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# Homology and Analogy in Transmembrane Channel Design: Lessons from Synaptic Membrane Proteins†

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Transport of ions and small molecules across biological membranes is an essential process in living cells. Specific transport systems—pumps and channels—have evolved to allow translocation against and along existing solute concentration gradients. The highest rates of transport are achieved by channel proteins that in their activated ("open") state allow flux rates approaching that of free diffusion (Hille, 1984).

In the nervous system, channel proteins provide the molecular basis for electrical signaling and selective information transfer between excitable cells and thus are particularly abundant and diversified. Many neuronal channel proteins are enriched at synapses, i.e., the cell–cell contacts specialized for interneuronal communication. Voltage-sensitive ion channels control the propagation of action potentials along the neuronal plasma membrane and regulate the release of neurotransmitter from presynaptic nerve terminals. Neurotransmitter-activated channel proteins serve as receptors for rapid transmembrane signaling at the postsynaptic membrane of classical "chemical" synapses. Gap junction channels connect the cytoplasm of neighboring cells and provide direct electric coupling via "electrical" synapses.

During the past 5 years, a wealth of sequence information has been obtained by cDNA cloning on various members of different classes of synaptic channel proteins [reviewed in Miller (1989) and Jan and Jan (1989)]. Comparison of the primary structures and their hydropathy analysis disclosed common structural designs characteristic of functionally related protein superfamilies and facilitated identification of domains implicated in transport and/or activation and inactivation ("gating"). Here, the prominent structural features recognized thus far for three major classes of synaptic channels are illustrated by discussing selected examples of the different protein families. Attempts will be made to correlate structural motifs with functional properties and to delineate the evolutionary diversification creating the present set of channel gene products.

## Potassium Channel Subunits: Evolutionary Ancestors of the Voltage-Gated Channel Protein Family

Conduction of action potentials along excitable membranes depends on the sequential activation and inactivation of voltage-sensitive $Na^+$ and $K^+$ channels (Hodgkin & Huxley, 1952). Due to the availability of selective toxins from scorpions and marine organisms, $Na^+$ channels have been purified by several laboratories and shown to represent large transmembrane protein complexes containing a predominant high molecular weight $\alpha$-subunit (apparent $M_r$ 260K) associated with a variable set of smaller polypeptides, the $\beta$-subunits [(mammalian brain) $\beta$1, 36 kDa; $\beta$2, 33 kDa; (mammalian muscle) $\beta$1, 38 kDa; for review, see Catterall (1988)]. The primary structure of the $\alpha$-subunit of different vertebrate and *Drosophila* $Na^+$ channels has been deduced from cDNA sequences and found to contain four repeated domains of 300–400 amino acids with about 50% overall amino acid identity (Noda et al., 1984, 1986a; Salkhoff et al., 1987; Kayano et al., 1988; Auld et al., 1988; Trimmer et al., 1989). Each domain contains six largely hydrophobic segments (S1 to S6) long enough to form a membrane-spanning $\alpha$-helix. Most of these segments possess predominantly hydrophobic residues with few negative charges. The S4 segment, however, is unique in being highly positively charged by having an arginine or lysine residue at every fourth position of its sequence (Figure 2). This segment is thought to play a crucial role in channel gating by serving as a voltage sensor (see below).

On the basis of hydrophobicity analysis and secondary structure prediction, Noda et al. (1986a) and others [see Catterall (1988)] have proposed a transmembrane topology of the $Na^+$ channel $\alpha$-subunit as shown in Figure 1A. Accordingly, the four homologous domains are assumed to follow the same general transmembrane folding scheme with six membrane-spanning segments, each. Both the C- and N-

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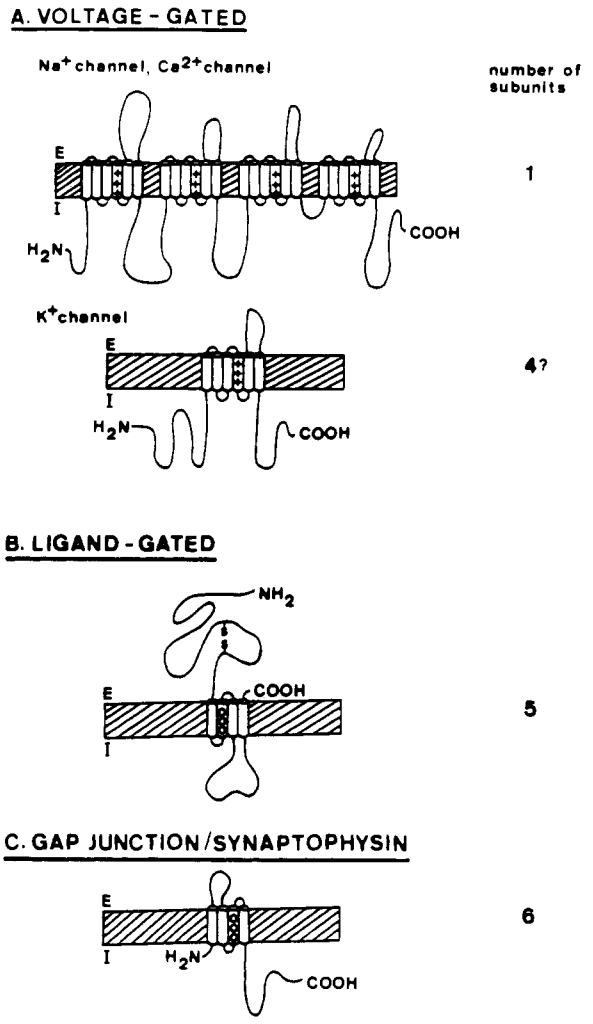


FIGURE 1: Proposed transmembrane topology and subunit number of different types of channel proteins. The protein-folding models shown represent idealized drawings deduced from various $Na^+$ channel, $Ca^{2+}$ channel, $K^+$ channel, nicotinic acetylcholine receptor, glycine receptor, $GABA_A$ receptor, synaptophysin, and gap junction protein sequences (see text). The length of individual extramembrane stretches may differ considerably between individual members of the channel protein families shown and do not correspond to a particular protein. E denotes the extracellular (or vesicular) and I the cytoplasmic sides of the membrane. The S4 segment of voltage-gated channels is indicated by (+) and the putative channel-forming M2 and M3 segments of ligand-gated channels and connexins and synaptophysin, respectively, by (O).

termini of the polypeptide then are located at the cytoplasmic side of the plasma membrane. An ion channel may be formed by symmetrically arranging the four repeat domains around a central axis perpendicular to the plane of the membrane, thus creating a pseudotetrameric transmembrane protein. Mapping of antibody epitopes and phosphorylation sites as well as protease digestion experiments are consistent with this model (Gordon et al., 1988; Catterall, 1988). Presently, however, it is unclear which transmembrane segments contribute to the channel proper by lining the ion path. Segments S2, S3, and S4 together with an additional hypothetical intramembrane stretch, S7, all have been considered in theoretical models (Greenblatt et al., 1985; Noda et al., 1986a; Guy & Seetharamulu, 1986). Also, a synthetic peptide corresponding to segment S3 of domain I of a rat $Na^+$ channel $\alpha$-subunit has been shown to form cation-selective channels upon incorporation into lipid bilayers (Oiki et al., 1988).

Functional expression in *Xenopus* oocytes confirmed that the $\alpha$-subunit sequence of the $Na^+$ channel indeed contains the elements required for ion conduction and voltage-dependent gating (Noda et al., 1986b; Auld et al., 1988; Stühmer et al., 1989; Trimmer et al., 1989). Injection of in vitro transcribed $\alpha$-subunit cRNA directs the synthesis and incorporation into the oocyte membrane of depolarization-activated $Na^+$-selective channels which display the pharmacological characteristics of their counterparts in brain. Noteworthy, kinetic characteristics of channel inactivation are altered by coexpression of other brain mRNAs (Auld et al., 1988). This may indicate a regulatory role of associated $\beta$-subunits or modifying enzymes like protein kinases.

The delineation of $Ca^{2+}$ channel subunit sequences and, recently, their functional expression disclosed similar structural principles. Dihydropyridines antagonize $Ca^{2+}$ currents in skeletal and heart muscles and find wide therapeutic applications in various human diseases. The dihydropyridine receptor has been purified from skeletal muscle [reviewed in Catterall (1988)], and cDNAs of its major antagonist binding subunit have been isolated from both muscle and heart (Tanabe et al., 1987; Ellis et al., 1988; Mikami et al., 1989). The deduced primary structures show four homologous repeats as found in the $Na^+$ channel $\alpha$-subunit and display considerable sequence similarity to the latter, in particular within the highly charged S4 segment (Figure 2); major variations concern the N- and C-terminal intracellular regions. Thus, a common transmembrane topology and probably a similar tertiary structure of the channel-forming domains seem to be shared by these voltage-gated cation channels.

The striking conservation of the S4 segment between different members of the voltage-gated channel protein family has raised speculations on its possible function. From elec-

| | | |
|---|---|---|
| *Electrophorus* $Na^+$ channel | DLRNVSALRTFRVLRALKTITIFP | Domain I |
| " " " | NMQGMSVLRSLRLLRIFKLAKSWP | Domain II |
| " " " | ELGAIKNLRTIRALRPLRALSRFE | Domain III |
| " " " | LFRVIRLARIARVLRLIRAAKGIR | Domain IV |
| Rat brain $Na^+$ channel, type I | LFRVIRLARIGRILRLIKGAKGIR | Domain IV |
| Rabbit muscle $Ca^{2+}$ channel | SSAFFRLFRVMRLIKLLSRAEGVR | Domain IV |
| *Drosophila* $K^+$ channel | ILRVIRLVRVFRIFKLSRHSK | - |
| Rat brain $K^+$ channel | ILRVIRLVRVFRIFRLSRHSK | - |

FIGURE 2: S4 segment of voltage-gated ion channels. Amino acid sequences shown are taken from the following: *Electrophorus* $Na^+$ channel, amino acids 202–225, 649–672, 1090–1113, and 1415–1438 (Noda et al., 1984); rat brain $Na^+$ channel, type I, amino acids 1634–1657 (Noda et al., 1986); rabbit muscle $Ca^{2+}$ channel, amino acids 1231–1254 (Tanabe et al., 1987); *Drosophila* $K^+$ channel, amino acids 432–452 (Tempel et al., 1987); rat brain $K^+$ channel, amino acids 290–310 (Baumann et al., 1988). Positively charged residues are indicated in boldface letters.

trophysiological measurements it was known that channel activation involves translocation of charges through the protein which are detectable as "gating current" (Armstrong, 1981). Different investigators have proposed that alterations of the transmembrane electrical field might induce movements of the charged S4 segments, which could quantitatively account for this gating current, thus producing a conformational change resulting in channel opening (Noda et al., 1984, 1986a; Guy & Seetharamulu, 1986; Catterall, 1988). Recently, this hypothesis has been verified by mutagenesis of up to three of the basic residues within the S4 segments of a brain $Na^+$ channel $\alpha$-subunit (Stühmer et al., 1989). Upon expression of the mutant mRNAs in the oocyte system, a direct correlation was found between the net positive charge in the first S4 segment and the steepness of the potential dependence of activation. The same study also confirmed that, as previously deduced from intracellular protease and anti-peptide antibody treatments (Armstrong et al., 1973; Vassilev et al., 1988), the cytoplasmic domain linking the homology repeats III and IV is involved in channel inactivation.

$K^+$ channels comprise the most diverse family of voltage-sensitive channel proteins (Hille, 1984). They are found in most cells of the body and control many cellular functions including duration of action potentials, neurotransmitter release from presynaptic nerve terminals, and cardiac pacemaking. Cross-linking and binding studies using "facilitatory" polypeptide toxins from snake and bee venom indicate that putative voltage-sensitive $K^+$ channel proteins in vertebrate brain are large complexes ($M_r$ about 400000) which contain at least two types of polypeptides (Rehm & Lazdunski, 1988; Schmidt & Betz, 1989). A major subunit species ($M_r$'s about 70K–90K) carries binding sites for different polypeptide toxins (Rehm & Betz, 1983; Black & Dolly, 1986; Schmidt et al., 1988; Rehm et al., 1988); the role of smaller accessory polypeptides (28–38 kDa) seen upon purification (Rehm & Lazdunski, 1988) or cross-linking (Schmidt & Betz, 1989) is not clear. However, reconstitution experiments show that this set of polypeptides can form functional $K^+$ channels upon incorporation into a planar bilayer (Rehm et al., 1989b). Thus, vertebrate $K^+$ channels apparently do not contain the high molecular weight subunit characteristic of voltage-gated $Na^+$ and $Ca^{2+}$ channel proteins.

A breakthrough in the elucidation of $K^+$ channel structure came from molecular genetic analysis of a neurological mutant of *Drosophila*, the "*shaker*" fly. Electrophysiological analysis of this mutant had indicated that its phenotype, continuous trembling of appendages, resulted from alterations in a voltage-sensitive $K^+$ current (Salkhoff & Wyman, 1981). Mutational breakpoint localization and chromosome walking techniques led to the identification of a large transcription unit that covers >100 kb of genomic DNA (Papazian et al., 1987; Kamb et al., 1987; Baumann et al., 1987). Analysis of the corresponding transcripts revealed a set of alternatively spliced gene products which displayed striking homology to $Na^+$ and $Ca^{2+}$ channel sequences (Tempel et al., 1987; Schwarz et al., 1988; Kamb et al., 1988; Pongs et al., 1988). The deduced protein sequences are characterized by six hydrophobic putative transmembrane regions including a typical S4 segment (Figure 2). Although diverging in their 5′ and 3′ coding regions, all fully processed mRNAs contained a central stretch encoding these predicted transmembrane domains. In other words, the *shaker* proteins apparently correspond to individual repeat domains of $Na^+$ and $Ca^{2+}$ channel $\alpha$-subunits but vary strikingly in their putative cytoplasmic N- and C-terminal regions (Figure 1). $K^+$ channels therefore are thought to consist of tetrameric assemblies of subunits, a view supported by genetic evidence indicating that *shaker* gene products interact with one another (Salkhoff & Wyman, 1983; Timpe & Jan, 1987). $Na^+$ and $Ca^{2+}$ channel proteins then may have evolved from *shaker*-related sequences by gene duplication and fusion events. This is consistent with the phylogeny of $K^+$ channels (Hille, 1984). Accordingly, $K^+$ channels belong to the evolutionarily oldest ion channels known.

Upon expression in *Xenopus* oocytes, *shaker* mRNAs generate functional $K^+$ channels, presumably by assembly of homooligomeric protein complexes. Their activation and inactivation kinetics differ for the various *shaker* transcripts, indicating a role of the variable C- and N-terminal regions in the determination of channel characteristics (Timpe et al., 1988a,b; Iverson et al., 1988). Up to now, five or more functionally different $K^+$ channel subunits have been shown to result from regulated splicing of the primary transcript of the *shaker* gene. Whether mixing of different subunits at variable stoichiometries further amplifies channel diversity in vivo is presently unclear.

In *Drosophila* and vertebrates, $K^+$ channel diversity may indeed be much larger than previously anticipated. Recently, a set of cDNAs encoding *shaker*-related proteins (*shab*, *shaw*, and *shal*) has been described, which all may correspond to functional $K^+$ channel subunits (Butler et al., 1989). These gene products exhibit high (>50%) homology to *shaker* transcripts within the central domain containing the putative six membrane-spanning segments. Interestingly, the number of positively charged residues within different S4 segments of *shaker*, *shab*, *shaw*, and *shal* is variable, suggesting that the corresponding channels may differ in activation kinetics (Butler et al., 1989).

Availability of *Drosophila* $K^+$ channel cDNA probes has allowed cloning of their homologues in the vertebrate central nervous system. From both mouse and rat brain libraries, *shaker*-related recombinants have been isolated which correspond to transcripts of different mammalian genes (Tempel et al., 1988; Baumann et al., 1988; McDonald et al., 1989). Also, a rat brain cDNA resembling *shab* has been recently obtained by expression cloning (Frech et al., 1989). Polypeptides of 55–95 kDa are predicted from these sequences. Rat proteins have been expressed in *Xenopus* oocytes and shown to functionally differ from the *shaker* proteins in that they form slowly inactivating $K^+$ channels of the delayed rectifier type prevalent in mammalian neurons and other excitable cells (Stühmer et al., 1988; Frech et al., 1989). The expressed channels were sensitive to $K^+$ channel blockers and, in one case, to facilitatory neurotoxins, and thus probably correspond to putative $K^+$ channel proteins identified in toxin binding studies (see above). Indeed, antibodies raised against sequences of a mouse *shaker* homologue recognize the purified mammalian dendrotoxin receptor (Rehm et al., 1989a). In other words, the structural features deduced from *shaker* transcripts appear to be generally valid for voltage-gated $K^+$ channel proteins.

## Inhibitory Glycine Receptor, an Archetypic Ligand-Gated Chloride Channel

Signal transmission at chemical synapses requires specific receptors that transduce neurotransmitter binding into electrical signals, e.g., alterations of membrane potential. Receptors containing integral ion channels mediate rapid (in the less than or equal to millisecond range) transduction events, whereas receptors activating G-protein-coupled channels operate at slower time scales (in the millisecond to second range). At resting membrane potential, excitation is generated by

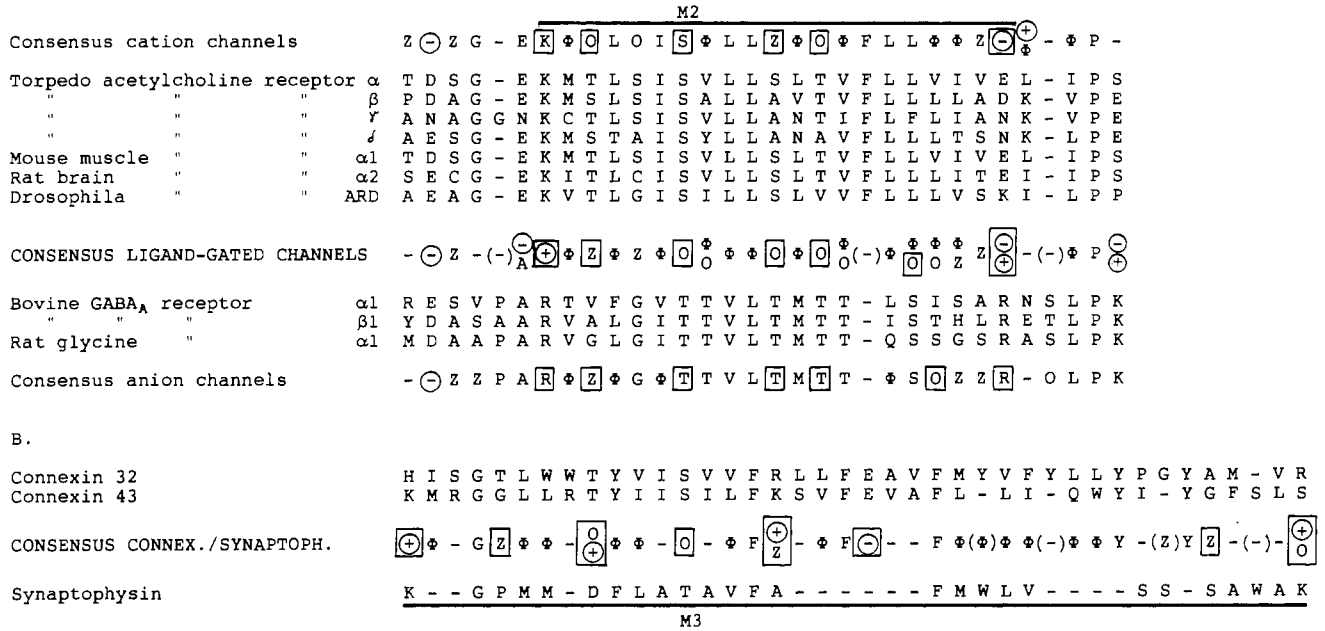


FIGURE 3: Comparison of putative channel-lining transmembrane segments. (A) M2 segments of different subunits of cation- and anion-conducing ligand-gated channel proteins. The sequences are taken from the following: *Torpedo* nicotinic acetylcholine receptor (Noda et al., 1983); mouse muscle nicotinic acetylcholine receptor (Boulter et al., 1985); rat brain nicotinic acetylcholine receptor (Wada et al., 1988); *Drosophila* nicotinic acetylcholine receptor (Hermans-Borgmeyer et al., 1986); bovine GABA$_A$ receptor (Schofield et al., 1987); rat glycine receptor (Grenningloh et al., 1987). (B) M3 segments of synaptophysin and different connexins. Sequences are from the following: connexin 32 (Paul, 1986); connexin 43 (Beyer et al., 1987); synaptophysin (Leube et al., 1987). The following symbols are used in addition to the IUPAC single-letter amino acid code: (O) hydroxylated side chain; (Φ) large hydrophobic residue; (Z) small or hydroxylated side chain; (+ and −) positively and negatively charged amino acids. Consensus sequences are aligned above or between the corresponding channel protein families. Charged, small, and hydroxylated residues at about every fourth position of the consensus sequences are boxed.

cation influx, but inhibition of neuronal firing results from increased $Cl^-$ permeability.

The nicotinic acetylcholine receptor at the neuromuscular junction initiates muscle contraction; due to its abundance in fish electric organ, it is the best characterized cation-conducing channel protein known [reviewed in Changeux et al. (1984)]. The primary structures of its subunits have been determined in different species (Noda et al., 1983; Claudio et al., 1983; Devilliers-Thiery et al., 1984), and homologous cDNAs have been isolated from vertebrate and *Drosophila* brain (Boulter et al., 1986; Goldman et al., 1987; Deneris et al., 1988; Nef et al., 1988; Hermanns-Borgmeyer et al., 1986; Bossy et al., 1988). The major inhibitory neurotransmitters at central synapses, glycine and γ-aminobutyric acid (GABA), gate chloride channel-forming receptors of similar conductance properties (Bormann et al., 1987), but distinct pharmacology. For example, the convulsive alkaloid strychnine antagonizes postsynaptic inhibition by glycine, the predominant inhibitory neurotransmitter in the brain stem and spinal cord, whereas benzodiazepines and barbiturates modify inhibitory GABA$_A$ receptor responses in many regions of the central nervous system.

The glycine receptor was the first ligand-gated channel protein to be isolated from mammalian nervous tissue (Pfeiffer et al., 1982). Affinity-purified preparations of this receptor contain two glycosylated integral membrane proteins of $M_r$ = 48K (α) and 58K (β), respectively (Pfeiffer et al., 1982; Graham et al., 1985; Becker et al., 1986). These polypeptides are thought to form the chloride channel of the receptor (Pfeiffer et al., 1984; Betz & Becker, 1988). A copurifying peripheral membrane protein of 93 kDa associated with cytoplasmic domains of the postsynaptic glycine receptor (Schmitt et al., 1987; Becker et al., 1989) has been implicated in its synaptic localization and/or anchoring to cytoskeletal elements.

Peptide mapping (Pfeiffer et al., 1984) and cDNA sequencing (Grenningloh et al., 1987a; Grenningloh et al., unpublished results) of the α and β glycine receptor subunits revealed a high homology between these proteins. Both possess a cleavable signal sequence and, in the C-terminal half of the polypeptide, four hydrophobic segments (M1 to M4) long enough to form transmembrane α-helices. This arrangement resembles that of nicotinic acetylcholine receptor (Noda et al., 1983; Changeux et al., 1984) and GABA$_A$ receptor proteins (Schofield et al., 1987; Levitan et al., 1988), suggesting that all channel-forming receptors are composed of subunits sharing a common transmembrane topology (Figure 1). Furthermore, significant amino acid sequence homology exists between the subunits of different ligand-gated ion channels (Grenningloh et al., 1987a,b; Schofield et al., 1987). Thus, these receptors constitute a protein superfamily that evolved by gene duplication from a common ancestor early in phylogeny.

The membrane-spanning segments M1 to M3 are highly conserved between glycine and GABA$_A$ receptor subunits, pointing to their potential importance in chloride channel function (Grenningloh et al., 1987b). Segment M2 contains many uncharged polar amino acid residues and therefore is thought to provide the hydrophilic inner lining of the chloride channel. Here, eight consecutive amino acid residues are identical in GABA$_A$ and glycine receptor subunits (Figure 3A). Interestingly, transmembrane segment M2 of nicotinic acetylcholine receptor proteins is known to be involved in cation transport and channel blocker binding (Giraudat et al., 1986; Hucho et al., 1986; Imoto et al., 1986). Segment M2 thus seems to be a common structural determinant of ligand-gated ion channel function. Indeed, comparison of both "cationic" and "anionic" M2 sequences unravels some common periodicity where a bulky hydrophobic residue at roughly every fourth position, i.e., on the same side of a potential transmembrane α-helix, is followed by a small or polar side chain (Figure 3A).

Having the latters arranged toward the channel lumen, a pore sufficiently large and polar for passage of permeating ions may be created in the center of only five symmetrically arranged $\alpha$-helices (Bormann et al., 1987). In support of a structural model assuming that homologous M2 segments from each receptor subunit associate to form the lining of the channel (Hucho, 1986), exchange of up to three serine residues within different M2 transmembrane segments of nicotinic acetylcholine receptor subunits was found to decrease single channel outward currents and to accelerate channel blocker dissociation (Leonard et al., 1988).

The M2 segments of anion-selective glycine and $GABA_A$ receptor proteins terminate with positively charged residues at both ends (Figure 3A). Furthermore, positively charged residues surround the M1 to M3 sequences both intra- and extracellularly. Cation-conducing nicotinic acetylcholine receptors in contrast have negatively charged side chains in addition to positive charges bordering transmembrane segment M2 (Figure 3A). Patch clamp data indicate two sequentially occupied anion binding sites in both glycine and $GABA_A$ receptor channels (Bormann et al., 1987). The terminal charged residues of the M2 segments may be the structural correlates of these sites at the presumptive inner and outer mouths of receptor ion channels, and thus provide their ion selectivity filter. Indeed, mutation of the negatively charged residues bordering the M2 regions of nicotinic acetylcholine subunits has been found to modulate the conductivity of this cation channel (Imoto et al., 1988). From single-channel analysis of the expressed mutant receptors, three rings of negatively charged and glutamine residues neighboring the M2 segments have been identified (see Figure 3A), which serve as major determinants of the rate of cation transport through the nicotinic receptor. Also, a synthetic peptide corresponding to segment M2 of the glycine receptor $\alpha$-subunit (Figure 3A) has been shown to produce randomly gated "channels" upon incorporation into planar lipid bilayers (D. Langosch, K. Hartung, E. Grell, E. Bamberg, and H. Betz, unpublished results). Interestingly, the ion selectivity of these channels was changed upon inversing the terminal charges of the peptide. As outlined above, an accumulation of hydroxylated residues is highly conserved within the M2 regions of the anion channel forming glycine and $GABA_A$ receptor subunits (Figure 3A). The high positive ion potential of the hydroxyl terminus may stabilize permeating anions and thus additionally contribute to ion selection in these channels.

A proline residue in segment M1 of glycine and $GABA_A$ receptor polypeptides is also present in nicotinic acetylcholine receptor proteins. Proline residues causing a bend in transmembrane-spanning $\alpha$-helices may provide the structural flexibility required for reversible conformational transitions of intramembrane regions of transport proteins (Brandl & Deber, 1986). Their conservation thus may point to a conserved machinery of different ligand-gated channels for transforming external ligand binding into activation of a presumptive intramembrane gating mechanism.

Besides considerable conservation of transmembrane sequences, homology also exists in the putative extracellular N-terminal domain. Remarkable are two precisely conserved cysteine residues, which also are present in nicotinic acetylcholine receptor polypeptides. For the acetylcholine receptor, these cysteines have been proposed to form a disulfide bridge essential for receptor tertiary structure (Mishina et al., 1985; Stroud & Finer-Moore, 1985). Similar folding patterns thus may exist in the extracellular portion of channel-forming receptor proteins.

Photoaffinity-labeling experiments using [$^3$H]strychnine have localized the ligand binding site of the glycine receptor on its $\alpha$-subunit (Graham et al., 1983). From theoretical considerations, a stretch of charged residues preceding the first transmembrane segment has been proposed to be part of the binding pocket (Grenningloh et al., 1987a). Interestingly, a corresponding region containing two neighboring cysteine residues is known to be important for acetylcholine binding to the $\alpha$-subunits of the nicotinic acetylcholine receptor (Kao et al., 1984). Here, however, many other extracellular residues are, in addition, labeled by covalent acetylcholine analogues and derivatives (Dennis et al., 1989). This suggests that agonist binding to ligand-gated channels generally may involve multiple interactions with an extended extracellular domain of the respective ligand binding subunits.

Analysis of the subunit composition of the glycine receptor indicates a pentameric channel core probably composed of three $\alpha$-subunits and two $\beta$-subunits, respectively (Langosch et al., 1988). This subunit stoichiometry resembles that of the nicotinic acetylcholine receptor, which also contains five membrane-spanning subunits (Changeux et al., 1984; Stroud & Finer-Moore, 1985). In view of the sequence homology and similar predicted transmembrane topology of the different receptor proteins discussed above, a quasisymmetrical pentameric complex of transmembrane polypeptides around a central ion channel is proposed as the common quaternary structure of all members of the ligand-gated ion channel superfamily (Langosch et al., 1988).

Expression of individual agonist binding subunits of glycine and $GABA_A$ receptors in *Xenopus* oocytes and mammalian cell lines generates functional ligand-gated channels that display most of the typical pharmacology of their corresponding receptors (Pritchett et al., 1988; Schmieden et al., 1989; Sontheimer et al., 1989). Although the efficiency of assembly of such homooligomeric receptors is low (Sontheimer et al., 1989), their formation clearly indicates that individual receptor subunits must have similar exchangeable oligomerization sites. This may be exploited in vivo for generating functional diversity from a limited set of subunit subtypes.

Recent biochemical and cDNA sequence data have established subtype diversity as a general phenomenon for brain nicotinic acetylcholine (Goldman et al., 1987; Deneris et al., 1988; Wada et al., 1988), $GABA_A$ (Levitan et al., 1988; Pritchett et al., 1989; Ymer et al., 1989), and glycine (Becker et al., 1988; Hoch et al., 1989) receptor subunits. In the case of $GABA_A$ and neuronal nicotinic acetylcholine receptors, expression of different subtype combinations has been shown to produce functionally and/or pharmacologically distinct receptor entities (Levitan et al., 1988a; Pritchett et al., 1989; Deneris et al., 1988). A particularly well-investigated example of variable subunit composition is the nicotinic acetylcholine receptor in vertebrate skeletal muscle. There, developmentally regulated embryonic and adult isoforms differing in channel properties are generated by exchange of a single subunit within the receptor complex (Mishina et al., 1986). A similar mechanism may underly glycine receptor heterogeneity in neonatal and adult spinal cord (Becker et al., 1988); here, however, neonatal receptors may be homooligomers of a single developmentally regulated subunit (Hoch et al., 1989).

## Synaptophysin, an Intracellular Analogue of Gap Junction Proteins

Neurotransmitter release from presynaptic nerve terminals involves rapid exocytosis of low molecular weight transmitters and neuropeptides from their storage compartments, synaptic vesicles. Fusion of secretory vesicles with specialized plasma

membrane domains is commonly assumed to underly exocytosis of hormones and neurotransmitters (Cecarelli & Hurlbut, 1980). To identify the molecules involved in vesicle–plasma membrane interaction, peripheral and integral membrane proteins of both synaptic vesicle and presynaptic plasma membrane fractions have lately been studied with great intensity [reviewed in Kelly (1988)].

Synaptophysin has orginally been identified as a major transmembrane glycoprotein of apparent $M_r$ = 38K in synaptic vesicle preparations isolated from rat brain (Wiedenmann & Franke, 1985; Jahn et al., 1985). Its detergent binding properties (Rehm et al., 1986) and amino acid composition deduced from the cDNA sequence (Buckley et al., 1987; Leube et al., 1987; Südhoff et al., 1987) classify synaptophysin as a hydrophobic integral membrane protein. On the basis of hydropathy analysis, synaptophysin is thought to span the vesicle membrane four times, with both its N- and C-termini on the cytoplasmic side of the vesicular membrane (Figure 1). This transmembrane topology is supported by protease digestion and epitope mapping experiments (Leube et al., 1987; Johnston et al., 1989) and resembles that of gap junction proteins (or "connexins"), in particular the well-characterized junction protein connexin 32 from liver (Paul, 1986; Kumar & Gilula, 1986) (Figure 1). Although synaptophysin exhibits no significant overall sequence homology to any other known protein, there is a common motif of hydrophobic amino acids in all predicted transmembrane regions of synaptophysin that reappears in one of the hydrophobic segments of junction proteins (Thomas et al., 1989). Furthermore, synaptophysin and connexins share extended C-terminal cytoplasmic tail regions containing many charged amino acid residues. These tail regions may mediate interactions with cytoskeletal elements; in the case of synaptophysin, the cytoplasmic C-terminal domain has been shown to contain a $Ca^{2+}$ binding site (Rehm et al., 1986).

Biochemical and reconstitution studies indicate that the similarity in presumed transmembrane topology of both synaptophysin and the connexins reflects common structural and functional properties. Liver gap junctions consist of paired connexons (Unwin & Zampighi, 1980). Each connexon forms a transmembrane channel composed of six symmetrically arranged copies of connexin 32. Synaptophysin also is a homooligomeric membrane protein containing six identical subunits (Thomas et al., 1988). Ultrastructural analysis of negatively stained synaptophysin preparations revealed rosette-like 7.8-nm particles that closely resemble other transmembrane channels, i.e., the nicotinic acetylcholine receptor, the voltage-gated $Na^+$ channel, and hepatic gap junction preparations. Upon incorporation into planar lipid bilayers, synaptophysin displays voltage-sensitive channel activity of average conductance $\approx$ 150 pS (Thomas et al., 1988). This value is similar to the conductance of electrically coupled cells and gap junction protein containing membranes. Thus, synaptophysin not only resembles gap junction connexons in predicted transmembrane topology and hexameric structure but also forms channels of comparable size and possibly corresponding function. The occurrence of such channels in intracellular membranes may appear surprising. However, many channel activities have recently been disclosed in cell organelles by the patch clamp method. Among others, a channel resembling reconstituted synaptophysin in conductance and opening time has been described in synaptic vesicles isolated from *Torpedo* electric organ (Rahamimoff et al., 1988).

The channel-forming domains of synaptophysin and the connexins are not identified. From helical modeling of connexin 32, transmembrane segment M3 has been postulated to line the channel proper (Milks et al., 1988). A large portion of this segment of different gap junction proteins is characterized by having charged, polar, or small side chains repeated at every fourth position adjacent to large hydrophobic residues (Figure 3B). This organization is shared by transmembrane segment M3 of synaptophysin and reminiscent of the consensus sequence of the M2 segment of ligand-gated channel proteins (Figure 3A). Thus, common structural features of pore-forming transmembrane $\alpha$-helices appear to be present in different channel protein families.

On the basis of high-resolution electron microscopic data, rotational sliding of entire subunits has been proposed in opening and closing of gap junction connexons (Unwin & Ennis, 1984). Gating of synaptophysin channels may involve similar subunit rearrangements within the hexameric membrane protein. Presently, the physiological trigger for synaptophysin channel activation is unknown; in reconstituted planar bilayers, positive transmembrane voltages are required. These may be sensed by the charged cytoplasmic tail regions characterizing synaptophysin and most members of the connexin family and, in analogy to the variable cytoplasmic N- and C-terminal domains of voltage-gated $Na^+$ and $K^+$ channels, modify the kinetics of channel opening. Alternatively, ion binding to these domains may be important; as outlined above, synaptophysin has a cytoplasmic $Ca^{2+}$ binding site. Also, gap junction conductances are known to be regulated by changes in internal pH, $Ca^{2+}$, and cAMP levels.

The physiological role of synaptophysin in the synaptic vesicle membrane is presently unknown. It may act as a transmembrane channel that allows exchange of low molecular weight components between the cytoplasm and vesicle interior. Such a function has been proposed for vacuolar $Ca^{2+}$-regulated channels in plants which are thought to serve for metabolite transport (Hedrich & Neher, 1987). Alternatively, synaptophysin might be involved in transport processes across two apposed lipid bilayers, in analogy to gap junction proteins. A tempting speculation is that, during exocytosis, synaptophysin may couple its associated vesicle to a related channel ("vesicle docking protein") in the presynaptic membrane, thus mediating the formation of a transient two-membrane channel or "fusion pore" (Thomas et al., 1988, 1989). The occurrence of such gap junction-like structures in the initial step of exocytosis in mast cells has indeed been indicated by electrophysiological experiments (Breckenridge & Almers, 1987). There, these pores are assumed to represent transitory structures preceding vesicle plasma membrane fusion. In the case of synaptic vesicles containing low molecular weight neurotransmitters, a channel mechanism of vesicular release may not only account for initiation of exocytosis but explain all features of the secretory event by assuming reversible pore formation via docking and undocking of vesicular and plasma membrane channels.

## Conclusions and Perspectives

The present structural data on different synaptic channel protein superfamilies indicate that their functional diversity largely arose by divergent evolution of common ancestral building blocks or subunits. These originally may have assembled into homooligomeric transmembrane structures being the precursors of modern $K^+$ channels, or of glycine and $GABA_A$ receptor homooligomers, which still are detected upon expression of a single subunit of these proteins. For creating stably assembled entities, the corresponding gene segments then were duplicated and eventually fused to generate single-subunit proteins of the voltage-gated $Na^+$ or $Ca^{2+}$ channel

prototype. On the other hand, with functional specialization being the primary goal, divergence of the duplicated genes allowed generation of heterooligomeric complexes of variable subunit composition. This seems to be realized to a large extent in the ligand-gated channel protein family where exchange of single subunits was shown to drastically modify receptor properties.

Although divergent evolution certainly accounts for the different voltage- and ligand-gated channel proteins of synaptic membranes, related channel structures probably also arose via convergence of unrelated polypeptide sequences. In case of the synaptophysin/connexin hexameric channel protein superfamily, similar transmembrane organization and functional properties are found for proteins barely related in amino acid sequence. Thus, as in the case of various soluble proteins, common folding designs may originate from distinct primary structures.

Despite great differences in sequence and transmembrane organization displayed by the various synaptic channel protein superfamilies, some common principles of transmembrane channel architecture emerge from their comparison: (a) All channel proteins are assembled from homologous "blocks" or subunits of transmembrane-spanning sequences. (b) Each subunit or homology domain contributes a membrane-spanning $\alpha$-helix to the lining of the channel. (c) The organization of these channel-lining $\alpha$-helices appears to follow some general rules. Commonly, small or polar (or even charged) residues adjacent to bulky hydrophobic side chains are accumulated on that side of the $\alpha$-helix which is assumed to be exposed to the channel lumen. (d) The pore size of the channel is largely determined by the number of homology blocks or subunits, i.e., of available lining segments [see Unwin (1986)]. (e) Rings of charged residues bordering the channel-lining $\alpha$-helices are major determinants of ion selectivity and current flow rates, probably by acting as transiently occupied binding sites for the transported ion species.

Apart from these rather general deductions, most of the pertinent structure–function relationships of channel proteins are not understood. In particular, the domains involved in channel activation are still poorly defined. Although extended cytoplasmic and extracellular domains certainly are important determinants of gating in voltage- and ligand-gated ion channels, the transduction of conformational changes in these regions into movement of transmembrane segments resulting in opening and closing remains enigmatic. Determination of the crystal structure of representative members of the different channel protein superfamilies hopefully will lead to a more detailed picture of this important class of membrane proteins.

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## *Accelerated Publications*

# Evidence from X-ray Absorption Fine Structure Spectroscopy for Significant Differences in the Structure of Concanavalin A in Solution and in the Crystal†

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ABSTRACT: We have used X-ray absorption fine structure spectroscopy (XAFS) to study and compare the structure of concanavalin A in crystals and in aqueous solution. Significant differences were found between crystal and solution in the configuration of the transition-metal site of the protein. The metal has six ligands in solution but only five in the crystal. The ligand bond lengths are shorter in the crystal than in solution. The vibrational disorder in the crystal and possibly the corresponding bond length show a negative temperature dependence whereas in solution they vary normally with temperature. The anomalous temperature dependence in the crystal suggests that as the temperature decreases the protein molecules are subject to additional stresses, which are transmitted as a tensile stress at the metal site leading to distorted geometry and lengthening and weakening of metal-ligand bonds.

Protein structure in solution and in the crystal has not been probed with the same technique to atomic resolution. Lack of such a direct comparison sometimes leads to a presumption that there may not be consequential differences in these two states. It has been noted recently that structures of some small proteins resolved by solution 2-D NMR can differ from their X-ray crystallographic counterparts [Wuthrich et al. (1989) and references cited therein]. However, there is the possibility

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