Channel Protein Engineering: A Novel Approach Towards the Molecular Dissection of Ligand-regulated Channels

Our goal is to establish the molecular determinants that account for the pore properties of ligand-regulated channel properties. We are modeling hypothesized pore forming structures from acetylcholine receptors using semi-empirical potential energy functions. The M2 segment of the nicotinic acetylcholine receptor from Torpedo californica is a candidate for the channel lining structure. It forms ionic channels in human erythrocyte membranes and in lipid bilayers. Furthermore, we designed and synthesized a tethered tetramer containing four M2-oligopeptides. The complete 101 residue protein forms channels in lipid bilayers with properties that resemble those of authentic cholinergic receptors. The general validity of this approach is currently being assessed.

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SUBJECT TERMS

protein structure, protein design, acetylcholine receptor ionic channels, biological membranes, signal transduction

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RESEARCH OBJECTIVE: Our goal is to establish the molecular determinant of the pore properties in ligand-regulated channel proteins.

PROGRESS (Year 1):
1. Molecular modeling of the pore forming structures of ligand-gated channel proteins.
   We pursued the modeling of the pore forming structures of two channel proteins with different primary structures and oligomeric number; namely, the voltage sensitive sodium channel and the nicotinic cholinergic receptor. Low-energy arrangements of \( \alpha \)-helical bundles were calculated by semi-empirical potential energy functions and optimization routines and were further refined using molecular dynamics. The ion-conducting pore is considered to be a symmetric or pseudosymmetric homooligomer of 3-5 amphipathic \( \alpha \)-helices arranged such that the polar residues line a central hydrophilic pathway and the apolar residues face the hydrophobic bilayer interior. The channel lining exposes either charged (Asp, Glu, Arg, Lys) or polar-neutral (Ser, Thr) residues. A bundle of 4 parallel helices constrained to \( C_4 \) symmetry, the helix axis aligned with the symmetry axis, and the helices constrained to idealized dihedral angles, produces a structure with a pore of the size inferred for the sodium channel protein (area \( \sim 16 \text{ Å}^2 \)). Similarly, a pentameric array optimized with constraints to maintain \( C_5 \) symmetry and backbone torsions characteristic of \( \alpha \)-helices adopts a structure that appears well suited to form the lining of the nicotinic cholinergic receptor (pore area \( \sim 46 \text{ Å}^2 \)). Thus, bundles of amphipathic \( \alpha \)-helices satisfy the structural, energetic, and dynamic requirements to be the molecular structural motif underlying the function of ionic channels.

2. The M28 transmembrane domain of the nicotinic cholinergic receptor forms ion channels in human erythrocyte membranes.
   We examined the notion that a synthetic peptide with the sequence of the M28 segment of the nicotinic acetylcholine receptor from \textit{Torpedo californica} forms ionic channels in biological membranes. For this purpose we selected human erythrocyte membranes and assayed channel formation by determining both hemoglobin and \( K^+ \) release. Indeed, this peptide forms a permeability pathway with an apparent cross-sectional diameter of 7-9 Å. The M28 pore is oligomeric and a pentamer is the species that accounts for the properties of the permeation path. Peptides that mimic other identifiable segments of the \textit{Torpedo} acetylcholine receptor, M18 and MIR, do not form channels in erythrocytes under the same conditions.

3. Synthesis of tetrameric synthetic channel proteins was achieved.
   We implemented the design principles outlined by Mutter and synthesized tethered tetramers containing the channel-forming domains of the \textit{Torpedo californica} acetylcholine receptor (AChR) \( \delta \) subunit...
transmembrane segment 2 (M2) attached to a carrier template. The carrier is a nine-aminoacid backbone with sequence K*KK*PGK*EK*G with K* containing Nα-N-tert-butylcarbonyl (tbooc), Nε-9-fluorenlymethoxycarbonyl (fmoc) to generate four branch points. Oligopeptides are then attached to template in a stepwise manner at the four base-deprotected lysine sidechains. A parallel array of the 4-oligopeptides is determined by tethering them to the carrier.

The complete 101 residue protein does indeed form channels in lipid bilayers which reproduce several features that are characteristic of authentic AChR channels, such as single channel conductance, cation selectivity, transitions between closed and open states in the millisecond time range. An analogue protein, in which the serine residue in position 8 is replaced for alanine in each of the four M28 23-mer peptides, also forms channels which, however, exhibit lower single channel conductance. By contrast, a similar tethered tetramer with M18-peptides does not form channels in accord with expectations. The general validity of this strategy to other channel sequences and oligomeric number is currently being explored. Thus, this novel class of synthetic channel proteins enriches our armamentarium directed towards the elucidation of structure-function relationships.

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PUBLICATIONS AND REPORTS (Year 1)


THE M25 TRANSMEMBRANE DOMAIN OF THE NICOTINIC CHOLINERGIC RECEPTOR FORMS ION CHANNELS IN HUMAN ERYTHROCYTE MEMBRANES

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SUMMARY. A synthetic peptide with the sequence of the M25 segment of the nicotinic acetylcholine receptor from Torpedo californica forms pores in human erythrocyte membranes as determined by hemoglobin and potassium release. This peptide forms a permeability pathway with an apparent cross-sectional diameter of 7-9 Å. The M25 pore is oligomeric and a pentamer is the species that accounts for the properties of the permeation path. Peptides that mimic other identifiable segments of the Torpedo acetylcholine receptor, M16 and M18, do not form channels in erythrocytes under the same conditions.

INTRODUCTION. The nicotinic acetylcholine receptor (AcChoR) of Torpedo californica is composed of four glycoprotein subunits (α, β, γ, δ) with stoichiometry α2βγδ (1, 2). A high degree of amino acid sequence homology exists among the four subunits, and all exhibit four putative transmembrane regions designated as M1, M2, M3 and M4 (3). In vivo, the AcChoR pentamer acts as a ligand activated cation channel (4) with an effective pore diameter of ~7 Å (5). The specific assignment of subunits involved in channel lining has been a subject of intense investigation. Evidence suggests that M2 is the segment which lines the pore (4, 6-8). Significantly, a synthetic 23-mer peptide with the sequence of M25 forms ion channels in lipid bilayers with single channel properties that emulate those of authentic AcChoR ion channels (8). Here we provide evidence of channel formation by the AcChoR M25 peptide in biological membranes.

MATERIALS AND METHODS

Peptides. Peptides were synthesized by solid phase methods on an Applied Biosystems model 430 peptide synthesizer, purified by HPLC and sequenced, essentially as previously described (8, 9). The amino acid sequences of the peptides studied are: M25 - EKMSTAISVLLAQAVFLLLTSQR (8), M16 - LFYVINFITPCVLISFLASLAF (8), MIR (a segment of the main immunogenic region) - VNQVETNRV (10), and a peptide composed of 23 serine residues (poly S).

Hemolysis Assay. Hemolysis was assayed according to Tosteson et al. (11). Briefly, freshly outdated blood from the Veteran's Administration Medical Center Blood Bank (La Jolla, CA) was washed three times with 0.3 M sucrose buffer (0.3 M sucrose, 0.01 M Tris-Hepes, 0.001 M EGTA).

The abbreviations used are: AcChoR, acetylcholine receptor, MIR, main immunogenic region, TFE, trifluoroethanol, Hb, hemoglobin.
An erythrocyte suspension was made 1% (v/v), and 2.5 ml samples were used for all assays. Synthetic peptides were dissolved in trifluoroethanol (TFE; Aldrich, Milwaukee, WI) and added to erythrocyte suspensions with immediate vortexing. Suspensions were incubated at 22 ± 2°C for desired time periods, whereupon two 1 ml aliquots were withdrawn and layered over 0.1 ml dibutylphthalate (Aldrich, Milwaukee, WI). Samples were immediately centrifuged for 5 minutes in an Eppendorf Model 5414 centrifuge, and the absorbance of the supernatants at 540 nm was recorded. The effect of equivalent amounts of pure TFE (5-30 μl) without peptide were subtracted from the lysis produced by peptides. Total lysis was obtained by solubilizing erythrocytes with Triton X-100 at a final concentration of 0.5% (v/v). K⁺ was measured on a Perkin-Elmer Atomic Absorption Spectrophotometer Model 5000 (λ = 766.5 nm). Supernatants were combined, and 1 ml aliquots diluted to a final volume of 5 ml with distilled water.

RESULTS AND DISCUSSION

Synthetic M28 peptide has hemolytic activity. Erythrocytes suspended in sucrose buffer are lysed by the synthetic M28 peptide. Figure 1 illustrates the time course of hemoglobin (Hb) release produced by three different concentrations of M28. Initially, Hb release increases linearly with time, leveling off at a steady state value. K⁺ release from erythrocytes is also a measure of cell lysis as illustrated in Figure 2B. The time courses of Hb and K⁺ release are similar, although the initial rate of K⁺ release is faster than that for Hb.

M28 forms channels in lipid bilayers (8). Therefore, a likely mechanism of lysis is that M28 creates a pore through which the high levels of intracellular K⁺ exit the cell. Accordingly, K⁺ efflux generates an osmotic imbalance leading to cell lysis. This model implies that external sucrose is too large to pass through the M28 pore. Therefore, sucrose in the buffer was replaced by Tris (at 0.15 M), and no lysis was obtained (Figs. 1 and 2A). Tris was selected for this assay because it is known to permeate through both the authentic AcChoR (5) and the M28 pore (8). Presumably, Tris' equilibrates with intracellular K⁺, and no osmotic imbalance is created. The dimensions of Tris⁺ (8Å × 7Å × 6Å) (12) and sucrose (11Å × 9Å × 8Å) (13) predict an effective cross-sectional diameter of the permeability path formed by the M28 pore of 7-9 Å, in agreement with the apparent cut-off size of the synthetic M28 (8) and authentic AcChoR (5) channels.

![Graph](image-url)
The M28 pore is oligomeric. A plausible structural model for the ion-conductive pore formed by M28 postulates a pentameric array of 5 amphipathic α-helices arranged such that the polar residues line a central hydrophilic pathway, and the apolar residues interact with the apolar core of the bilayer (8). Information about the size of oligomeric channels is obtainable from membrane conductance measurements in planar lipid bilayers (14). Double logarithmic plots of conductance vs. concentration of channel forming peptide have a slope equal to the number of peptides per channel. Since the rate of hemolysis is dependent on membrane conductance, a double logarithmic plot of the initial rate of hemolysis vs. concentration of M28 will have a slope equal to the size of M28 oligomers involved in the rate limiting step of the hemolysis pathway. Accordingly, Figure 3 shows that the assembly of a trimer is the rate limiting step in the formation of a functional ion channel. However, based on Tris permeability (5, 8), it is likely that pentamers are the dominant species responsible for the M28 conductive pore.

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**Fig. 2.** Hb release (A) and K* release (B) from erythrocytes suspended in sucrose buffer (●, □) or in Tris buffer (○, △) and exposed to 15 μM M28 (●, ○) or 15 μM polyserine, n = 23 (■, □).

**Fig. 3.** Plot of log (initial rate of hemolysis) vs. log [M28]. Initial rates are initial slopes from a plot of % Hb release vs. time. Initial rates for 5, 10 and 15 μM M28 were determined in duplicate, and the mean rates were plotted vs. [M28]. Slope of 3.0 was calculated by linear regression.
Specificity of the assay. To test the specificity of M28 hemolytic activity, synthetic peptides with sequences derived from other AcChoR structural domains were studied. M15 (3, 8) is a putative transmembrane segment (15), whereas the extramembranous synaptic domain of the α subunits contains a hydrophilic segment—the main immunogenic region (MIR) (10). Neither M15 nor MIR (α) peptides would be expected to be cytolytic, and indeed they show no activity when tested under the same conditions as M28 (Fig. 4). Figure 2A,B also show that a polyserine 23-mer has no cytolytic activity. This is significant because serines are postulated to line the polar face of M28 (4, 8) and lack of cytolysis by polyserine supports the notion that amphiphilicity is a hallmark of channel forming α-helices (7, 8).

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REFERENCES
Molecular Engineering of Channel Proteins

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INTRODUCTION AND SCOPE

A central goal in membrane biology is to understand how channel proteins work in terms of their underlying protein structures. Ionic channels are symmetric (or pseudosymmetric) transmembrane protein assemblies organized around a central aqueous pore. The two key functional elements are the ionic channel - the actual polar pathway that permits the selective passage of ions across the low dielectric constant apolar core of the membrane lipid bilayer and the sensor - the structure that detects the stimulus and couples it to the opening or closing (gating) of the channel. The current excitement in membrane protein science emerges precisely from structural information that is providing clues about the molecular determinants of function. Powerful and sensitive techniques have been developed and are now intensely used to tackle questions about structure-function relationships (cf. 60,74). Molecular cloning and sequencing led to the elucidation of the primary structures of several multi-member gene families (cf. 4,8,36,43,68,74); channel proteins have been purified and reconstituted in lipid bilayers with full retention of function (cf. 62); the properties of many channel proteins have been characterized at the single channel level (83); cDNA or RNA transcripts have been expressed in oocytes as functional proteins (5,32,54,74); molecular modeling has predicted specific peptide segments to form the channel lining that when synthesized by solid phase methods proved to be indeed channel formers in lipid bilayers (77-79). This latter approach - molecular engineering - (66) will be reviewed briefly with particular emphasis on the progress we have made in designing peptides that emulate the pore structure of two prototype proteins of two major gene families in the brain, namely the voltage sensitive sodium channel and the nicotinic cholinergic receptor (77-79).
Figure 1 illustrates in the form of a flow chart, the strategy towards the molecular dissection of functional determinants in channel proteins (66).

**CHANNEL PROTEIN ENGINEERING: STRATEGY**

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Primary Structure
- Empirical Secondary Structure Prediction
  - Hypothetical Structure Model
  - Design of "Functional Peptide": Channel Sensor
    - Synthesis of "Functional Peptide"
    - Functional Assay of Synthetic Channel in Lipid Bilayers
    - Synthesis of Analogues (Specific Amino Acid Substitution)

Comparison with Authentic Channel
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**FIG. 1. Channel Protein Engineering: The Strategy.**

Given the amino acid sequence for a channel protein, the next step consists of applying empirical secondary structure predictors in order to postulate a structural model of the protein. Secondary structure prediction methods (16,21,28,30,50,56) are empirical and, therefore, are inherently uncertain. Their value resides in their use as a guide to the design and interpretation of experiments. To demonstrate the existence of such functional segments is to design a "functional peptide" that would mimic the predicted structural element: the "channel segment" or the "sensor." To test directly this prediction the functional peptide is synthesized by solid phase methods (3.58,59,92) and its ability to form ion channels is tested by incorporating the peptide into a synthetic lipid bilayer (63,89). The channel activity of the peptide is characterized in detail in the lipid bilayer in terms of ion conduction and channel gating (77-79). This characterization is compared with the specific features of the authentic channel which, in turn, assist in the redesign of the peptide to match the anticipated characteristics of the authentic channel (66). In addition, the identification of a specific residue thought to be critical for the function under study can then be substituted for an inert one without altering secondary structure in a drastic way. Such analogues provide valuable information to assign functional significance to specific residues (66). The consistency in the structure function relationships is the measure of the reliability of this strategy. This is basically the philosophy behind this approach: the hypothesis is formulated, and the consequences are evaluated experimentally. If any of the predictions is not fulfilled, the model is readjusted and reevaluated.

A remarkable feature inferred from the amino acid sequences of channel proteins is the high homology conservation among members of this family of proteins. Furthermore, protein segments that could be organized as amphipathic transmembrane α-helices are identifiable in all the channel proteins for which sequence information is available (4,8,31,36,68,74,87). This is significant because amphipathic α-helices are structural entities that in a membrane milieu may self-assemble into oligomers. In addition, image analysis of electron micrographs obtained from ordered arrays of channel proteins in their native membrane environment produced low resolution images (approximately 18 Å) that are consistent with symmetric or pseudosymmetric protein assemblies organized around a central aqueous pore (96,97). These key features raise the notion that a uniting motif in the biological design of ion channels is an oligomeric array of transmembrane amphipathic α-helices arranged such that polar residues face a central hydrophilic pore and apolar residues face the hydrophobic bilayer interior (12,21,23,25-27,30,33,34,67). Such structure accounts for the geometric and symmetry constrains of channel proteins, offers a rationale for the extensive homology conservation and provides a basis for the diversity of channel proteins in so far as this would be determined by sequence specificity and oligomer size.

It is worthy of note that several peptides, both of natural origin and synthetic, form channels in lipid bilayers (for reviews see 7,94). Among the natural peptides, the 26-residue peptides melittin (22,95) and Staphylococcus aureus δ-toxin (22,57,93) have been studied in detail both from the structural as well as from the channel action points of view. The synthetic channel peptides characterized range from relatively simple sequences containing only leucine and serine residues, for example (LSSLLSL)₈ and (LSSLSSL)₈ (52) or leucine, serine and glycine (LSLG)₉ (45) to more complex, for example, GFLMITLLILFSQFLPLMLR, a peptide that emulates a segment (residue 16 to 57) of subunit 8 of the H⁺-ATP-synthetase of yeast mitochondria (61). A common structural feature of these polypeptides is that they can adopt an amphipathic helical conformation in the bilayer nonpolar interior. If they aggregate, they would have a tendency to form clusters with a central pore involving the polar faces of the helices (e.g. 52,57). This raises the notion that amphipathic peptides of
length sufficient to traverse the width of the bilayer hydrophobic core and organized as bundles of α-helices provide a structural basis for ion-conducting channels (12,21,23,30,33,34).

THE VOLTAGE SENSITIVE SODIUM CHANNEL

Given the primary structure of the sodium channel proteins, several proposals were suggested concerning the folding of the α polypeptide chain across the bilayer membrane (30,34,48,68,71,73,74). We proposed a model for the folding of the polypeptide chain of the sodium channel within the lipid bilayer (30). It consists of four homologous regions, each containing 8 membrane-spanning structures, probably α-helical. The tertiary structure is pseuodoradially symmetric. The model suggests the existence of four amphipathic transmembrane helices (1 α-helix contributed by each one of the four homologous repeats) which meet with their hydrophilic faces inward to form a putative ion channel. The central channel created by the boundary of the four helices would be about 4.2 Å across its narrowest dimension, thus accounting for the effective cut off size of the sodium channel (37).

The amino acid sequences of segments postulated to be involved in channel lining for rat brain I, rat brain II (69,70,88), rat brain IA (2), rat brain III (44,91), E. electricus (71), and Drosophila sodium channels (84,85) show high degree of homology conservation. We focus here on the structure and results obtained with a synthetic channel peptide with sequence DPWNLDFITIFAYVTEFVDL which corresponds to the rat brain I channel segment of homologous repeat 1 (referred to as Sc1 (30)). Secondary structure predictors suggest that the main structural feature of the synthetic peptide is an α-helix (16,19,28,82,86). Since the spacing between residues is 1.5 Å in an α-helix, such a segment would be about 33 Å long, sufficient to traverse the hydrocarbon core of the membrane (9,30). Circular dichroism spectra of the Sc1 peptide in trifluorethanol show the characteristic features of α-helices, namely, the double minima at 208 and 222 nm and the maximum at 195 nm (14,90). As reported, the synthetic amphipathic 22-mer peptide Sc1, does indeed form transmembrane ionic channels in lipid bilayers (77,78). This is illustrated in Figure 2. The single channel conductance, γ, recorded in symmetric 0.5 M NaCl at 100 mV, is 25 pS for the authentic brain sodium channel (35) and 20 pS for the synthetic channel peptide (77,78). Similar γ values are reported for the authentic electric eel (80) and rabbit skeletal muscle (24) sodium channels.

It is clear, that the synthetic channel peptide reproduces several features which are characteristic of the authentic brain sodium channel, such as the similarity in γ and transitions between the closed and the open state in the millisecond time range (Fig. 2 and Table 1). In contrast, the synthetic channel peptide differs from the authentic brain channel in lacking discrimination between Na⁺ and K⁺ ions as well as being insensitive to the applied electric field (39). This disparity was anticipated from the model which considers that the pore is formed of four distinct amphipathic helices corresponding to the specific chemical sequences of each of the four repeats, and because the voltage sensor is assigned to other distinct transmembrane segments of the array (30).
Model of a Plausible Channel Structure

Energetic considerations suggest that a bundle of four amphipathic α-helices is a plausible channel structure. This emerged from a theoretical search of stable low-energy conformations for the tetrameric α-helical bundles (98) performed by empirical potential energy functions and energy minimizations routines (10,15,47). Figure 3 shows a computer-generated molecular model of the pore-forming structure produced by a bundle of four parallel and aligned α-helices (78). The interior of the structure has an excess of negatively charged residues (D and E) that provide a conductive pathway for cations which is adequate to span the non-polar interior of the bilayer as the hydrophobic residues located on the exterior of the array provide favorable and extensive lipophilic boundaries. The sodium channel selectivity, as postulated (30), appears to result from the location of the residues in the channel lining. The effective pore area of the four α-helical cluster is in agreement with the apparent dimension of the authentic sodium channel selectivity filter of 16 Å² (Table 1) (for reviews see 6,11,38). Thus, the tetrameric bundle appears to account for the geometric constrains of the sodium channel.

THE NICOTINIC ACETYLCOLINE RECEPTOR

The nicotinic acetylcholine receptor (AcChoR) of Torpedo californica is composed of four glycoprotein subunits (α,β,γ,δ) with stoichiometry α₂βγδ (81). A high degree of amino acid sequence homology exists among the four subunits, and all exhibit four putative transmembrane regions designated as M1, M2, M3 and M4 (36,72,74). In vivo, the AcChoR pentamer acts as a ligand activated cation channel (cf. 38) with an effective pore diameter of approximately 7 Å (1,18). The specific assignment of subunits involved in channel lining has been a subject of intense investigation. Affinity labeling with non-competitive channel blockers, such as chlorpromazine (13,29) and triphenylmethylphosphonium (33,40,75,76), identified serine 262 in M2 of the δ subunit as a reactive site. Analysis of the single-channel conductance of AChRs, expressed in Xenopus oocytes containing chimeric Torpedo-bovine δ

### TABLE 1. Ionic Conductance Characteristics of Authentic AChR and Sodium Channel Proteins and of Corresponding Synthetic Channel Peptides

<table>
<thead>
<tr>
<th>Property</th>
<th>Torpedo ACHR[a]</th>
<th>M2δ Peptide[b]</th>
<th>Sodium Channel[c]</th>
<th>Sc1-Peptide[d]</th>
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<tbody>
<tr>
<td>γNa⁺ (pS)</td>
<td>45</td>
<td>40</td>
<td>25</td>
<td>20</td>
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<td>γK⁺/γNa⁺</td>
<td>1.1</td>
<td>1.2</td>
<td>0.13</td>
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<tr>
<td>γTris⁺/γNa⁺</td>
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<tr>
<td>γCl⁻/γNa⁺</td>
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<td>≤0.03</td>
<td>≤0.01</td>
<td>≤0.1</td>
</tr>
<tr>
<td>Pore Area (Å²)</td>
<td>44[a]</td>
<td>46</td>
<td>16[a]</td>
<td>16</td>
</tr>
<tr>
<td>Oligomeric Structure</td>
<td>5-mer</td>
<td>5-mer</td>
<td>4-mer</td>
<td>4-mer</td>
</tr>
</tbody>
</table>

N.D., not determined

[a] Determined in symmetric 0.5 M NaCl, KCl, TrisCl and from reversal potential measurements of Torpedo ACHR (51,65; and unpublished results).

[b] Refers to the most frequent event determined in symmetric 0.5 M NaCl, KCl or TrisCl and from reversal potential measurements (79).

[c] Determined in symmetric 0.5 M NaCl and KCl and from reversal potential measurements of batrachotoxin-modified sodium channels from rat brain (35,49,46).

[d] Refers to the most frequent event determined in symmetric 0.5 M NaCl and KCl and from reversal potential measurements (78).

[97; for review, please see 38. (Modified from ref. 79.)

FIG. 3. A bundle of 4 amphipathic α-helices is a plausible structure for the synthetic channel peptide Sc1. Stereo, end-view of a computer generated molecular model of the pore-forming structure. Orthographic projection of the pore showing the four α-helices (blue), the acidic (red), polar-neutral (yellow) and lipophilic (purple) residues. The dimension of the central pore is 4 Å X 4 Å (reproduced with permission from ref. 78).
Model of a Plausible Channel Structure

The pentameric parallel helical array of the model channel has an internal pore with a diameter ranging from approximately 4 Å at its narrowest point to approximately 6 Å at its widest extent. Nonpolar residues are predominantly on the outside and polar residues predominantly on the inside, except at the two ends of the helices. Residues S4, T5, S8, A12, F16 and T20 face the lumen of the pore. The R23 side chains bridge the C-termini of neighboring helices forming a ring at one end of the pore. At the other end, E1 bridges the amino groups of the N-terminus and K2 within each helix. This symmetric pentamer conforms to the general features of the packing arrangement postulated for the αβγδ AChR pentamer: The outside is lipophilic, the inside more hydrophilic, and S8 (corresponding to S262 of the AChR 8 subunit) is exposed on the channel lumen in accord with chemical labeling experiments.

Recently, AChRs with mutations at various sites in the M2 segment, were expressed in Xenopus oocytes after injection of cloned mRNAs and the single channel properties analyzed (42,53). Focusing on M2α a change in E1 (E 255) for Q produced AChRs with a drastic reduction in γ (2 fold) and in the sensitivity of outward and inward channel currents to intracellular and extracellular [Mg2+] respectively (42). The αβγδ stoichiometry of AChRs enables the addition or deletion of serine residues at the conserved position corresponding to S8 in the M2α-mimicking peptide yielding mutant AChRs with 0,1,2, or 4 serine residues at this site. The binding affinity of mutant AChRs for the QX-222 channel blocker increased with the number of serine residues (53). This result is in gratifying accord with affinity labeling and sequencing results (29,76), the expectation of residues containing OH as contributors to the structure lining the channel of a water-filled pore (cf. 20), the transmembrane arrangement of M2 (42) and the channel activity of the M2α mimicking peptide (79).

An issue that must be addressed concerns the specificity of the channel formation by synthetic peptides. All models postulate (e.g. 21,33,36,74) very hydrophobic transmembrane segments with an excess of non-polar residues such as V, I, L, F and A as components of the array. These segments occur at the interface between homologous regions or between subunits providing favorable and extensive hydrophobic boundaries. For the sodium channel rat brain I the sequence of such a segment, Sh1, is: IFFVLYIFLGSFYLIN LILAVV (30). The synthetic Sh1 peptide was incorporated into lipid bilayers and, indeed, it does not form channels (Oiki, S., Danhu, W. and Montal, M., unpublished results).

For the Torpedo AChR the sequence of such a segment, M1δ, is: PLFYVINFITPCVLISFLASLAFYLP A. This peptide does not form channels (79). These results support the reliability of the approach.

The results and interpretations outlined above for both the voltage 
sensitive sodium channel and the AChR synthetic channel peptides represent a starting point upon which to build up the structure of the authentic channel. The ionic conduction characteristics of authentic Torpedo AChR and rat brain sodium channel proteins and of the corresponding synthetic channel peptides, M2δ and Sc1, respectively, are compared in Table 1 (78,79). The peptide that mimics the AChR M2δ-segment forms channels in bilayers with properties remarkably similar to those of the authentic protein. In contrast, the sodium channel peptide mimetic lacks the selectivity between Na+ and K+ that is so distinctive of the authentic channel as well as its exquisite regulation by voltage (39). Therefore, selectivity and gating are our immediate aims and understanding their origin our guide.

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ABBREVIATIONS USED

AChR, acetylcholine receptor; ACh, acetylcholine; V, applied voltage; γ, single channel conductance; τ, open channel lifetime.

When referring to specific amino acids, standard one-letter codes are used:
A: alanine; R: arginine; N: asparagine; D: aspartate; C: cysteine; E: glutamate; Q: glutamine; G: glycine; H: histidine; I: isoleucine; L: leucine; K: lysine; M: methionine; F: phenylalanine; P: proline; S: serine; T: threonine; W: tryptophan; Y: tyrosine; V: valine.

REFERENCES

We have identified a cDNA corresponding to a channel protein that is present in the brain. This protein was identified using a screen for cDNA clones that hybridize to a genomic library of mouse brain. The cDNA is 2.8 kilobases long and encodes a protein of 170 amino acids. The protein is expressed in a variety of cell types, including neurons and glia. The protein is found in the plasma membrane of these cells, suggesting that it may be involved in the regulation of ionic fluxes. We have characterized the electrophysiological properties of the channel and found that it is activated by depolarizing potentials.

We have developed a method for culturing primary cultures of mouse spinal cord that express the necroticisoform of the NMDA receptor. This receptor is expressed in the central nervous system and is thought to be involved in neuronal death following injury. We have found that the expression of this receptor is regulated by the availability of glutamate in the extracellular space. We have also found that the expression of this receptor is regulated by the activity of the kinase Akt.

367.3 HUMAN GABA_A RECEPTORS ASSEMBLED FROM DIFFERENT COMBINATIONS OF CLONED SUBUNIT SUBUNITS HAVE DIFFERENT ELECTROPHYSIOLOGICAL PROPERTIES. R. G. Heiligenberg, J. M. Flavell, and M. Manzur. UCSF, San Francisco, CA 94143.  
We have expressed human GABA_A receptors in Xenopus oocytes and found that different combinations of cloned subunit subunits have different electrophysiological properties. We have found that the receptor expressed from the full-length receptor is more sensitive to GABA than the receptor expressed from the truncated receptor. We have also found that the receptor expressed from the full-length receptor is more resistant to desensitization than the receptor expressed from the truncated receptor.
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