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Montal, Mauricio

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Our goal is to establish the molecular determinants that account for the pore properties of voltage-gated and ligand-gated channel proteins. We are modeling hypothesized pore-forming structures of sodium channels, calcium channels, potassium channels and acetylcholine receptors. We succeeded in designing a calcium channel protein according to our model. Accordingly, a protein was synthesized by solid-phase methods. The synthetic channel protein forms channels in lipid bilayers that emulate the pore properties of the authentic channel. The key role of specific residues in determining the activity of these channel proteins is currently being assessed. These studies are guided by the results on "computer mutations."

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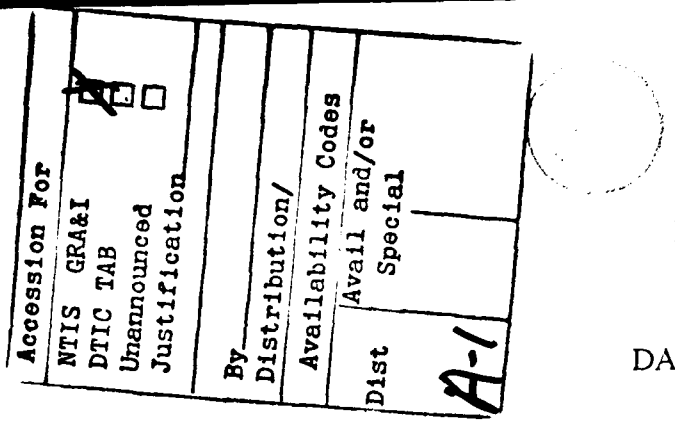

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PROGRESS REPORT ON CONTRACT N00014-89-J-1469

PRINCIPAL INVESTIGATOR: Dr. Mauricio Montal

CONTRACTOR: University of California, San Diego

CONTRACT TITLE: Channel Protein Engineering: A Novel Approach Towards the Molecular Dissection Determinants in Ligand-regulated Channels

START DATE: 1 February 1989

RESEARCH OBJECTIVE: To identify the structures forming the channel lining of ligand-gated channels; to characterize the single channel properties of the pore-forming structure; and to model the structure using molecular mechanics and dynamics.

PROGRESS (Year 2)

1. Molecular modeling of the pore forming structures of voltage-gated and ligand-gated channel proteins.

We pursued the modeling of the pore forming structures of the sodium channel and the acetylcholine receptor (Proteins 8:226-236, 1990, enclosed reprint). Further, we calculated the energy profiles for cations and found them to be consistent with the permeation properties observed experimentally. We introduced the use of "computer mutations" whereby specific residues are substituted and thereby various molecular contributions to the binding sites and energy barriers are deduced. Notably, for the acetylcholine receptor channel, an innermost site is found to be attributable to -OH groups of serine residues, consistent with our previous modeling and more recent experiments. However, a most external site is found to arise from a ring of highly conserved phenylalanine residues, not usually regarded as providing favorable interactions with ions. This site may provide favorable interactions with amphipathic channel blockers such as local anesthetics. Calculations are currently being extended to include a molecule of QX-222, a quarternary ammonium derivative of the local anesthetic lidocaine, within the lumen of the pore. Specific predictions for possible experimental mutations are made which can serve to test both the proposed structure, as well as the computational inferences we have drawn from it (Progr. Cell Res. 1:195-211, 1990, enclosed reprint).

We are in the process of calculating the pore structure of the rat brain acetylcholine receptor α_4, a neuronal receptor with presumed tetrameric structure. The M2 transmembrane segment may form the bundle that lines the pore: it shows extensive homology to the M2 segment of the muscle receptor, it has the same two potential binding sites exposed to the lumen of the pore, namely a serine and a phenylalanine spaced eight-residues apart and is also amphipathic. Modeling is also carried out on a tetrameric potassium channel (of the delayed rectifier type) and on a tetrameric calcium channel (of the dihydropyridine receptor family). The results on the calcium channel are more extensive and very provocative. We modeled the inner bundle that lines the calcium channel as a tetramer of S3 segments contributed by the four homolgous repeats. The bundle is funnel-shaped with the narrowest end pointing to the N- terminus. The exterior of the bundle is hydrophobic and, presumably, is in contact with the apolar membrane interior. The lumen of the pore is lined with polar/neutral residues and two clusters of acidic residues spaced ten residues

apart. The pore diameter at its narrowest extent is 7.4 Å and occurs at the level of a conserved aspartic acid. Thus, this motif satisfies the structural and energetic requirements for the function of the inner bundle that forms the pore of calcium channel proteins. We are currently investigating the binding of QX-222 and how the structurally related dihydropyridine enantiomers of BayK 8644 [(+) - antagonist and (-) - agonist] may cause opposite actions. Binding sites for drugs are evaluated using the docking subroutine in the INSIGHT program package (Biosym Technologies, CA.), monitoring electrostatic and Van der Waals interactions, simultaneously. We identified a potential binding site for the 1, 4-dihydropyridine located in the middle of the bundle and formed by a serine residue of one helix and stabilized by a cysteine in an adjacent helix. We are evaluating the model by "computer mutations" and "experimental mutations". As described below, we succeeded in designing a synthetic calcium channel protein according to this model. Therefore, the current excitement is based on the availability of the structure model and of the synthetic pore protein, which should prove valuable in assessing the functional role of key residues and should facilitate the conceptual design of drugs that alter the pore by blocking it from the aqueous pathways or via the hydrophobic access to the protein from the bilayer interior.

2. Synthesis of tetrameric synthetic channel proteins and demonstration of channel blockade by a local anesthetic.

We have extended our synporin strategy (Proc. Natl. Acad. Sci. USA 87:6929-6933, 1990. enclosed reprint) to design and synthesize pore proteins based on sequence information and oligomeric number. This concerted approach encompassing the technologies of molecular modeling, synthetic chemistry and single channel recordings in lipid bilayers is proving to be valuable in identifying and further examining the functional pore entities of an entire channel protein (FASEB J. 4:2623-2635, 1990, enclosed reprint).

Thus far, we have designed and synthesized four homotetrameric proteins which imitate the sequence of a highly conserved segment predicted to line the pore of channel proteins.

A. For the nicotinic acetylcholine receptor from *Torpedo californica* a muscle-like receptor. the sequence is:

EKMSTAISVLLAQAVFLLLTSQR

B,C. For the nicotinic acetylcholine receptor from brain, a neuronal acetylcholine receptor. the sequence for the α_3 or α_4 subunit is:

EKVTLCISVLLSLTVFLLLITE

and for the β_2 subunit is:

EKVTLAISVLLALTVFLLLISK

Where A is replacing the authentic cysteine in this position.

D. For the dihydropyridine-sensitive L-type calcium channels, the sequence is:

DPWNVFDFLIVIGSIIDVILSE

This sequence is identical in L-type calcium channels from skeletal and cardiac muscle, brain, aorta and lung (Soc. Neurosci. 16(2): 957a, 1990, abstract enclosed).

As control proteins for the first three cholinergic receptor channels, we synthesized a similar tethered tetramer with M1 peptides; for the calcium channel, the control protein is a tethered tetramer of the S5 segment of repeat IV. None of the control proteins form channels in lipid bilayers, in accord with expectations.

The synthetic proteins emulate the pore properties of the authentic channels, including single channel conductance, cation selectivity, transitions between closed and open states in the millisecond time range and sensitivity to channel blockers. In membranes composed of phosphatidylcholine and phosphatidylethanolamine, the single channel conductance in symmetric 0.5M KCl or 0.5M NaCl of the muscle-like channel protein, is 20 pS and 15 pS, respectively. For the neuronal-like channel protein, the corresponding values are 28 pS and 14 pS respectively. The apparent K_d for channel blocking by QX-222 in 0.5M KCl is $\approx 10\mu M$. Thus, the M2 synthetic channel proteins exhibit the sensitivity to local anesthetic channel blockers characteristic of the authentic cholinergic receptor. For the calcium channel protein, the apparent selectivity ratio inferred from single channel conductance ratios is: $Ba^{2+}>Ca^{2+}>Sr^{2+}>Na^{+}>K^{+}>>Cl^{-}$, in agreement with measurements obtained on authentic calcium channels. To assess the integrity of the designed pore protein we examined its pharmacology. Remarkably, the synthetic protein exhibits the pharmacological specifity that is characteristic of authentic calcium channels and at similar effective concentrations: Nifedipine, verapamil, QX-222, Cd^{2+} and Ca^{2+}, all block ion conduction through the synthetic pore protein. A critical test of the model involved the action of the enantiomeric forms of the 1, 4- dihydropyridine BayK 8644. We showed that the (-) BayK 8644 acts as agonist and increases the frequency of openings and prolongs the channel opentime, whereas the (+) enantiomer acts as a powerful blocker and drastically reduces the number of openings and their open lifetime. Hence, the stereospecific activity of 1,4-dihydropyridines on the synthetic channel matches that exerted on authentic channels.

WORKPLAN (Year 3): We are in the process of examing the key role of specific residues in determining the activity of these channel proteins and their sensitivity to local anesthetic blockers by designing and synthesizing analogues where such residues are substituted. These studies are guided by the results of the "computer mutations" previously described.

PUBLICATIONS AND REPORTS:

1. Two (2) copies of reprints of research supported by the award are enclosed. These include:

Montal, M. Molecular engineering of channel proteins. In: *Membrane Technology in Clinical Pathology, Biochemistry and Pharmacology*. Verna, R., ed., Serono Symposia Publications, Vol. 64:9-22, Raven Press, New York, (1989).

Montal, M., M .S. Montal and J. Tomich. Synporins: Synthetic proteins that emulate the pore structure of biological ionic channels. Proc. Natl. Acad. Sci. USA, 87: 6929-6933 (1990).

Montal, M. Molecular anatomy and molecular design of channel proteins. FASEB J., 4:2623-2635 (1990).

Oiki, S., V. E. Madison and M. Montal. Bundles of amphipathic transmembrane a-helices as a structural motif for ion-conducting channel proteins: Studies on sodium channels and acetylcholine receptors. In: PROTEINS: Structure, function and Genetics., 8:226-236 (1990).

Montal, M. Molecular anatomy and molecular design of channel proteins. July 20-August 3, 1990. Tenth International Biophysics Congress, Vancouver, Canada. S9.4, p.24 (1990).

Grove, A., J. M. Tomich and M. Montal. Design, synthesis and single channel characterization of a dihydropyridine-sensitive calcium channel protein. Soc. Neurosci. 16(2):957a. (1990).

2. A progress report was submitted on June 15, 1990.

TRAINING: Two trainees are involved in the project.

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Proc. Natl. Acad. Sci. USA
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Biophysics

Synporins—synthetic proteins that emulate the pore structure of biological ionic channels

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Communicated by Bruno Zimm, May 3, 1990 (received for review February 27, 1990)

ABSTRACT **A class of proteins that mimic the fundamental pore structure of authentic ionic channels has been designed, synthesized, and characterized. The design is based on our earlier result that a 23-mer peptide with the sequence of the M2 segment of the *Torpedo californica* acetylcholine receptor δ subunit—Glu-Lys-Met-Ser-Thr-Ala-Ile-Ser-Val-Leu-Leu-Ala-Gln-Ala-Val-Phe-Leu-Leu-Leu-Thr-Ser-Gln-Arg—forms cation-selective channels in lipid bilayers, presumably by self-assembly of conductive oligomers. Accordingly, a tethered parallel tetramer was synthesized with four M2δ peptides attached to a carrier template—a 9-amino acid backbone with four attachment sites. As expected, the complete 101-residue protein does form channels in lipid bilayers reproducing several features that are characteristic of authentic acetylcholine receptor channels, such as single-channel conductance, cation selectivity, transitions between closed and open states in the millisecond time range, and sensitivity to local anesthetic channel blockers. An analogue protein, in which the serine residue in position 8 is replaced with alanine in each of the four M2δ 23-mer peptides ([Ala[8]]M2δ), also forms channels that, however, exhibit lower single-channel conductance. By contrast, a similar tethered tetramer with M1δ peptides does not form channels, in accord with expectations. The general validity of this strategy to other channel sequences and oligomer numbers is anticipated. Thus, synporins—a term coined to identify this class of synthetic pore proteins—enrich our armamentarium directed toward the elucidation of structure–function relationships.**

Ionic channels are transmembrane protein arrays organized around a central aqueous pore (for review, see ref. 1). A key issue has been to account for the known permeation properties of channels, specifically the pore size and ionic selectivity of the open channel, in terms of structural elements predicted from the deduced amino acid sequences.

In one approach to this problem, a hypothesis is first formulated about the existence of such functional segments. The design of a functional peptide that mimics the predicted structural element is followed by its chemical synthesis using solid-phase methods, and the peptide's ability to form ion channels is tested by incorporating it into a synthetic lipid bilayer. The channel activity of the peptide is characterized in terms of ion conduction and channel gating. This characterization is compared with the specific features of the authentic channel. The comparison, in turn, leads to a redesign of the peptide to match the anticipated characteristics of the authentic channel. Finally, the identification of a specific residue thought to be critical for the function under study can then be tested by substitution (2, 3).

A test case using this approach has been the nicotinic acetylcholine receptor (AcChoR). The *Torpedo californica* AcChoR is composed of four glycoprotein subunits ($\alpha,\beta,\gamma,\delta$) with stoichiometry $\alpha_2\beta\gamma\delta$ (4). *In vivo*, the AcChoR pentamer acts as a ligand-activated cation channel with an effective pore diameter of ≈7 Å (see ref. 1). A high degree of amino acid sequence homology exists among its four subunits, and each exhibits four presumed transmembrane regions designed as M1, M2, M3, and M4 (5). A synthetic peptide with a sequence that corresponds to M2δ forms discrete channels in lipid bilayers. The channel conductance and selectivity sequence are comparable to those characteristic of the authentic AcChoR channel (2).

This molecular-engineering approach has the great virtue of simplicity. The design, however, dictates that these peptides self-assemble in the membrane to generate discrete conductive oligomers, expressed as channel units. We show here that this lack of control over the final product (oligomeric number) can be circumvented by synthesizing a tethered parallel tetramer with four *Torpedo* AcChoR M2δ peptides attached to a carrier template—a 9-amino acid backbone with four attachment sites (6). When incorporated in lipid bilayers, the complete 101-residue protein forms channels that reproduce several features that are characteristic of authentic AcChoR channels: single-channel conductance (γ), cation selectivity, transitions between closed and open states in the millisecond time range, and sensitivity to a local anesthetic channel blocker. Thus, synthetic pore proteins—synporins—provide a strategy to investigate structure–function relationships in channel proteins. A preliminary account of this research was presented elsewhere (7).

MATERIALS AND METHODS

Materials. 1,2-Diphytanoyl-*sn*-glycero-3-phosphocholine (PC), 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine (POPC), and 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphoethanolamine (POPE) were obtained from Avanti. All other chemicals were of the highest purity available commercially.

Synthesis of Proteins. Synthesis of the four-helix bundles was accomplished by a two-step procedure. A common 9-amino acid template was synthesized using automated solid-phase synthetic techniques starting with 0.5 mmol of *t*-Boc-glycine-PAM resin with a substitution of 0.79 mmol/g (where *t*-Boc is N^{α},*N-tert*-butyloxycarbonyl; Applied Biosystems). Amino acids (2.0 mmol) were added as the preformed hydroxybenzotriazole (HOBt) esters on an ABI model 431 peptide synthesizer (2, 3). Coupling efficiencies were monitored at all steps by using quantitative ninhydrin assays (8). Coupling steps were repeated as required to generate coupling efficiences ≥99% per residue. Accordingly, the template NH_2-K*K[‡]K*PGK*E[†]K*G-PAM resin was generated, where E[†] is L-glutamic acid, γ-benzyl ester, K[‡] is N^{ϵ}-2-chlorobenzyloxycarbonyl-L-lysine, and K* is N^{ϵ}-

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Abbreviations: γ, single-channel conductance; AcChoR, acetylcholine receptor; TFE, trifluoroethanol; PC, 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphoethanolamine; POPC, 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine.

¶To whom reprint requests should be addressed.

S9.3 IONOPHORES: STRUCTURE AND INTERACTION IN RELATION TO TRANSMEMBRANE ION-TRANSPORT. K.R.K. Easwaran, Molecular Biophysics Unit, Indian Institute of Science, Bangalore - 560 012, India.

Much of the current understanding of the molecular mechanism of transmembrane ion-transport has come essentially due to the discovery of certain transport molecules, *Ionophores*, which act as carriers or channels in selectively enhancing the ionic permeability across natural and model membranes. There are essentially two aspects of the problem which need a detailed study to understand the process of carrier or channel mediated ion-transport across membranes at the molecular level, namely, a) structure and conformation of ionophores and their cation complexes b) the mode of interaction of these ionophores with model and biological membranes. Our results on the structure and conformation of carrier ionophores and their cation complexes using NMR and CD techniques in solution and X-ray crystallography in the solid state studied under different conditions clearly showed that the ion selectivity of the ionophore depends not only on the nature of the ligands but also on the conformational state of the whole molecule and that the conformation is very much dependant on the size and charge of the cation and the nature of solvent and anion used. The studies on the interaction of ionophores with model membranes, in particular phospholipids, using DSC, ESR, ^{1}H and ^{31}P NMR gave information on the preferred location of the ionophores in the lipid bilayer and the mode of insertion in the lipid matrix. These results will be presented in relation to the transmembrane ion transporting properties of ionophores.

S9.4 MOLECULAR ANATOMY AND MOLECULAR DESIGN OF CHANNEL PROTEINS. Mauricio Montal, Departments of Biology and Physics, University of California, San Diego, La Jolla, California 92093-0319.

A central goal in membrane biophysics 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 apolar core of the lipid bilayer and the *sensor* - the structure that detects the stimulus and couples it to the opening or closing (gating) of the channel. Molecular cloning and sequencing led to the elucidation of the primary structures of several multi-member gene families. Given this structural information, molecular modeling has been used to postulate structural models of the protein with emphasis on the identification of structural elements: the *channel* and the *sensor*. To test the models the putative functional peptides are synthesized by solid phase methods and their ability to form ion channels is assessed by incorporating them into synthetic lipid bilayers. This approach - *molecular engineering* - will be briefly described with particular emphasis on the progress we have made in the design and synthesis of peptides and proteins 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 (Supported by NIH, NIMH and ONR).

395.9

FUNNEL-WEB SPIDER TOXIN (FTX) SELECTIVELY BLOCKS THE SUSTAINED, BUT NOT THE TRANSIENT, CALCIUM CURRENT IN RETINAL HORIZONTAL CELLS

J.M. Sullivan* and E.M. Lasater Depts. of Physiology and Ophthalmology, Univ. of Utah School of Medicine, Salt Lake City, Utah, 84108

Using the whole-cell patch-clamp technique, we have identified two separate Ca currents--one sustained ($I_{Ca}S$), the other transient ($I_{Ca}TR$)--in cultured horizontal cells (HCs) isolated from adult white bass retinas. Ca currents were enhanced using 10 mM extracellular Ca, while Na^+ and K^+ currents were pharmacologically supressed. The large transient Ca current is similar, but not identical, to the T-Current described in other preparations. $I_{Ca}TR$ activates above -60 mV, is inactivated at a holding potential of -40 mV and is carried less well by Ba^{++} than Ca. Unlike the T-Current, $I_{Ca}TR$ is not preferentially blocked by Ni^{++}. This is the first time that a large transient Ca current has been seen in HCs, or in a non-mammalian vertebrate preparation. The sustained Ca current is similar, but not identical, to the L-Current described in many preparations. $I_{Ca}S$ activates above -30 mV, is larger when Ba^{++} replaces Ca and is enhanced by the dihydropyridine agonist BAY K 8644 (Calbiochem). Unlike the L-Current, $I_{Ca}S$ is not preferentially blocked by Cd^{++}, nor is it reduced by ω-conotoxin fraction GVIA (a gift from Dr. B. M. Olivera). FTX (a gift from Dr. R. Llinas), a factor isolated from the venom of the funnel-web spider (Llinas et al., *P.N.A.S.*, 86:1689, 1989), selectively blocks $I_{Ca}S$ at very low concentrations: a 1:300,00 dilution of the column fraction containing FTX blocked 60% of the sustained current, while a 1:10,000,000 dilution blocked 10% of this current. This block was maintained even after washing with FTX-free Ringer for up to 20 min. These results strongly suggest that the sustained and transient Ca currents are carried through two different and unique types of channels, and support the notion that there is a wide variety of Ca channels among different species and tissue types.

This work was supported by N.I.H. Grant EY05972 to E.M.L.

395.10

DISTINCT RAT BRAIN CLASS-C CALCIUM CHANNELS ARE GENERATED BY ALTERNATIVE SPLICING.

W.J. Tomlinson*, M.M. Gilbert* and T.P. Snutch. Biotechnology Laboratory, Univ. of British Columbia, Vancouver, B.C., Canada V6T 1W5.

Rat brain class C Ca channel cDNAs (rbC) hybridize on Northern blots to two mRNAs of approx. 8 and 12 kilobases (kb). The deduced primary structure of two overlapping clones (rbC-61 and rbC-30) shows that the class C gene product is >90% identical to the rabbit cardiac dihydropyridine receptor/Ca channel (designated rbC-I). Analysis of a number of other cDNAs reveals distinct class C coding sequences (designated rbC-II). Further, in 1400 amino acids compared, rbC-I and rbC-II transcripts differ at only two sites. In the first instance, rbC-II transcripts have a three amino acid insertion (ProAlaArg) corresponding to the putative cytoplasmic loop between homology domains II and III. The second site, a 28 amino acid region which includes the third putative transmembrane segment (S3) of domain IV of rbC-I, is replaced by a different 28 amino acid hydrophobic stretch. By Southern blot analysis, rbC-I and rbC-II specific probes are shown to hybridize an identical pattern of rat genomic DNA fragments. In addition, polymerase chain reaction analysis of rat genomic DNA shows that the rbC-I and rbC-II transcripts are generated from the same genomic DNA fragment. These results suggest that in addition to the distinct genes which encode the rat brain class A, B, C and D Ca channels, alternative splicing provides a further source of Ca channel diversity in the CNS.

395.11

Biochemical Characterization of a High Affinity [^{3}H]Ryanodine Receptor From Rabbit Brain Membranes.

P.S. McPherson and K.P. Campbell Howard Hughes Medical Institute and Program in Neuroscience and Dept. of Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, IA 52242.

The skeletal muscle receptor for the plant alkaloid ryanodine has been shown to be identical to the Ca^{2+} release channel of the sarcoplasmic reticulum. High affinity [^{3}H]ryanodine binding has been recently demonstrated in isolated brain membranes (Ashley, R.H., J. Memb. Biol., 111:179, 1989). We have shown that [^{3}H]ryanodine binding is enriched in membranes from the hippocampus but is significantly lower in membranes from the brain stem and spinal cord. Approximately 50% of [^{3}H]ryanodine labeled receptor is solubilized from brain membranes using CHAPS phosphatidylcholine containing 1 M NaCl. The brain [^{3}H]ryanodine receptor comigrates through sucrose gradients with the skeletal receptor as a large complex (~30S). Solubilized receptor is specifically immunoprecipitated by sheep polyclonal antibodies against purified skeletal muscle ryanodine receptor coupled to protein A Sepharose. [^{3}H]Ryanodine labelled receptor binds to heparin-agarose with high affinity, but does not bind to wheat germ agglutinin lectin. [^{3}H]Ryanodine labeled receptor is isolated using heparin-agarose chromatography followed by sucrose density gradient centrifugation. A protein of ~400,000 Da is enriched in peak [^{3}H]ryanodine binding fractions of heparin-agarose eluates and sucrose gradients, and is cross-reactive with antibodies raised against the skeletal muscle ryanodine receptor. We propose that the ~400,000 Da protein is the brain form of the high affinity ryanodine receptor and that it functions as a Ca^{2+} release channel in brain endoplasmic reticulum. Kevin P. Campbell is an Investigator of the Howard Hughes Medical Institute.

395.12

DIVERSE ω-CONOTOXIN GVIA BINDING PROTEINS IN ELECTRIC ORGAN AND BOVINE BRAIN

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Binding sites for the marine snail toxin, ω-conotoxin GVIA (ωCgTx), have been identified in a variety of neuronal tissue types, and have been assigned exclusively to voltage-dependent calcium channels. ωCgTx has been shown to bind with high affinity (K_D=3 pM) to relatively few receptor sites (0.4 pmoles/mg protein) in bovine brain (Yamaguchi et al., *JBC* 263, 9491), and with low affinity (K_D= 1 μM) to a greater number of receptor sites (290 pmoles/mg protein) in synaptosomes prepared from the electric organ of *Discopyge ommata* (Miljanich et al., *Brain Res* 453, 247). Our results suggest that these binding sites represent a heterogeneous population of ωCgTx binding proteins and that, in electric organ, not all are actually calcium channels. Crosslinking experiments with the bomobifunctional imidoester, dimethylsuberimidate (DMS), reveal that ^{125}I-ωCgTx specifically labels two proteins in bovine brain with M_r values of 330 and 220 kD as determined by SDS-PAGE, and three proteins in electric organ synaptosomes with M_r values of 170, 60, and 45 kD. We have demonstrated that the 45 kD protein of electric organ is the α-subunit of the nicotinic acetylcholine receptor (nAChR) and that it accounts for a significant fraction of the total number of ωCgTx binding sites. Furthermore, it can be separated from both the 170 and 60 kDa proteins by immunoprecipitation with a Mab against the nAChR. All three electric organ ωCgTx binding proteins share similar pharmacological properties, in that half maximal displacement of ^{125}I-ωCgTx occurs in the range of 0.1-1.0 μM ωCgTx, and the binding of ^{125}I-ωCgTx is not inhibited by other calcium entry blockers, such as nitrendipine, or by α-bungarotoxin and other agents known to interact with the nAChR. ωCgTx binding both in brain and electric organ is characteristically inhibited by higher concentrations of Ca^{2+} (0.5-1.0 mM) whereas in brain preparations we demonstrate that ωCgTx binding is actually stimulated up to 3-fold at lower Ca^{2+} concentrations (0.1-1.0 μM). It is noteworthy that this stimulatory effect occurs over the same concentration range as Ca^{2+} binding to sites controlling permeation and inactivation of calcium channels.

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DESIGN, SYNTHESIS, AND SINGLE CHANNEL CHARACTERIZATION OF A DIHYDROPYRIDINE-SENSITIVE CALCIUM CHANNEL PROTEIN.

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The major protein component of a dihydropyridine-sensitive calcium channel from skeletal muscle was cloned and sequenced (Tanabe, T. et al. 1987, *Nature* 328, 313-318). The primary structure suggests the occurrence of four internal repeats, each containing six presumably α-helical transmembrane segments. We designed a protein that mimics a pore-forming structure of the calcium channel; a carrier template (KKEKPGTGEKO) (Mutter, M. et al. 1988, *Tetrahedron* 44, 771-785) was used to direct the assembly of a bundle comprised of four identical 22-mer peptides with sequences corresponding to transmembrane segment IVS3 (DFWNVFDFLIVIGSIIDVILSE). This synthetic protein forms cation-selective channels in lipid bilayers. At pH 7.2, the single channel conductances in symmetric 50 mM $CaCl_2$, $BaCl_2$ and $SrCl_2$ are 7.2 ± 1.3, 9.5 ± 1.2 and 6.4 ± 0.4 pS, respectively, and 10.8 ± 1.9 pS in 500 mM NaCl. Channels are blocked by 10^{-7} M nifedipine, 10^{-6} M verapamil and 10^{-4} M of the local anesthetic analogue of lidocaine QX-222. A different synthetic protein containing four peptides with sequences corresponding to segment IVS5 (YVALLIVMLPFTYAVIGMQMFGK) does not form discrete channels.

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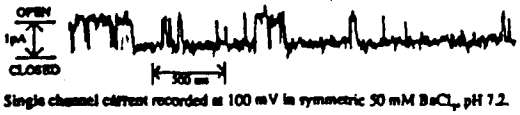

Single channel current recorded at 100 mV in symmetric 50 mM $BaCl_2$, pH 7.2.

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Bundles of Amphipathic Transmembrane α-Helices as a Structural Motif for Ion-Conducting Channel Proteins: Studies on Sodium Channels and Acetylcholine Receptors

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ABSTRACT Channel proteins are transmembrane symmetric (or pseudosymmetric) oligomers organized around a central ionic pore. We present here a molecular model of the pore forming structures of two channel proteins with different primary structures and oligomeric size: the voltage-sensitive sodium channel and the nicotinic cholinergic receptor. We report low-energy arrangements of α-helical bundles calculated by semiempiricial potential energy functions and optimization routines and further refined using molecular dynamics. The ion-conducting pore is considered to be a symmetric or pseudosymmetric homooligomer of 3–5 amphipathic α-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 four 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 ~ 16 Å^2). Similarly, a pentameric array optimized with constraints to maintain C_5 symmetry and backbone torsions characteristic of α-helices adopts a structure that appears well suited to form the lining of the nicotinic cholinergic receptor (pore area ~ 46 Å^2). Thus, bundles of amphipathic α-helices satisfy the structural, energetic, and dynamic requirements to be the molecular structural motif underlying the function of ionic channels.

Key words: protein structure, ionic pores, neural membranes, protein design, energy minimization, molecular dynamics

INTRODUCTION

Channel proteins are a special class of membrane proteins that mediate the selective passage of ions across the low dielectric constant apolar core of the membrane lipid bilayer (for review see 1). Understanding their mode of action has progressed with the advent of recombinant DNA technology that resulted in the elucidation of the primary structure of several channel proteins.[2–4] A remarkable feature inferred from the amino acid sequences is the high homology conservation among members of this family of proteins. They are composed of homologous subunits or domains which contain several transmembrane segments. Further, segments that could be folded into amphipathic transmembrane α-helices are identifiable in all channel proteins for which sequence information is available.[5–9] This is significant because amphipathic α-helices could form a bundle with one side facing an internal hydrophilic pore and the other facing hydrophobic helices or the membrane core. In addition, image analysis of electron micrographs obtained from ordered arrays of channel proteins in their native membrane environment produced low resolution images (~ 18 Å) that are consistent with radial symmetric or pseudosymmetric protein assemblies organized around a central aqueous pore.[10,11] These key features, homology and symmetry, raise the notion that a unifying motif in the biological design of ion channels is an array of transmembrane α-helices arranged such that amphipathic helices face a central hydrophilic pore whereas hydrophobic helices sur-

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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).