms and $\tau_{o2}=78 \pm 5$ ms, and $\tau_{c1}=1.4 \pm 0.2$ ms and $\tau_{c2}=28 \pm 3$ ms ($n=3$). The efficiency of channel formation by T_5M2δ is considerably lower (~20%) than that observed with the tetrameric T_4M2δ [25,26] or with monomeric M2δ [10,25,26]. Channel activity of purified T_5M2δ is highly sensitive to aging and heat inactivation: activity is lost within 24 h at 4°C or after incubation at 50°C for 5 min.

3.4. *Comparison with authentic AChR channels*

We have previously shown that synthetic M2δ peptides (Fig. 1A and B; Fig. 2, lane 2) form channels in lipid bilayers of heterogeneoeus conductance and lifetimes, resulting from the self-assembly of conductive oligomers of distinct sizes [10]. The design of tethered oligomeric proteins was introduced to generate proteins of defined oligomeric number and predetermined orientation of peptide modules relative to one another [25–27]. This approach has led to the generation of homotetrameric (T_4M2δ) and homopentameric (T_5M2δ) proteins that exhibit homogeneous conductances. Accordingly, whereas M2δ peptide forms channels in POPE/POPC bilayers with primary conductances of 20 pS and 40 pS (0.5 M KCl), T_4M2δ and T_5M2δ form homogeneous channels with unitary conductances of 20 pS [25] and 40 pS (Fig. 3), respectively. This suggests that the $\gamma=20$ pS and $\gamma=40$ pS events recorded with M2δ peptide arise from non-covalently bonded tetrameric and pentameric arrays, respectively [10].

The pore area calculated for a pentameric cluster of α-helices, 42 Å^2 [11], is consistent with the observed channel conductance of T_5M2δ. The 40 pS conductance events recorded with T_5M2δ approximate the single channel conductance of authentic, purified *Torpedo* AChR reconstituted in POPE/POPC bilayers ($\gamma=45$ pS in symmetric 0.5 M KCl [35]). Whereas the *Torpedo* AChR is a heteropentamer, T_5M2δ is a homopentamer. This feature may explain the observed ~10% difference in single channel conductances. Thus far, we have focused on the design of pore-forming structures without the ligand-binding domain or other modulatory structures of the receptor [14,25–27]. The design of functional homomeric channel proteins sets us now in position to undertake the challenging task of the step-by-step reconstitution of a channel protein from its elementary functional units [14].

3.5. *Conclusion*

The synthesis of a pentameric protein designed to function as a transmembrane ionic pore was achieved. T_5M2δ displays several pore properties of the purified *Torpedo* AChR channel reconstituted in lipid bilayers, namely, single channel conductance, cation selectivity and channel open lifetimes in the millisecond time range [7,32]. These results support the notion that a five helix bundle may represent the structural motif underlying the inner bundle that forms the pore of the pentameric AChR channel.

Acknowledgments We thank R. Blewitt for his help with molecular graphics, and A. Grove and A.V. Ferrer-Montiel for comments. This work was supported by grants from the US Public Health Service (MH-44638 to M.M. and GM-43617 to J.M.T.), the Department of the Army Medical Research (DAMD 17-89-C-9032 to M.M.), the Office of Naval Research (ONR N000 14-89-J1489 to M.M.) and a Research Scientist Award from the Alcohol, Drug Abuse and Mental Health Administration (MH-00778) to M.M.

REFERENCES

[1] Unwin, N. (1993) Cell 72/Neuron 10, 31–41
[2] Changeux, J.-P., Galzi, J.-L., Devillers-Thiery, A. and Bertrand, D. (1992) Quart. Rev. Biophys. 25, 395–432.
[3] Karlin, A. (1991) Harvey Lect 85, 71–107.
[4] Toyoshima, C. and Unwin, N. (1990) J. Cell Biol. 111, 2623–2635.
[5] Reynolds, J. and Karlin, A. (1978) Biochemistry 17, 2035–2038.
[6] Numa, S. (1989) Harvey Lect. 83, 121–165.
[7] Hille, B. (1992) Ionic Channels of Excitable Membranes, Sinauer, Sunderland, MA.
[8] Adams, D.J., Dwyer, T.N. and Hille, B. (1980) J. Gen. Physiol. 75, 492–510.
[9] Dwyer, T.M., Adams, D.J. and Hille, B. (1980) J. Gen. Physiol. 75, 469–492.

[10] Oiki, S., Danho, W., Madison, V. and Montal, M. (1988) Proc. Natl. Acad. Sci. USA 85, 8703–8707.
[11] Oiki, S., Madison, V and Montal, M. (1990) Proteins Struct Funct. Genet. 8, 226–236
[12] Giraudat, J., Dennis, M., Heidman, T, Chang, J-Y. and Changeux, J.P. (1986) Proc Natl. Acad. Sci. USA 83, 2719–2723.
[13] Hucho, F, Oberthur, W. and Lottspeich, F. (1986) FEBS Lett. 205, 137–142
[14] Montal, M (1990) FASEB J. 4, 2623–2635
[15] Furois-Corbin, S. and Pullman, A., (1989) Biochim. Biophys. Acta 984, 339–350.
[16] Bechinger, B., Kim, Y., Chirlian, L.E., Gesell, J., Neumann, J.-M., Montal, M, Tomich, J., Zasloff M and Opella, S.J. (1991) J. Biomol NMR. 1, 167–173.
[17] Eisenman, G., Villarroel, A., Montal, M. and Alvarez, O. (1990) Prog. Cell Res 1, 195–211.
[18] Imoto, K., Busch, C, Sakmann, B., Mishina, M., Konno, T., Nakari, J., Bujo, H., Mori, Y, Fukuda, K. and Numa, S. (1988) Nature 335, 645–648
[19] Imoto, K., Konno, T., Nakai, J., Wang, F., Mishina, M. and Numa, S. (1991) FEBS Lett. 289, 193-200
[20] Villarroel, A., Herlitze, S., Koenen, M. and Sakmann, B. (1991) Proc. R. Soc. Lond. B 243, 69–74
[21] Charnet, P., Labarca, C., Leonard, R.J., Vogelaar, N J., Czyzyk, L., Ganin, A., Davidson, N. and Lester, H.A. (1990) Neuron 2, 87–95.
[22] Revah, F., Bertrand, D., Galzi, J.L., Devillers-Thiery, A., Mulle, C., Hussy, N., Bertrand, S., Ballivet, M. and Changeux, J.P. (1991) Nature 353, 846–849.
[23] Sakmann, B. (1992) Science 256, 503–512.
[24] Villarroel, A and Sakmann, B. (1992) Biophys. J. 62, 196–208.
[25] Montal, M., Montal, M.S and Tomich, J M. (1990) Proc. Natl. Acad. Sci. USA 87, 6929–6933.
[26] Grove, A., Iwamoto, T., Montal, M.S., Tomich, J.M. and Montal, M. (1992) Methods Enzymol. 207, 510–525.
[27] Grove, A., Tomich, J.M and Montal, M. (1991) Proc. Natl. Acad Sci. USA 88, 6418–6422
[28] Merrifield, R.B. (1965) Science 150, 178–185.
[29] Mutter, M. and Vilieumier, S. (1989) Angew. Chem. Intl. Ed. Engl. 28, 535–554.
[30] Laemmli, U.K. (1970) Nature 227, 680–685.
[31] Suarez-Isla, B.A, Wan, K., Lindstrom, J. and Montal, M. (1983) Biochemistry 22, 2319–2323.
[32] Montal, M., Anholt, R. and Labarca, P. (1986) in: Ion Channel Reconstitution (Miller, C., Ed.) pp. 157–204, Plenum Press, New York.
[33] Labarca, P., Lindstrom, J. and Montal, M. (1984) J Gen. Physiol 83, 473–496
[34] Labarca, P., Lindstrom, J. and Montal, M. (1984) J. Neurosci. 4, 502–507
[35] Ferrer-Montiel, A ,V., Montal, M.S., Diaz-Munoz, M. and Montal, M. (1991) Proc. Natl. Acad. Sci. USA 88, 10213–10217.