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Transmembrane Auxiliary Subunits of Voltage-dependent Ion Channels*

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Auxiliary subunits of voltage-dependent ion channels can be subdivided into two classes. One class consists of the entirely cytoplasmic intracellular subunits, which have no transmembrane domains such as the β subunit of the voltage-dependent Ca^{2+} channel and the β subunit of the voltage-dependent K^+ channel. The other class of auxiliary subunits contains at least one transmembrane domain and an extracellular glycosylated region. This review will concentrate on the transmembrane auxiliary subunits. For mammalian systems, these proteins include the $\alpha_2\delta$ and γ subunits of the voltage-dependent Ca^{2+} channel, the β_1 and β_2 subunits of the Na^+ channel, and the β subunit of the maxi-K or large conductance Ca^{2+}-activated K^+ channel. Most traditional voltage-dependent K^+ channels and Cl^- channels have not been shown to have transmembrane-containing associated proteins, although a family of membrane proteins including IsK,Cl (minK) may fit into this category. Recent genetic approaches using *Drosophila* and *Caenorhabditis elegans* have begun to identify evolutionarily related subunits as well as unique proteins that also profoundly alter ion channel properties and expression.

The interest in identifying and studying these primarily extracellular proteins lies in the diverse and often unknown means by which they modify ion channel function and expression. Many are capable of altering pharmacological interactions or bind drugs directly while others are likely ligands for extracellular matrix proteins and cell-cell interactions. Some are highly negatively charged and may screen surface charges or influence ion concentration near the pore region of the ion channel. All are glycosylated to some extent suggesting a role for these proteins in plasma membrane targeting and/or subunit folding.

Properties of Protein Structure

Transmembrane Topologies—Overall, there is very little if any sequence similarity between the different transmembrane auxiliary subunits. The Ca^{2+} channel $\alpha_2\delta$ subunit and the Na^+ channel β_1 and β_2 subunits are all type I transmembrane proteins, which consist of NH_2-terminal signal sequences and a transmembrane domain near their carboxyl terminus (1–3), whereas the Ca^{2+} channel γ subunit contains perhaps as many as four transmembrane domains (4) (Table I). Interestingly, a protein with significant homology to the Ca^{2+} channel α_2 protein has recently been identified as the *unc-36* locus in *C. elegans*, although its transmembrane topology has not yet been determined.[1]

The β subunit found in association with the maxi-K channel of bovine tracheal smooth muscle has two transmembrane domains with the majority of its mass facing the extracellular space (5). Similarly, the TipE protein of *Drosophila,* which dramatically affects expression of the Na^+ channel *para* α subunit gene, also has two transmembrane domains (6). TipE appears to be structurally and evolutionarily unrelated to the mammalian Na^+ channel β subunits.

Controversy surrounds the small single transmembrane glycoprotein IsK,Cl (minK), which induces slowly activating voltage-dependent K^+ and Cl^- currents in *Xenopus* oocytes. Initially, these proteins were considered to form voltage-gated K^+ channels by themselves based on experiments that demonstrated that mutations in the transmembrane region altered gating and relative ionic permeabilities (7, 8). However, cytoplasmic and extracellular IsK peptides lacking the transmembrane domains have recently been shown to activate slow K^+ and Cl^- currents (9) giving support for the hypothesis that IsK,Cl forms channels by associating with either endogenous factors or preexisting silent channels. The nature of the endogenous pore-forming subunit with which it associates, if any, is unknown, and therefore our discussion of these potential transmembrane auxiliary subunits is limited.

Significantly, the transmembrane topologies of several of these proteins severely restrict the functional role of the cytoplasmic amino acids. For instance, the $\alpha_2\delta$ subunit has only 5 cytoplasmic amino acids (10), two amino acids of which are positively charged suggesting that they may function primarily to stabilize the transmembrane domain. Although the Na^+ channel β_1 subunit has a slightly larger cytoplasmic carboxyl terminus (36 amino acids), truncations of the entire carboxyl terminus do not affect β_1 subunit modulation of inactivation kinetics (11).

In contrast, the cytoplasmic residues of the maxi-K β subunit may be of importance for two reasons. The maxi-K channel is regulated by cyclic AMP-dependent protein kinase A (12), of which a single consensus site for PKA[2] phosphorylation exists on the cytoplasmic face of the β subunit. While a second consensus sequence for PKA phosphorylation is present on the *Drosophila slopoke (slo)* α subunit and mutations in this site abolish PKA regulation in *Xenopus* oocytes (13), these experiments were done in the absence of β subunit coexpression, and it remains uncertain whether the β subunit may further contribute to regulation by PKA. A second reason for examining the cytoplasmic sequences is that coexpression of the maxi-K β subunit significantly alters Ca^{2+} sensitivity of the channel. Although there are no identifiable Ca^{2+} binding motifs on the 28 cytoplasmic amino acids of the β subunit, these regions may interact with and alter Ca^{2+} binding sites on the carboxyl terminus of the α subunit.

Posttranslational Modifications—One common feature of these auxiliary subunits is that they are all highly *N*-glycosylated (Table I). As for many glycosylated proteins, the function of the extensive carbohydrate linkages of these subunits remains largely unknown. *N*-Linked oligosaccharides may allow proper folding and assembly of channel subunits in the endoplasmic reticulum (14). For example, tunicamycin application to rat brain neurons in primary culture inhibited the synthesis of Na^+ channel α and β_2 subunit complexes and the expression of functional channels (15). In addition, deglycosylation of Ca^{2+} channels expressed in *Xenopus* oocytes resulted in a marked reduction in the current stimulation induced by the $\alpha_2\delta$ subunit (10). Perhaps the carbohydrate of these subunits plays an important role in stabilizing the channel complex or in modification of surface charge. To support this, sialic acid residues have been shown to contribute to surface charge of the glycosylated Na^+ channel α subunit (16).

Interestingly, the structures of both Ca^{2+} channel $\alpha_2\delta$ subunits and Na^+ channel β_2 subunits are significantly affected by reducing agents. The Na^+ channel β_2 subunit is the only protein known to form a disulfide linkage to a pore-forming α subunit (17). The α/β_2 association has been shown to occur late in processing and may be a rapid regulatory event in stimulating surface expression of preformed α subunits (15). In contrast, the Ca^{2+} channel $\alpha_2\delta$ subunit

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[1] L. Lobel and H. R. Horvitz, personal communication.

[2] The abbreviation used is: PKA, protein kinase A.

TABLE I
Major transmembrane auxiliary subunits of voltage-dependent ion channels

Protein	Molecular mass	Tissue expression	TM[a]	Sites of *N*-gly[b]	GenBank accession number	Ref.
	kDa					
Ca^{2+} channel						
$\alpha_2\delta$	175,[c] 150	Muscle, heart, brain	1	18	P13806	1
Unc-36α_2 (*C. elegans*)	?[d]	Brain, ?	?	?		Lobel and Horvitz
γ	32	Skeletal muscle	4	2	M32231	4
Na^+ channel						
β_1	36	Skeletal muscle, heart, brain	1	4	P14860	2
β_2	33, 250[c]	Brain	1	4	U37026	3
TipE (*Drosophila*)	50	Brain	2	5	U27561	6
K^+ channel						
maxi-K β	31	Smooth muscle, ?	2	2	L26101	5

[a] Transmembrane domains.
[b] Potential sites of *N*-linked glycosylation.
[c] Non-reduced.
[d] ?, unknown.

forms extensive disulfide linkages within itself, which allow it to retain association of the α_2 and δ components after their proteolytic cleavage. Truncation analysis of the $\alpha_2\delta$ subunit has shown that both α_2 and δ proteins are required for functional activity and that cotranslational formation of the disulfide-linked tertiary structure is necessary for proper $\alpha_2\delta$ structure (10). The Ca^{2+} channel γ subunit also shifts in molecular weight upon addition of reducing agents (4), suggesting the presence of internal disulfide bonds.

Ligand Binding and Modification of Ion Channel Ligand Interactions—Original interest in studying these auxiliary subunits was due to their ability to alter the binding of neurological and cardiovascular drugs to the ion channel pore-forming subunit, although recently gabapentin, a novel anticonvulsant medication, was shown to bind with high affinity directly to the Ca^{2+} channel $\alpha_2\delta$ subunit (18). Gabapentin may control neuronal excitability by modifying Ca^{2+} channel activity or expression, although its application does not appear to alter L-, N-, or T-type voltage-dependent Ca^{2+} channel currents (19). On the other hand, antibodies directed against the $\alpha_2\delta$ subunit block secretion from PC12 cells and electric organ (20–22), suggesting that the $\alpha_2\delta$ subunit may play a distinct role in neurotransmitter release.

Measurements of effects of these auxiliary subunits on binding activity are useful in determining the possible role of these proteins in channel assembly and give an indication of proper folding. Coexpression of the Na^+ channel β_1 subunit along with the rat brain type IIA α in Chinese hamster ovary cells results in a 2–4-fold increase in cell surface expression as measured by both saxitoxin binding and functional Na^+ channel activity (23). The β_1 subunit does not appear to have an effect on toxin binding affinity. Although there are no reports of β_2 subunit contribution to ligand binding, it is clear that disulfide linkage of the β_2 subunit to the α subunit correlates with an increase in Na^+ channels expressed at the surface (24).

The temperature-sensitive paralytic mutant *tipE* flies have a 40–50% reduction in [^{3}H]saxitoxin binding (25), which parallels a 40–50% reduction in Na^+ currents measured from cultured embryonic neurons (26). The *tipE* mutation results in a truncated protein, which likely has residual function (6), such that the null *tipE* phenotype is unknown. Saxitoxin binding may be reduced even further in the total absence of this auxiliary protein.

Characterizing the effects of the Ca^{2+} channel $\alpha_2\delta$ subunit on ligand binding is often complicated by coexpression of the β subunit, which has its own effects on ligand binding. While cotransfection of the Ca^{2+} channel $\alpha_2\delta$ subunit with the α_{1B} subunit in HEK293 cells was shown to have no effect on ω-conotoxin GVIA binding capacity, coexpression with α_{1B} and β_2 subunits resulted in a 2-fold increase in binding capacity (27). Larger effects of the $\alpha_2\delta$ subunit on binding capacity (>8-fold) were seen when subunit mRNA levels were optimized (28). In both studies there was a small but significant decrease in the affinity of the channels for ω-conotoxin GVIA when the $\alpha_2\delta$ subunit was coexpressed. Studies performed with Chinese hamster ovary cells stably expressing the α_{1C}, $\alpha_2\delta$, and β_2 subunits also demonstrated that expression of the $\alpha_2\delta$ subunit increased dihydropyridine binding sites without altering the affinity of the receptor for this ligand (29).

In contrast, transient coexpression of the Ca^{2+} channel $\alpha_2\delta$ subunit in COS.M6 cells resulted in a significant increase in the affinity of the cardiac α_1 subunit for dihydropyridines, suggesting that the $\alpha_2\delta$ subunit causes a conformational change in the structure of the α_{1C} subunit (30). In addition to being required for high affinity dihydropyridine binding, the $\alpha_2\delta$ subunit was also shown to be essential for reconstitution of allosteric regulation by Ca^{2+}, diltiazem, and (−)-D600 (30). Involvement in the allosteric regulation by Ca^{2+} ions is particularly interesting, as it suggests that either the $\alpha_2\delta$ subunit modifies Ca^{2+} binding sites on the α_1 subunit or binds Ca^{2+} ions itself possibly through sialic acid residues or extracellular negatively charged amino acids.

Recent studies of the *C. elegans* homologue of the Ca^{2+} channel α_2 subunit resulted in the discovery that the *unc-36* mutant phenotype also consists of a hypersensitivity to the dihydropyridine channel blockers.[1] This gives further evidence that the $\alpha_2\delta$ subunit can significantly modulate ligand binding either by directly contributing to the dihydropyridine binding site or by altering the drug binding site on the α_1 subunit.

The Ca^{2+} channel γ subunit has very minor effects on ligand binding. Small effects of this subunit were seen on [^{3}H]PN-200–110 binding affinity and on allosteric regulation by diltiazem and (−)-D600, although the effects of the β subunit and $\alpha_2\delta$ subunit predominate (30).

In the presence of cross-linking reagents, the maxi-K β subunit becomes covalently attached to charybdotoxin, suggesting that the β subunit may directly contribute to the formation of the receptor binding site on the α subunit (31). In addition, the β subunit is required for activation of the maxi-K channel by the agonist dehydrosoyasaponin I (32). Channels composed of only the α subunit are insensitive to this agent, while the open probability of native smooth muscle maxi-K channels increases by more than 50-fold in its presence (33).

Unique Structures—A well characterized motif identified within the Na^+ channel β_2 subunit amino acid sequence may be the most informative with regard to the potential function of these proteins *in vivo*. Sequence analysis of the β_2 subunit identified an extracellular region containing an immunoglobulin-like fold that has similarity to the neural cell adhesion molecule contactin (3). As contactin/F3 binds to the extracellular matrix protein tenascin, it was suggested that the immunoglobulin fold of the Na^+ channel β_2 subunits may likewise be involved in extracellular matrix interactions that result in the immobilization of these channels to nodes of Ranvier, axon hillocks, and other sites of high concentration. This finding complements the ability of the Na^+ channel α subunit to bind ankyrin (34) and gives evidence for both an intra- and extracellular network for localization. A similar V-type immunoglobulin fold was identified in the Na^+ channel β_1 subunit.

Highly negatively charged extracellular regions are found in both the Ca^{2+} channel $\alpha_2\delta$ subunit and the *Drosophila* TipE protein. Similar clusters of acidic amino acids were described in the extracellular region of the parathyroid Ca^{2+}-sensing receptor, where they were suggested to contribute to low affinity Ca^{2+} ion binding (35). Perhaps the negatively charged regions of the $\alpha_2\delta$ subunit are involved in the apparent allosteric regulation of dihy-

TABLE II
Functional effects/disease correlates of voltage-dependent ion channel transmembrane subunits

Protein	Functional expression	Modification of ion channel drug binding		Unique attributes	Human chromosomal localization (Ref.)	Disease involvement
		K_D	B_{max}			
Ca^{2+} channel						
$\alpha_2\delta$	↑	↓	—[a](DHP)[b]	Target of gabapentin	7q (41)	Malignant hyperthermia ?[c]
		↑	↑ (ω-cgtx)[d]			
Unc-36α_2 (*C. elegans*)	?	?	?	DHP hypersensitivity in mutants	NA[e]	Uncoordinated, slow pharyngeal pumping
γ	No change	—	—	?	17q23 (49)	?
Na^+ channel						
β_1	↑	—	↑ (STX)[f]	?	19 (50)	?
β_2	↑	—	↑ (STX)	↑ Membrane surface area	?	?
TipE (*Drosophila*)	↑	—	↑ (STX)	Required for *para* expression	NA	Temperature-sensitive paralysis
K^+ channel						
maxi-K β	No change	—	—	↑ Ca^{2+} sensitivity	5q34 (51, 52)	?

[a] —, no effect.
[b] DHP, dihydropyridine.
[c] ?, unknown.
[d] ω-Conotoxin GVIA.
[e] NA, not applicable.
[f] STX, saxitoxin.

dropyridine binding by Ca^{2+} as discussed above or contribute to the accumulation of Ca^{2+} ions near the pore. The *Drosophila* TipE protein is also highly negatively charged, although the significance of this is unknown (6).

Functional Significance

Enhancement of Current Amplitude—All of these auxiliary subunits, with the exception of the Ca^{2+} channel γ subunit and the maxi-K β subunit, significantly increase functional expression of ionic currents (Table II). Coexpression of either Ca^{2+} channel $\alpha_2\delta$ subunit or Na^+ channel β_1 and β_2 subunits in *Xenopus* oocytes or mammalian cells frequently results in at least a 2-fold increase in current amplitude over expression of the α subunit alone. The largest enhancement of Na^+ currents results when both β_1 and β_2 are coexpressed with the α subunit (3). This is similar to the Ca^{2+} channel, where coexpression of both β and $\alpha_2\delta$ subunits are required for maximal functional expression.

Interestingly, expression of the *Drosophila* Na^+ channel α subunit encoded by the paralytic (*para*) locus in *Xenopus* oocytes results in no measurable current in the absence of the TipE protein (36). Thus, when TipE was cloned (6) it was suggested to function as a chaperone-like protein, which may aid in the correct folding and surface expression of the para α subunit. Therefore, TipE may primarily be involved in Na^+ channel synthesis and would not be a true auxiliary subunit as it might not be a stable component of the channel at the cell surface. In fact, genetic analysis has suggested that this protein only needs to be expressed transiently in the *Drosophila* during pupal development in order to rescue the adult paralysis (6). One advantage of the genetic approach of identifying auxiliary ion channel subunits is that proteins will be discovered that are involved in other stages along the entire pathway necessary for ion channel formation.

The mechanism of action of these auxiliary subunits on enhancement of current amplitude is either through increased surface expression or increased single channel activity or conductance. Toxin binding experiments, single channel recordings, measurements of gating charge movement, and biochemical estimates of protein expression have all been used to address the role of these subunits in channel expression.

Saxitoxin binding studies support the role of the Na^+ channel β_1 subunit in mediating a 2–4-fold increase in surface expression (23), and disulfide linkage of the β_2 subunit to the α subunit has been correlated with an increase in channels at the cell surface (15). Thus, it appears that both Na^+ channel auxiliary subunits may play an important role in regulating trafficking of Na^+ channels to the cell surface. Few studies have yet investigated the role of the Na^+ channel β subunits at the single channel level.

Data in support of a role for the Ca^{2+} channel $\alpha_2\delta$ subunit in regulating channel surface expression have come from several experiments, including a biochemical analysis of the amount of α_{1C} protein expressed at the plasma membrane of *Xenopus* oocytes. When oocytes were coinjected with RNA encoding the $\alpha_2\delta$ subunit, the amount of ^{35}S-labeled α_{1C} protein located at the plasma membrane was tripled (37). Subsequent studies demonstrated that coexpression of $\alpha_2\delta$ with the α_{1C} and β_2 subunits in HEK 293 cells approximately doubles the maximal amount of gating charge moved, suggesting that the $\alpha_2\delta$ subunit increases the number of functional L-type Ca^{2+} channels at the cell surface (38). In addition, coexpression of the Ca^{2+} channel $\alpha_2\delta$ subunit has been shown to increase the number of ω-conotoxin GVIA binding sites at the surface of transiently transfected HEK 293 cells (28), also suggesting a role for the $\alpha_2\delta$ subunit in membrane targeting or channel stabilization. When comparisons were made, however, between the increase in current enhancement and the increase in binding capacity in these cells, it was noted that the increase in current stimulation was much greater than could be accounted for by the change in B_{max}. This suggested that the $\alpha_2\delta$ subunit may also affect Ca^{2+} channel function by changing microscopic channel properties such as the open channel probability or the single channel conductance. Indeed, coexpression of the $\alpha_2\delta$ subunit has been shown to result in an increase in the single channel open probability of the α_{1C} subunit expressed in *Xenopus* oocytes (37).

Unique Functional Effects—The maxi-K β subunit contributes significantly to the Ca^{2+} dependence of channel activity. While early studies demonstrated a role for the carboxyl terminus tail domain of the maxi-K α subunit in determining Ca^{2+} sensitivity (39), coexpression of the β subunit was shown to result in K^+ currents that were more sensitive to both Ca^{2+} and voltage. In fact, coexpression of the β subunit with the maxi-K channel in *Xenopus* results in an approximately 10-fold increase in Ca^{2+} sensitivity (32, 40), which approaches the sensitivity for native maxi-K channels from brain and smooth muscle.

Capacitance measurements as well as electron microscopy were used to demonstrate the expansive effect of the Na^+ channel β_2 subunit on the cell surface membrane (3). Concurrent with the increase in plasma membrane surface area, intracellular membrane vesicles appeared to be reduced, suggesting a possible role for the β_2 subunit in fusion of intracellular vesicles to the surface membrane. Perhaps there is a correlation between the extracellular adhesion-like character of the Na^+ channel β_2 subunit and its ability to augment the cell surface membrane of *Xenopus* oocytes. This functional effect on cell capacitance has not been reported for any other ion channel auxiliary subunit.

Disease Correlates—Defects in ion channel pore-forming subunits have recently been implicated in several human genetic disorders including long QT syndrome, hypo- and hyperkalemic periodic paralysis/paramyotonia congenita, polycystic kidney disease type II (Ca^{2+}/Na^+ channel like molecule), myotonia congenita, episodic ataxia/myokymia syndrome, and many others. As auxiliary subunits significantly alter the function of the ion channel α subunits, it is likely that many of these and other neurological and

cardiovascular disorders may be shown to result from mutations in these genes.

There have been few direct implications of these transmembrane auxiliary ion channel proteins in human genetic disease. The Ca^{2+} channel $\alpha_2\delta$ subunit gene has recently been linked to the inheritance of malignant hyperthermia in one European family (41), although mutations within this gene have not yet been identified. The skeletal muscle ryanodine receptor is linked to approximately half of malignant hyperthermia-susceptible European families. How mutations in either the ryanodine receptor or Ca^{2+} channel $\alpha_2\delta$ subunit might result in the same disease remains unknown, although both Ca^{2+} channels are involved in excitation-contraction coupling and have been shown by electron microscopy to be in close approximation to each other in skeletal muscle (42). Perhaps mutations in the $\alpha_2\delta$ subunit could influence the behavior of the α_1 subunit as a voltage sensor for excitation-contraction coupling.

Autoimmune anti-Ca^{2+} channel antibodies directed against extracellular epitopes may play a role in the pathogenesis of Eaton-Lambert myasthenia syndrome (43) and amyotrophic lateral sclerosis (44). While any Ca^{2+} channel subunit or associated protein may be the inciting antigen in these diseases, the presence and extracellular presentation of the $\alpha_2\delta$ subunit in small cell carcinoma cells (45) may render autoantibodies directed against it physiologically significant as a basis for the alteration in channel activity and neuronal function.

New genetic approaches of studying neurologic mutants in *Drosophila* and *C. elegans* are beginning to shed light on possible human diseases due to mutations in the transmembrane auxiliary subunits. The discovery of homologous ion channel auxiliary subunits in *C. elegans*, including the Ca^{2+} channel α_2 subunit specified by the *unc-36* locus,[1] indicates an important requirement for these proteins throughout over a billion years of evolution. Furthermore, the fact that loss of function mutations in the α_2 *unc-36* auxiliary subunit result in a phenotype that is almost identical to that resulting from mutations in an α_1 subunit (unc-2) (46) suggests that the auxiliary subunits are as intrinsic to ion channel function as the pore-forming subunits themselves.

In the absence of human models, gene-targeted knock-out mice will be an invaluable aid in understanding the importance of these ion channel auxiliary subunits. Thus far, there has been only one gene-targeted knock-out of a voltage-dependent ion channel subunit, in addition to the naturally occurring dysgenic mouse. Ablation of the skeletal muscle Ca^{2+} channel β subunit (47) resulted in a phenotype similar to the dysgenic mouse, which lacks the skeletal muscle α_1 subunit (48). These mice die shortly after birth of respiratory paralysis due to a lack of functional diaphragmatic skeletal muscle. Investigators in this field look forward to further characterization of these proteins, which will arise from examination of these proteins in non-mammalian systems and knock-out animals.

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