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Targeting Mechanisms of High Voltage-Activated Ca^{2+} Channels

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Functional voltage-dependent Ca^{2+} channel complexes are assembled by three to four subunits: α_1, β, $\alpha_2\delta$ subunits (C. Leveque *et al.*, 1994, *J. Biol Chem.* **269**, 6306–6312; M. W. McEnery *et al.*, 1991, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 11095–11099) and at least in muscle cells also γ subunits (B. M. Curtis and W. A. Catterall, 1984, *Biochemistry* **23**, 2113–2118). Ca^{2+} channels mediate the voltage-dependent Ca^{2+} influx in subcellular compartments, triggering such diverse processes as neurotransmitter release, dendritic action potentials, excitation–contraction, and excitation–transcription coupling. The targeting of biophysically defined Ca^{2+} channel complexes to the correct subcellular structures is, thus, critical to proper cell and physiological functioning. Despite their importance, surprisingly little is known about the targeting mechanisms by which Ca^{2+} channel complexes are trausported to their site of function. Here we summarize what we know about the targeting of Ca^{2+} channel complexes through the cell to the plasma membrane and subcellular structures.

KEY WORDS: Ca^{2+} channels; channel targeting; α_1 and β subunits; heterologous expression systems; striated muscle; hippocampal neurons.

INTRODUCTION

Voltage-dependent Ca^{2+} channels are composed of a pore-forming α_1 subunit and the ancillary β and $\alpha_2\delta$ subunits. Recently γ subunits homologous to those in skeletal muscle were identified in brain, although it is not yet clear whether they assemble with Ca^{2+} channel complexes. The α_1 subunit is structurally organized into four domains, each containing six transmembrane regions with the voltage sensor and a hairpin structure, which most likely forms the pore of the channel (Catterall, 1998). The domains are connected to each other by peptide loops, which are targets for intracellular protein–protein interactions and therefore for channel modulation, sorting, and clustering. Presently, five types of voltage-dependent Ca^{2+} channels have been shown to be expressed in the central nervous system (CNS), heart, and skeletal muscle, L-, N-, P/Q-, R-, and T- type (Ca_v 1-3), each characterized with distinct pharmacological and electrophysiological properties and subcellular distribution.

The genome projects and molecular cloning have identified more than 20 putative genes which may encode functional Ca^{2+} channel subunits and assemble to the different Ca^{2+} channel types (Catterall, 1998; Jones, 1998). Heterologous expression of some of these genes in *Xenopus* oocytes or HEK293 cells indicates that $\alpha_1$1.1 (formerly α_{1S}), $\alpha_1$1.2 (formerly α_{1C}) and $\alpha_1$1.3 (formerly α_{1D}) encode the L-type channels, $\alpha_1$2.1 (formerly α_{1A}) encodes the ω-agatoxin-sensitive P/Q-type channel, $\alpha_1$2.2 (formerly α_{1B}) eucodes the ω-conotoxin GVIA-sensitive N-type channel, and $\alpha_1$2.3 (formerly α_{1E}) encodes the R-type channel, while $\alpha_1$3.1-3 (formerly α_{1G-I}) encode the low-threshold T-type Ca^{2+} channels. In addition to the α_1 subunits, several β subunits have been identified. β subunits modify the gating properties of high voltage-activated Ca^{2+} channels probably through their direct interaction with the pore forming α_1 subunit (Birnbaumer *et al.*, 1998). In general, it seems that β subunits lead to a

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higher open probability of the channel at lower potentials and sharpen the Ca^{2+} signal by varying the response window of the channel complex to a voltage change. Thus, the ancillary β subunits confer subunit-specific properties to each of the high voltage-activated α_1 subunits, creating channel complexes with defined biophysical properties, which may differ significantly within one subgroup of high voltage-activated Ca^{2+} channels. Minute changes in the biophysical properties of a channel can cause severe malfunctions within an animal like migraine or epilepsy (Hans *et al.*, 1999; Jen, 1999; Kraus *et al.*, 1998, 2000). The density of the Ca^{2+} channel complexes, their precise localization, and their interaction with modulatory proteins will also critically affect the function of neuronal circuits. Thus, the precise targeting of a biophysically defined Ca^{2+} channel complex to a subcellular structure like the presynaptic terminal or the triad in skeletal and heart muscles is crucial for the specific function of a neuron or muscle cell. Understanding the mechanisms of sorting, assembly, and regulation of Ca^{2+} channel complexes in subcellular domains is therefore essential for understanding higher order processes such as neuronal networks, heart beat, or skeletal muscle contraction.

A major advantage of studying the targeting of ion channels is that the precise localization of channel complexes can simultaneously be analyzed with fluorescent tags and markers in biochemical and cellular assays in combination with measuring their underlying currents and physiological responses in a native environment (e.g., EC-coupling or synaptic transmission). Thus, targeting events can be visualized, measured, and quantified.

In this review we will summarize the results for targeting of $\alpha_1\beta$, $\alpha_2\delta$ and γ subunits in several cell types and how these subunits affect the targeting of the high voltage-activated Ca^{2+} channel complex. We will first discuss the results obtained in heterologous expression systems and will compare them to what we learned about their targeting in their physiological environment, e.g., striated muscle and neuronal cells.

FORMING CA^{2+} CHANNEL COMPLEXES IN HETEROLOGOUS EXPRESSION SYSTEMS: THE IMPORTANCE OF CA^{2+} CHANNEL β SUBUNITS

Early studies of recombinant Ca^{2+} channel complexes expressed in heterologous expression systems revealed that the whole cell current amplitude as well as the number of drug-binding sites measured in *Xenopus* oocytes or HEK293 cells is drastically increased, when pore-forming Ca^{2+} channel α_1 subunits are coexpressed with the ancillary Ca^{2+} channel β subunits. These early results suggested that α_1 and/or β subunits are critical for efficient transport of the Ca^{2+} channel complex. Several groups addressed the question of how Ca^{2+} channel complexes are directed to the cell membrane using heterologous expression systems and recombinant channel subunits.

Targeting of L-type Channels and P/Q-Type Channels in Heterologous Expression Systems

The group of Marlene M. Hosey and Annette C. Dolphin analyzed the transport and localization of L-type and P/Q-type channels in heterologous expression systems. M. M. Hosey concentrated first on the effect of β_{2a} subunits on the localization of L-type channel subunit $\alpha_1$1.2 (Chien *et al.*, 1995). Using cell fractionation they first described that β_{2a} subunits when transiently expressed in HEK293 cells were detected in the membrane and not the cytosolic fraction of the cell lysate. Using antibodies against β_{2a}, the immunofluorescence of β_{2a} in HEK293 cells confirmed the membrane localization, since strong plasma membrane staining and diffuse cytosolic staining were detected by confocal microscopy. In contrast, $\alpha_1$1.2 subunits expressed alone in HEK293 cells revealed a predominantly intracellular and perinuclear staining. However, coexpression of β_{2a} caused plasma membrane staining of $\alpha_1$1.2. Comparison of the protein levels indicated that β_{2a} did not increase the overall protein concentration of $\alpha_1$1.2 within the cell but rather transported more protein to the plasma membrane. This became also obvious in DHP binding studies. Binding of DHP to the $\alpha_1$1.2 subunit could only be quantified in the presence of β_{2a} subunits but not when expressed alone. Despite the detection of $\alpha_1$1.2 protein very little DHP binding was detected. These results implied that β subunits also induce maturation of DHP binding sites on the L-type channel subunit $\alpha_1$1.2. Pulse chase studies determined that the half life of the $\alpha_1$1.2 subunit was not altered in the presence of β_{2a} subunits for whole cell extracts (half life of $\alpha_1$1.2 approximately 3 h). In a further experiment the group showed that β subunits also increased L-type currents in HEK293 cells in a time-dependent manner. Currents were approximately 2–4-fold larger in α_1/β cotransfected cells than α_1 alone, reaching a maximum after 40–50 h and then decaying slowly. Thus, these results indicate that the Ca^{2+} channel ancillary subunit β_{2a} target the pore forming $\alpha_1$1.2 subunit to the plasma membrane and is probably involved in the maturation process of the L-type channel complex.

The transport of β_{2a} subunits to the plasma membrane is independent of the α_1 subunit and was studied in more detail. The β_{2a} subunit is the only protein among

the β subunits which is palmitoylated raising the question of whether palmitoylation is responsible and sufficient for membrane localization. Palmitoylation occurs at two cysteine residues in the N-terminus of β_{2a} (Cys3 and 4) and was reduced by mutations in the α_1 subunit interaction domain (BID) and the SH3 domain from β_{2a} (Fig. 1). Substitution of Cys3 and Cys4 in the N-terminus of β_{2a} for Ser abolished palmitoylation. Both proteins, wild type and mutated β_{2a} subunits, were identified in the membrane fraction by immunoprecipitation. However, mutated β_{2a} subunits revealed a dramatic decrease of whole cell current when coexpressed with $\alpha_1$1.2, with no decrease in the size of charge movement, indicating that the same amount of $\alpha_1$1.2 subunits were in the plasma membrane (Chien *et al.*, 1996). The subcellular localization of nonpalmitoylated β subunits revealed that palmitoylation deficient β_{2a} subunits (rat) as well as the nonpalmitoylated β_{1b} and β_3 (both rat) and β_{2a} and β_{2b} (rabbit) subunits exhibited a diffuse intracellular staining pattern. All nonpalmitoylated β subunits were fractionated with the membrane but not with the cytosolic fraction suggesting that these β subunits are associated with intracellular membrane systems. Exchange of the N-terminus of β_{1b} and β_3 against the N-terminus of β_{2a} transferred palmitoylation to β_{1b} and β_3. However, exchange of the N-terminus was not sufficient for plasma membrane localization indicating that palmitoylation is insufficient for membrane targeting (Chien *et al.*, 1998). Thus, palmitoylation of β_{2a} is pivotal for plasma membrane localization of this particular subunit, but other structural parts of the protein must be involved for plasma membrane targeting.

In a further study the group analyzed the effect of other β subunits on the distribution of $\alpha_1$1.2 and which domain of the β subunit mediates the targeting of the α_1 subunits. Gao *et al.* (1999) showed that a redistribution of $\alpha_1$1.2 containing channels was accomplished by all β subunits (β_{1-4}). Expression of $\alpha_1$1.2 and any of the β subunits (in the absence of $\alpha_2\delta$) resulted in membrane staining and punctate channel clusters. Interestingly, the conserved core region of the β subunit was sufficient for membrane targeting and clustering of $\alpha_1$1.2 subunits in tsA201 cells (HEK cell line). Mutations within the interaction domain of β for α_1 subunits revealed perinuclear staining of the α_1 subunit as a result of the disrupted interaction between α_1 and β as shown in immunoprecipitation studies. Mutations in the second domain within the SH3-motif abolished the interaction between $\alpha_1$1.2 and β subunits. These mutations, as expected, resulted in loss of membrane targeting of $\alpha_1$1.2 subunits.

Following the characterization of the β subunit protein domains necessary for targeting Gao *et al.* (2000b) examined the influence of the C-terminus of $\alpha_1$1.2 subunits on L-type channel localization. C-terminal deletion mutants were introduced into the full length $\alpha_1$1.2 subunit and analyzed with immunocytochemical methods to understand the subcellular distribution of the deletion mutants in HEK293 cells in the presence of cotransfected β subunits. Subsequent truncation of the C-terminus reduced membrane staining. Two mutants were detected which completely abolished membrane staining when coexpressed with β subunits, i.e., $\alpha_1$1.2-Δ1623 and $\alpha_1$1.2-Δ1623-1673, indicating that this region contains a critical protein domain for channel targeting (note: the C-terminus of $\alpha_1$1.2 is 664-amino-acids long. It starts at amino acids position 1507 and ends at 2171.). A smaller deletion within this region ($\alpha_1$1.2-Δ1623-1666) reduced membrane staining by 90%. None of these mutant channels expressed functional Ca^{2+} channels. Interestingly, this site overlaps with the calmodulin binding domain suggesting that calmodulin binding is involved in trafficking. However, several studies showed that calmodulin binding deficient L-type channels express Ca^{2+} currents (Peterson *et al.*, 1999; Qin *et al.*, 1999; Zuhlke *et al.*, 1999; Zuhlke and Reuter, 1998). Additional sites were detected, which reduced membrane staining, but increased intracellular accumulation. These sites are located at the more C-terminal end of the $\alpha_1$1.2 subunits. Interestingly, the intracellular accumulated mutants did not show differences in PN200 binding and exhibited Ba^{2+} currents indicating that these mutant channels were functional. The authors suggested that the increase in intracellular staining might be due to an alteration of the constitutive recycling of the channel complex between the intracellular compartments and the plasma membrane. Thus, the C-terminus of the L-type channel subunit $\alpha_1$1.2 is involved in targeting of the channel complex to the membrane.

Studies in COS-7 cells by A.C. Dolphin's group revealed that β subunits target P/Q-type channels to the plasma membrane (Bogdanov *et al.*, 2000). Using depolarization sensitive $\alpha_1$2.1 antibodies directed against an exofacial epitope close to the extracellular mouth of the pore to detect membrane staining the authors found that coexpression of β and $\alpha_1$2.1 but not expression of $\alpha_1$2.1 alone resulted in localization of the channel complex at the cell membrane. While expression of β_3 and β_4 subunits alone did not reveal membrane staining, β_{1b} and β_{2a} subunits reached the plasma membrane by itself. The subcellular localization of β_{1b} in COS-7 cells differed from the subcellular localization in tsA201 cells, where β_{1b} was localized intracellulary and not at the membrane. The authors suggested that cell-type-specific proteins may change the targeting mechanisms of the β_{1b} subunit. By engineering chimeras between the β_{1b} (membrane attached) and β_3 (not at the membrane) subunits the group of A. Dolphin

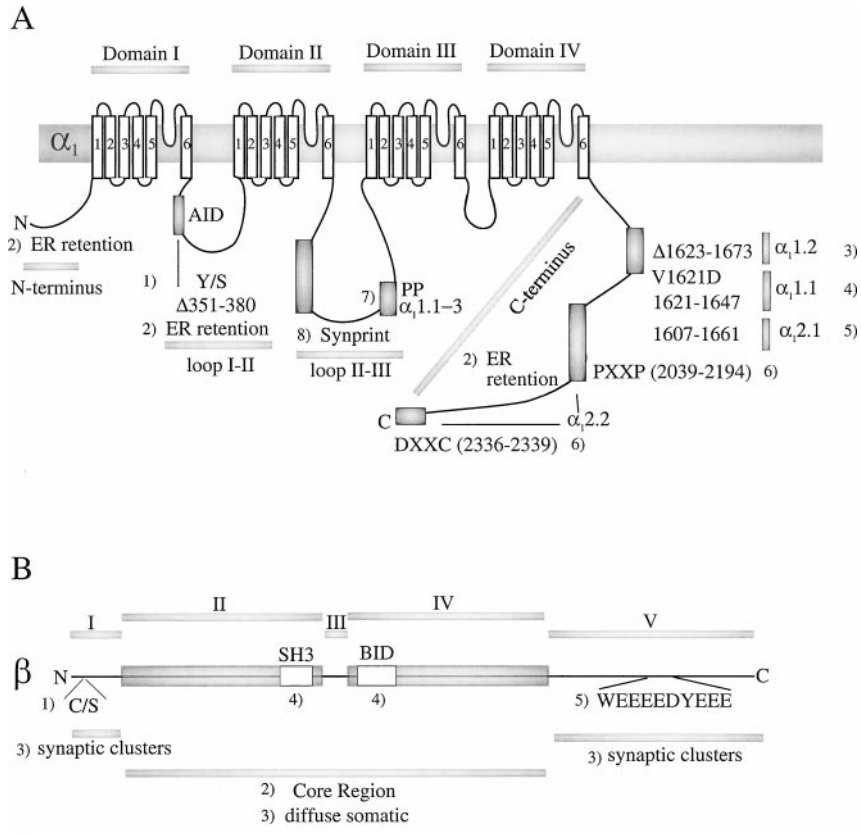

Fig. 1. Schematic representation of Ca^{2+} channel α_1 and β domains involved in targeting of the Ca^{2+} channel complex. A) Schematic representation of the α_1 subunit and the domains involved in targeting. The Ca^{2+} channels α_1 subunits consists of four domains, which are connected via intracellular peptide loops. (1) The interaction between $\alpha_1$1.2 and β subunits is necessary for membrane transport of $\alpha_1$1.2 subunits. The point mutation Tyr/Ser (Y/S) in the intracellular loop I-II of $\alpha_1$1.2, which disrupts the interaction between α_1 and β subunits (Pragnell *et al.*, 1994), abolishes membrane staining of α_1/β transfected cells (Gerster *et al.*, 1999). In addition, the Y/S mutation and deletion of the β interaction domain of the α_1 subunit (AID) (Δ351–380) in the skeletal muscle type $\alpha_1$1.1 subunit abolish the clustering of β_{1a}-GFP subunits in dysgenic myotubes and redistribution of β subunits in HEK293 cells (Neuhuber *et al.*, 1998a,b). (2) The $\alpha_1$2.1-3 and $\alpha_1$1.2 subunits contain an ER reteution signal within the loop I-II, which is shielded by the β subunit for complex formation and plasma membrane transport (Bichet *et al.*, 2000; Cormet *et al.*, 2002). In addition, ER retension was observed for the N-terminus and C-terminus of $\alpha_1$2.1 (Cornet *et al.*, 2002). (3) The C-terminal region in the $\alpha_1$1.2 subunit (Δ1623–1673) completely abolishes membrane staining of α_1 subunit in the presence of β subunits. This region overlaps, with the Ca^{2+} calmodulin binding region of L-type channels (Gao *et al.*, 2000b). (4+5) A 27-amino-acid-long sequence (1621–1647) in the C-terminus of $\alpha_1$1.1, which contains a consensus PDZ binding motif, binds to proteins of the skeletal muscle and abolishes localization to the triads in dysgenic myotubes once mutated (Proenza *et al.*, 2000). A similar region of 55 amino acids length (1607–1661) was identified by Flucher *et al.* (2000). (6) Two regions within the N-type channel $\alpha_1$2.2 C-terminus are involved in the interaction with the adaptor proteins CASK and Mint-1. CASK interaction involves a proline-rich sequence (PXXP; amino acids 2039–2194). Mint interaction involves the last amino acids of the C-terminus (DXXC-COOH; 2336-2339)(Maximov *et al.*, 1999). Truncation of the last 300 amino acids of the C-terminus of $\alpha_1$2.2a and mutations in the CASK and Mint1 interaction domains diminish or abolish synaptic clustering of N-type channels. (7) A poly-proline motif within the C-terminal half of the loop II-III of the $\alpha_1$1.2 subunit is involved AKAP79 mediated trafficking of L-type channels (Altier *et al.*, 2002). (8) The deletion of the synprint peptide of $\alpha_1$2.1 subunit results in loss of presynaptic targeting of heterologously expressed P/Q-type channels in superior cervical ganglion neurons (Mochida *et al.*, 2003a,b). B) Schematic representation of the β subunit and the domains involved in targeting. The β subunit consists of 5 domains according to their sequence homology. Domain I, III and V are low homology domains, while domain II and IV are highly conserved among the members of the β subunit family (Birnbaumer *et al.*, 1998; Chien *et al.*, 1998). (1) Palmitoylation of β_{2a} subunits occurs at Cys3 and 4 and is effected by mutation within the α_1 interaction domain of β subunit (BID) and the SH3 domain from β_{2a}. Substitution of Cys3 and Cys4 by Ser abolished palmitoylation (Chien *et al.*, 1998). (2) The core region of β subunits is sufficient for membrane targeting and clustering of $\alpha_1$1.2 in tsA201 cells (Gao *et al.*, 1999) and is sufficient to assemble with $\alpha_1$2.1 and $\alpha_2\delta$ to functional channels (De Waard *et al.*, 1994; Wittemann *et al.*, 2000). However, this domain is not sufficient for synaptic transport and clustering of the GFP-tagged construct in hippocampal neurons (Wittemann *et al.*, 2000). (3) The nonconserved N- and C-termini distribute in synaptic clusters in cultured hippocampal neurons similar to wild type GFP-β_4 subunits alone, while the conserved core region of β_4 reveals a diffuse somatic staining in these neurons (Wittemann *et al.*, 2000). (4) Mutations in the SH3 domain and BID domain, which diminish or abolish the interaction between α_1 and β subunits result in loss of membrane targeting of α_1 subunits (Gao *et al.*, 1999). (5) A highly acidic motif (WEEEEDYEEE) in the C-terminus of β_{1b} is responsible for membrane association of this subunit in COS-7 cells (Bogdanov *et al.*, 2000).

(Bogdanov *et al.*, 2000) characterized an 11 amino acid motif responsible for membrane association. This highly acidic motif (WEEEEDYEEE) is localized in the nonconserved C-terminal region of the β_{1b} subunit. The authors suggested that this sequence motif may be important for clustering and specific localization of β_{1b} assembled channels.

The results demonstrate that $\alpha_1$1.2 containing L-type channels and $\alpha_1$2.1 containing P/Q-type channels need ancillary Ca^{2+} channel β subunits to reach the plasma membrane in heterologous expression systems. All 4 β subunits are capable of targeting α_1 subunits. Targeting involves several domains including the binding domain of the β subunit to the α_1 subunit and the C-terminus of α_1. β_{1b} and β_{2a} subunits when expressed alone contain subunit specific domains (palmitoylation domain and acidic sequence motif), which allow these β subunits to reach the plasma membrane by itself. Palmitoylation and depalmitoylation might be a mechanism for β subunit recruitment to the plasma membrane and therefore for modulation of Ca^{2+} channel function. Membrane association of β subunits in general might be a mechanism for modulating channel complex stability via anchoring Ca^{2+} channel complexes into subcellular structures.

Correlation of Targeting and Modulation of Ca^{2+} Channel β Subunits

Several studies addressed the question of whether Ca^{2+} channel targeting mediated by the β subunits is necessary for its modulatory activity or if these two functions of the β subunit are independent from each other. Yamaguchi *et al.* (1998) showed that β subunits participate in membrane trafficking and modulation of L-type channel complexes. Preincubated $\alpha_1$1.2 subunit expressing *Xenopus* oocytes were injected with purified his-tagged β_3 subunits (final concentration in oocytes was 300 nM) and whole cell currents were measured 1–4 h after injection. The injection of β_3 subunits caused a fast, β_3 specific change in the voltage dependence of activation and the kinetics of the L-type channel current (1–2 h). The biophysical effect was followed by an increase in whole cell membrane current (2–4 h). By using a HA-tagged $\alpha_1$1.2 subunit and subsequent immunofluorescence detection, the group correlated the increase in whole cell current with an increase in the amount of $\alpha_1$1.2 subunits at the plasma membrane. Bafilomycin A_1, an inhibitor of intracellular glycoprotein transport, abolished the increase of membranous $\alpha_1$1.2 subunits after β_3 injection. (It has to be mentioned that Taurelius *et al.* (1997) reported the expression of an endogenous β subunit in *Xenopus* oocytes, which is highly homologous to the mammalian β_3 subunit.) The data were supported by Gerster *et al.* (1999). They first showed that $\alpha_1$1.2 subunits distribute to almost 80% in the endoplasmic reticulum (ER), while 15% were detected in the plasma membrane. The 15% membrane localization could probably be due to the transport of α_1 in the presence of endogenous β in HEK293 cells or spill over of the overexpression system. In the presence of β_{1a}, β_{2a}, and β_3 subunits plasma membrane localization was drastically increased from 50 to 80% depending on the β subunit. To demonstrate that the targeting effects were mediated by the direct interaction between α_1 and β subunits a point mutation was introduced into the $\alpha_1$1.2 subunit. The exchange of Tyr against Ser within the intracellular loop I-II has been described to disrupt the interaction between α_1 and β subunits (Neuhuber *et al.*, 1998a,b; Pragnell *et al.*, 1994). No increase in membrane staining was observed for this mutant, leaving 90% of the channel within the ER. An interesting observation was also that ER localization could not be observed for β subunits in the absence of α_1 subunits. However, in the presence of α_1 ER localization was approximately 20–30% for β_{1a} and β_3. β_{2a} always localized to the plasma membrane and localization was independent of the α_1 subunit. Gerster *et al.* (1999) further analyzed the biophysical properties of mutant and wild type $\alpha_1$1.2 channels in the presence and absence of β subunits. Both channel types showed no differences in the activation and inactivation times when expressed with or without β subunits, indicating that $\alpha_1$1.2 channels are intracellulary modulated by the β subunits. Coexpression of $\alpha_1$1.2 or mutated $\alpha_1$1.2 with β subunits increased the current amplitude several fold. In addition, no differences were observed on the single-channel level, when the mean open time and open probability were analyzed between β_{1a} assembled wild type and mutated $\alpha_1$1.2 channels. Interestingly, both parameters were increased in the presence of β_{1a} subunits.

Thus, these studies indicate that β subunits target $\alpha_1$1.2 subunits through the ER to the plasma membrane leading to an increase in the amount of functional channels. In addition, on the cytosolic surface of the cell membrane β subunits modulate the biophysical properties of the channel. Modulation of the biophysical properties by low-affinity binding of β subunits and targeting via a high-affinity interaction with the α_1 subunit has been suggested by several groups (Gao *et al.*, 1999; Neuhuber *et al.*, 1998a,b; Tareilus *et al.*, 1997). In fact, injection of various concentrations of β_3 subunits into *Xenopus* oocytes expressing $\alpha_1$2.2 subunits indicated different concentration dependencies of channel expression and channel modulation (Canti *et al.*, 2001). Analysis of changes in the voltage-dependent inactivation and noninactivating

currents for $\alpha_1$2.1 subunits expressed with β_{1b} and β_{2a} subunits suggested that β subunits are able to unbind from the α_1 subunit (Restituito *et al.*, 2001). More recently, Garcia *et al.* (2002) applied purified β_{1a} subunits to endogenous $\alpha_1$1.1 channels of spherical vesicles derived from skeletal muscle plasma membranes via the patch pipette. Diffusion of the β_{1a} protein to the cytoplasmic surface of the $\alpha_1$1.1 subunit increased the peak current and the slow component of the tail current without altering the activation and inactivation kinetics and current–voltage relation. In addition, no change in the gating currents were observed suggesting that β subunit action occurs without increasing the amount of channels in the plasma membrane. Since more than one β subunit binding site has been described on the Ca^{2+} channel α_1 subunit further studies will show whether β subunits have to permanently interact with α_1 to modulate the channel.

β Subunits Guide α_1 Subunits Through the ER in Heterologous Expression Systems

How do β subunits target α_1 subunits to the cell membrane? Ca^{2+} channel β subunits bind to several intracellular regions of the α_1 subunit. The binding region comprises the C-terminus of the α_1, the N-terminus and in particular the intracellular loop I-II. As we discussed earlier a mutation within the interaction domains between α_1 and β subunits interferes with plasma membrane localization, indicating that α_1/β interaction is necessary for channel targeting. By studying the trafficking of the intracellular loopI-II through the cell Bichet *et al.* (2000) found that this intracellular domain contains an endoplasmic reticulum (ER) retention signal, which may be shielded by the Ca^{2+} channel β subunit. They first demonstrated that the loop I-II of the P/Q-type channel α_1 subunit decreased the membrane expression level of a nonrelated channel, the *Shaker* potassium channel in *Xenopus* oocytes. This recombinant ion channel normally elicits a voltage gated potassium current in *Xenopus* oocytes. Fusion of the loop I-II, to the C-terminus of the *Shaker* channel decreased currents, while fusion of the Ca^{2+} channel β_3 subunits to the C-terminus had no effect on current size in comparison to wild type *Shaker*. Coexpression of the β_3 subunit together with the *Shaker*-loopI-II chimera rescued expression to levels of the wild type *Shaker* channel. These experiments indicated that the intracellular loopI-II of the $\alpha_1$2.1 subunit suppresses functional protein expression of an unrelated protein and that the Ca^{2+} channel β subunit which binds to the loopI-II antagonizes the suppressor effect of the loopI-II.

Since these experiments suggested that membrane localization is disturbed in the presence of the loop-I-II, the authors designed another set of experiments to show that membrane localization of a protein is disrupted by loop I-II. In order to prove this hypothesis the intracellular region of the transmembrane protein CD8 was removed against loop I-II, which now also contained an epitope-tag (*myc*) for localization of the construct using *myc*-tag specific antibodies. As expected fusion of the loop-I-II inhibited membrane localization of CD8 in COS-7 cells. In the presence of coexpressed Ca^{2+} channel β_3 subunits membrane expression of the *myc*-tagged CD8-loopI-II fusion protein was detected. In addition, the authors demonstrated that this chimera (*myc*-tagged CD8-loopI-II fusion protein) was restricted in its distribution to the ER and redistributed to the cell membrane once β_3 subunits were coexpressed. Using pulse chase experiments the authors also showed that even within a 5-min-metabolic labeling and chase time, β_3 and loopI-II immunoprecipitated. The result indicated that β_3 and loop I-II interact very early in the biosynthesis of Ca^{2+} channel complexes probably at the ER level and this interaction is necessary for membrane targeting of loop I-II. In a final experiment the authors showed that coexpression of loopI-II and the full-length rabbit $\alpha_1$2.1 subunits (BI-isoform) and β_3 subunits abolished Ca^{2+} current in *Xenopus* oocytes probably due to the competition for β_3 binding. Point mutations within the loop I-II which abolished binding to the β subunits and deletion of the intracellular loop I-II within the $\alpha_1$2.1 subunit were both insensitive to overexpression of loopI-II. In a more recent study the group of Michel De Waard identified further ER retension signals of the $\alpha_1$2.1 subunit (Cornet *et al.*, 2002). Using again CD8 fusion proteins between Ca^{2+} channel protein domains and the plasma membrane surface marker CD8 the group showed that the N-terminus and the C-terminus also contain ER retention signals. ER retention was also observed for the loopI-II of all high voltage-activated, non-L-type channels ($\alpha_1$2.1-3) and the L-type channel $\alpha_1$1.2, but was not detected for the skeletal muscle L-type loopI-II ($\alpha_1$1.1) and the T-type channel ($\alpha_1$3.1).

Thus, the results indicate that Ca^{2+} channel α_1 subunits mostlikely are transported through the ER via interaction with the ancillary β subunit. β subunits shield the ER retention signal of the Ca^{2+} channel α_1 subunit, which is localized in the intracellular domain I-II of the channel and is also the interaction site with the β subunit. However, other protein domains such as the N-terminus, the C-terminus and non-β-interacting domains within the loopI-II may contribute to ER retention of the full-length channel. Retention of channel subunits within the ER seems to be a general mechanism for controlling ion channel assembly. For example, K_{ATP} channels consisting of four pore-forming α and four regulatory β

subunits only pass the ER when assembled as an octameric complex. In this configuration the ER retention signals, which are found in both α and β subunits are shielded (Zerangue *et al.*, 1999). Similar mechanisms are described for $GABA_B$ receptor, NMDA receptors and CFTR (for review see Ma and Jan, 2002).

Small G Proteins bind to Ca^{2+} Channel β Subunits and May Antagonize the Interaction With α_1 Subunits

Recently a yeast two hybrid screen performed by Béguin *et al.* (2001) identified the small G protein kir/Gem as a potential interacting protein of Ca^{2+} channel β_3 subunits. They showed that kir/Gem interacted also with other β subunits, β_1 and β_2. kir/Gem-β binding was stronger in the presence of $GTP_\gamma S$ and was abolished by Ca^{2+}/Calmodulin, which also interacts with kir/Gem. Coexpression of kir/Gem with L-type channels ($\alpha_1 1.3/\beta_{1-3}$ and $\alpha_1 1.2/\beta_3$) in *Xenopus* oocytes and coexpression of kir/Gem with P/Q-type ($\alpha_1 2.1/\beta_1/\alpha_2\delta$) and N-type ($\alpha_1 2.2/\beta_1/\alpha_2\delta$) channels in BHK cells drastically reduced the Ca^{2+} currents for channels expressed with the α_1 and β subunits, but not when $\alpha_1 1.2$ was expressed alone. Confocal microscopy revealed that coexpression of $\alpha_1 1.2/\beta_3$ and kir/Gem reduced $\alpha_1 1.2$ surface expression causing an intracellular aggregation of $\alpha_1 1.2$ in HEK293 cells. Since kir/Gem showed high to moderate mRNA levels in pituitary, adrenal cells, and pancreatic islets the authors tested the function of kir/Gem on secretion in PC12 and MIN6 cells. Overexpression of kir/Gem reduced L-type Ca^{2+} currents and secretion in both cell types. The authors suggested a model for Ca^{2+} channel expression. In this model Ca^{2+}/Calmodulin activated kir/Gem binds Ca^{2+} channel β subunits, which interferes with α_1/β interaction and reduces the number of functional Ca^{2+} channels in the plasma membrane.

The Role of the $\alpha_2\delta$ Subunit in Membrane Targeting is Controversial

Several groups also studied the role of the $\alpha_2\delta$ ancillary subunit for Ca^{2+} channel targeting. The $\alpha_2\delta$ subunit is encoded by one gene, which is posttranslationally cleaved and processed into two disulfide-linked proteins (α_2 and δ). The δ protein contains one transmembrane region responsible for anchoring the subunit into the membrane. The α_2 part is highly glycosylated and is located to the extracellular surface of the cell (Brickley *et al.*, 1995; Gurnett *et al.*, 1996; Wiser *et al.*, 1996). Controversial results exist for effects of $\alpha_2\delta$ on channel modulation and surface expression. Modulatory effects on biophysical properties of the channel were described for shift in voltage dependence of activation and inactivation and change in activation and inactivation kinetics (Bangalore *et al.*, 1996; Felix *et al.*, 1997; Gao *et al.*, 2000a,b; Klugbauer *et al.*, 1999; Singer *et al.*, 1991; Stephens *et al.*, 2000; Wakamori *et al.*, 1994). Felix *et al.* (1997) reported for L-type channels and P/Q-type channels that the modulatory effect on the biophysical properties was mediated by the δ portion of the protein. However, several studies reported no or only minor effects on Ca^{2+} channel modulation (e.g., Ellinor *et al.*, 1993; Mikami *et al.*, 1989; Welling *et al.*, 1993; Jones *et al.*, 1998). Similar controversial results exist for the role of $\alpha_2\delta$ in transport and expression of Ca^{2+} channel complexes. In *Xenopus* oocytes the coexpression of $\alpha_2\delta$ increased currents through N-type channels ($\alpha_1 2.2/\beta_3$), P/Q-type channels ($\alpha_1 2.1/\beta_4/\beta_1$), L-type channels ($\alpha_1 1.2$, $\alpha_1 1.2/\beta_{2\alpha}$ α_1 1.1), and T-type channels ($\alpha_1 3.1$) (Mori *et al.*, 1991; Singer *et al.*, 1991; Shistik *et al.*, 1995; Gurnett *et al.*, 1996; Gao *et al.*, 2000a; Hobom *et al.*, 2000). A correlation between an increase in the whole cell current and an increase of gating current was observed when $\alpha_2\delta$ was coexpressed with $\alpha_1 1.2$, $\alpha_1 2.1$, or $\alpha_1 2.3$ in HEK293 cells (Bangalore *et al.*, 1996; Brodbeck *et al.*, 2002; Jones *et al.*, 1998). Using the same system $\alpha_2\delta$ increased current density for $\alpha_1 1.2/\beta_{2a}$- and $\alpha_1 2.3/\beta_3$-assembled channels ($\alpha_2\delta$-1 and $\alpha_2\delta$-3) (Klugbauer *et al.*, 1999). Coexpression of $\alpha_2\delta$ increased L-type current ($\alpha_1 1.2$ alone) and PN2000 binding in tsA201 cells and also increased P/Q-type ($\alpha_1 2.1/\beta_4$) current (Gurnett *et al.*, 1997). Gurnett *et al.* (1996 and 1997) demonstrated that the extracellular part of $\alpha_2\delta$ in its glycosylated form interacted with the extracellular loops of the α_1 subunits ($\alpha_1 1.2$ and $\alpha_1 1.1$) and was necessary but not sufficient for increase in current density and drug-binding affinity (PN200), since deletion of the α_2 part and deglycosylation of the subunit abolished P/Q-type current stimulation and decreased the binding affinity. Shistik *et al.* (1995) described that $\alpha_2\delta$ but not β_{2a} increased the amount of L-type channel complexes measured as ^{35}S-labeled $\alpha_1 1.2$ protein in the membrane of *Xenopus* oocytes. No increases in current levels or drug binding were reported by other groups for $\alpha_1 2.3$ subunits coexpressed with $\alpha_2\delta$ (Stephens *et al.*, 1997; Wakamori *et al.*, 1994).

Only a few studies tried to visualize the targeting effects of the $\alpha_2\delta$ subunit on Ca^{2+} channel complexes. Gao *et al.* (1999) showed that expression of the $\alpha_2\delta$ subunit alone revealed plasma membrane staining. However, coexpression of $\alpha_2\delta$ with $\alpha_1 1.2$ did not target the α_1 subunit to the plasma membrane. Coexpression of all three subunits α_1, β and $\alpha_2\delta$ showed membrane staining for all

overexpressed subunits. Thus, $\alpha_2\delta$ subunits were not sufficient to transport the $\alpha_1$1.2 subunit to the plasma membrane. Comparable studies were performed by Brice and Dolphin (1999) in COS7 cells using P/Q-type channel α_1 subunits. Here, $\alpha_1$2.1 and $\alpha_2\delta$ subunits were expressed alone or in combination. No membrane staining of α_1 subunits was detected and expression of $\alpha_2\delta$ alone did not result in plasma membrane localization. The discrepancy between $\alpha_2\delta$ distribution between both studies was suggested to be due to the different glycosylation machinery between the two cell lines used. Since $\alpha_2\delta$ is highly glycosylated insufficient or incorrect glycosylation may result in reduction of membrane targeting (Puri *et al.*, 1997). In another study Yamaguchi *et al.* (2000) revealed that coexpression of $\alpha_2\delta$ with $\alpha_1$1.2 (an HA epitope-tagged $\alpha_1$1.2 was used) increased the membrane fluorescence by 86% and average peak current by 99%, while β_{2a} increased it by 225 and 548%, respectively. A cooperative effect between $\alpha_2\delta$ and β was observed because expression of all three subunits increased fluorescence intensity by 470% and whole cell current by 1027%.

The role of $\alpha_2\delta$ subunits for channel targeting is controversial. The described results may depend on the expression system and subunit combination used. However, it is interesting to speculate that $\alpha_2\delta$ and β subunit may cooperatively lead the channel through the cell and are necessary for maturation of the channel complex at the membrane.

γ Subunits Reduce the Expression and Protein Levels of α_1 Subunits

The γ subunit was first discovered in skeletal muscle. On the basis of the structural homology and sequence identity, seven other γ-like genes have been described, which may assemble with voltage-dependent Ca^{2+} channels in nonskeletal muscle tissue. Recently, the groups of Kevin Campbell and Annette Dolphin observed effects of the muscle γ_1 subunit and the related subunits γ_2 (stargazin) and γ_7 on expression of voltage-dependent Ca^{2+} channels (Kang *et al.*, 2001 Moss *et al.*, 2002). Coexpression of $\alpha_1$2.1/β_3/$\alpha_2\delta$ or $\alpha_1$2.2/β_3/$\alpha_2\delta$ subunits with γ_1 or γ_2 subunits in *Xenopus* oocytes reduced the Ca^{2+} current by approximately 30–35%. Reduction in expression was only observed in the presence of $\alpha_2\delta$ subunits (Kang *et al.*, 2001). A more drastic effect on N-type channel expression was described for the γ_7 subunit. Coexpression of $\alpha_1$2.2/β_{1b} and γ_7 subunits almost completely reduced N-type currents in COS-7 cells and *Xenopus* oocytes and reduced P/Q- and L-type currents in *Xenopus* oocytes. The effect of γ_7 was independent of the $\alpha_2\delta$ subunit and was specific for Ca^{2+} channels since coexpression of γ_7 with the voltage gated K^+ channel K_v3.1b did not reduce the K^+ current. No effect of γ_7 was observed for endogenous N-type Ca^{2+} channel expression in sympathetic ganglion neurons, indicating that the γ subunit does not influence the expression of preexisting N-type channels in these neurons. Immunocytochemical studies revealed that γ_7 when coexpressed with a GFP tagged $\alpha_1$2.2 subunit and the β_{1b} subunit reduced the GFP fluorescence of the α_1 subunit. Decrease in the α_1 fluorescence was explained by a decrease in $\alpha_1$2.2 but not β_{1b} protein levels in the presence of γ_7 in these cells (Moss *et al.*, 2002). Thus, γ_1, γ_2, and γ_7 subunits decrease the expression of voltage-dependent Ca^{2+} channels. Several mechanisms may account for the suppression effect of the γ subunits such as change in protein degradation, protein-folding, protein synthesis, or protein targeting to the plasma membrane.

AKAP79 Regulates L-Type Channel Expression

PKA-mediated phosphorylation of L-type channels increases L-type channel activity in skeletal muscle, heart, and brain (Bean *et al.*, 1984; Gray and Johnson, 1987, Sculptoreanu *et al.*, 1993). In heterologous expression systems PKA phosphorylation of L-type channels occurs only in the presence of A-kinase anchoring proteins. A recent study by Altier *et al.* (2002) described now the role of the postsynaptic-scaffolding protein AKAP79 for L-type channel expression. Expression of AKAP79 selectively increased whole cell currents for L-type ($\alpha_1$1.2β_{1b}/$\alpha_2\delta$ or $\alpha_1$1.2/$\alpha_2\delta$) but not P/Q-, R-, or T-type currents. The increase in current amplitude was not dependent on its role in PKA signaling and did not involve a change in the single-channel properties. Neither the deletion of the PKA interacting domain within AKAP79 nor inhibition or activation of PKA changed the L-type current increase. Insertion of a hemaglutinin epitope tag into an extracellular loop of the $\alpha_1$1.2 subunit allowed the monitoring of the cell surface expression of L-type channels via chemiluminescence intensity measurements. A sevenfold increase in chemiluminescence intensity was observed for coexpression of $\alpha_1$1.2 with β subunits and more importantly a twofold increase in the presence of AKAP79 suggesting that AKAP increases the amount of $\alpha_1$1.2 protein in the plasma membrane. By expressing chimeras between $\alpha_1$1.2 and $\alpha_1$2.3 subunits (R-type channels are not upregulated by AKAP79) and injecting intracellular peptide loops into *Xenopus* oocytes expressing AKAP79 and L-type channels the authors identified the C-terminal end of the intracellular loopII-III of the L-type channels as the region

responsible for AKAP79 regulation. The C-terminal half of loopII-III contains a poly proline (PP) motif, which is conserved in the L-type channel $\alpha_1$1.1-3 subunits. Deletion of this motif in $\alpha_1$1.2 increased whole cell L-type currents to levels of wild type channels expressed with AKAP79. Coexpression of AKAP with PP deleted L-type channels did not result in further channel upregulation. Thus, the PP region may act as a suppressor region for surface expression of L-type channels.

SORTING OF CA^{2+} CHANNELS IN POLARIZED EPITHELIAL CELLS

Polarized epithelial cell lines, like Madin-Darby canine kidney (MDCK) epithelial cell line, are widely used to study the differential distribution of neuronal proteins. Various labs tried to establish a correlation between basolateral versus apical sorting in epithelial cells and somatodendritic versus axonal targeting in neurons (Dotti and Simons, 1990; Jareb and Banker, 1998). Brice and Dolphin (1999) used this system to study the differential distribution of N-, P/Q-, and L-type channels. Using immunocytochemical studies they found that α_1 subunits ($\alpha_1$2.1, $\alpha_1$2.2, and $\alpha_1$1.2) as earlier described in COS-7 and HEK293 cells (Brice *et al.*, 1997) were only sufficiently targeted to the plasma membrane when coexpressed with the ancillary subunits $\alpha_2\delta$ and any of the four β subunits. The L-type channel in the combination $\alpha_1$1.2, $\alpha_2\delta$ and any of the β subunits distributed mainly to the basolateral side of the cell, while the N-type channel in the combination $\alpha_1$2.2, $\alpha_2\delta$ and any of the β subunits targeted mainly to the apical pole of the cell. Interestingly, the distribution of the P/Q-type channel ($\alpha_1$2.1 subunit) depended on the coexpression of the β subunit. β_{1b} and β_4 assembled P/Q-type channels targeted mainly to the apical side, β_{2a} assembled channels to the basolateral side, while β_3 containing channels distributed equally over the three distinct areas.

The results indicate that again β subunits target α_1 subunits to the plasma membrane. L-type channels and β_{2a} assembled P/Q-type channels appear to be localized at the basolateral membrane, which maybe comparable to somatodendritic targeting in neurons, while N-type channels and β_{1b} and β_4 assembled P/Q-type channels target to the apical membrane, which maybe comparable to axonal sorting of the proteins. This distribution would correlate with the function of these channel types in neurons. While N- and P/Q-type channels would prefer the presynaptic terminal to trigger synaptic transmission, L-type channels may remain at the soma to trigger excitation transcription coupling.

TARGETING OF CA^{2+} CHANNELS IN STRIATED MUSCLE

L-type channels in striated muscle, i.e. skeletal and cardiac muscle are responsible for excitation contraction coupling through activation of ryanodine-sensitive Ca^{2+} release channels (RyR; Ryanodine Receptor) of the sarcoplasmic reticulum (SR), which leads to release of Ca^{2+} from the SR and subsequent contraction of the muscle. Efficient excitation contraction coupling requires a close interaction of L-type channels located in the plasma membrane invaginations (surface membrane of the T-tubules) and RyR (Endo-Sarcoplasmic Reticulum) and therefore colocalization of the two receptor types (for review (Flucher and Franzini-Armstrong, 1996)). Cell lines derived from the dysgenic muscle (GLT and NLT) have been extensively used to study the precise targeting of exogenously expressed L-type channels in particular the $\alpha_1$1.1 subunit and its function in excitation–contraction coupling. In GLT cells no $\alpha_1$1.1 subunits are expressed, while transversal tubule are normally formed and RyR are regularly clustered in T-tubule/SR junctions. Thus, correct targeting of L-type channels should result in colocalization and coupling with the RyR, which then should restore excitation–contraction coupling.

$\alpha_1$1.1 Subunits Target β Subunits to the Triads in Muscle Cells

Two groups in parallel addressed the question of L-type channel targeting in dysgenic myotubes. The group of Kurt Beam compared the subcellular localization of four Ca^{2+} channel α_1 subunits ($\alpha_1$1.1, $\alpha_1$1.2, $\alpha_1$2.1 and $\alpha_1$2.2) (Grabner *et al.*, 1998). GFP was tagged to the N-terminus of the α_1 subunits. All GFP-α_1 subunits revealed functional channels when exogenously expressed (mononuclear injection of cDNAs) in dysgenic skeletal myotubes as indicated in their class specific macroscopic Ca^{2+} channel current properties. Confocal microscopy showed that both L-type channel α_1 subunits ($\alpha_1$1.1 and $\alpha_1$1.2) clustered in punctates, while P/Q- and N-type channels distributed perinuclear. In addition, $\alpha_1$1.1 and $\alpha_1$1.2 but not $\alpha_1$2.1 and $\alpha_1$2.2 subunits were able to restore the electrically evoked contraction in dysgenic skeletal myotubes. Comparable results were obtained by Flucher and coworkers (Neuhuber *et al.*, 1998). Their studies in dysgenic myotubes addressed the association of $\alpha_1$1.1 and β_{1a} subunits in skeletal muscle triads. Using GFP-tagged β_{1a} subunits and antibodies against the $\alpha_1$1.1 subunits, the authors demonstrated that coexpression of β_{1a}-GFP and $\alpha_1$1.1 caused redistribution of β_{1a}-GFP into clusters.

β_{1a}-GFP when expressed alone revealed a homogenous, diffuse distribution. This clustering depended on the interaction between $\alpha_1$1.1 and β subunits, since $\alpha_1$1.1 mutants ($\alpha_1$1.1-Y366S and $\alpha_1$1.1-Δ351–380), which disrupt the interaction with the β subunit, failed to cluster β_{1a}-GFP. It has to be noted that $\alpha_1$1.1-Y366S is localized in comparable clusters as wild type $\alpha_1$1.1, while $\alpha_1$1.1-Δ351–380 was localized in a dense reticular membrane system (probably ER). The clusters mostlikely represent the incorporation of $\alpha_1$1.1 and β subunits into the T-tubule/SR junctions since double labeling experiments with the RyR showed colocalization between the different channels and subunits. For both functional $\alpha_1$1.1 channel subunits ($\alpha_1$1.1-Y366S and wild type), β_{1a} significantly increased the L-type Ca^{2+} currents and evoked a depolarization induced Ca^{2+} transient indicating that both subunits are capable of voltage sensing necessary for EC coupling (Flucher *et al.*, 2000). The same result was observed when the $\alpha_1$1.1 subunit was C-terminally tagged with GFP. Ca^{2+} currents and EC coupling was restored due to the correct localization of the construct into the triad junction (Flucher *et al.*, 2000). Thus, $\alpha_1$1.1 subunits target β_{1a} subunits into the triad. The correct targeting of the L-type channel in dysgenic myotubes restores EC-coupling.

Following the study in dysgenic myotubes Neuhuber *et al.* (1998a,b) analyzed the effects of Ca^{2+} channel β_{1a} and β_{2a} on the assembly of $\alpha_1$1.1 containing channels in a heterologous expression system (tsA201 cells). Expression of GFP-tagged or untagged β_{1a} revealed a diffuse fluorescence, with weak or no fluorescence in the nucleus. GFP alone showed strong fluorescence in the nucleus and diffuse fluorescence in the cytoplasm/ER. In contrast $\alpha_1$1.1 subunits and its mutations ($\alpha_1$1.1-Y366S and $\alpha_1$1.1-Δ351–380) localized in a cytoplasmic membrane system with high density in the perinuclear region of the cell. $\alpha_1$1.1 staining was detected with an antibody against $\alpha_1$1.1. Coexpression of $\alpha_1$1.1 and GFP-β_{1a} resulted in the redistribution of GFP-β_{1a} to the cytoplasmic membrane system, while $\alpha_1$1.1 mutations, which disrupt the β subunit interaction domain, could not attract the β_{1a} into the membrane system. Expression of β_{2a} caused the localization of the subunit to the periphery of the cell, mostlikely the membrane. Coexpression of β_{2a} with $\alpha_1$1.1 induced the assembly of $\alpha_1$1.1 in discrete aggregates in the cell periphery, while the β subunit interaction deficient $\alpha_1$1.1 mutant subunits remained in the tubular reticular membrane system. In contrast, β_{1a} assembled $\alpha_1$1.1 or $\alpha_1$1.1-Y366S but not $\alpha_1$1.1-Δ351–380 (does not express functional channels) expressed Ca^{2+} channels with a higher frequency in comparison to β_{2a} coexpressed channels, indicating that β_{2a} can translocate $\alpha_1$1.1 to the plasma membrane but can not modulate $\alpha_1$1.1 channel function.

The β_{1a} Subunit is Important for L-Type Channel Expression and EC-Coupling in Skeletal Muscle Cells

The importance of the β subunit for targeting and function of skeletal muscle L-type Ca^{2+} channels can be drawn from the experiments performed by the group of Roberto Coronado. They used a β_1 deficient mouse for their studies, which is the only β subunit expressed in skeletal muscle. β_1 knockout mice die at birth from asphyxia (Gregg *et al.*, 1996). Primary cultures of embryonic skeletal muscle myotubes revealed a drastic decrease in L-type current, charge movement and lacked EC coupling. Using antibodies against $\alpha_1$1.1 showed that the levels of $\alpha_1$1.1 were decreased. Fractions of $\beta_1(-/-)$ myotubes also displayed no $\alpha_1$1.1 staining. More importantly, the $\alpha_1$1.1 subunit lacked the punctate distribution, while clustering was not affected for RyR (Gregg *et al.*, 1996; Strube *et al.*, 1998). Transient transfection of cDNAs encoding the β_{1a} or β_{2a} subunits both rescued the L-type Ca^{2+} current, which were not distinguishable on the single channel level. However, β_{1a} transfected myotubes revealed a higher density of charge movement and a larger amplitude of Ca^{2+} transients after voltage pulse. This indicates that EC coupling is more efficient in β_{1a} transfected cells probably since β_{1a} assembled channel couple either better to the RyR or are more efficiently transported and localized in the triads (Beurg *et al.*, 1999a,b). In a further study Beurg *et al.* (1999a,b) characterized the protein domains, which were responsible for the differential effects of β_{1a} and β_{2a} on charge movement and transient currents. They constructed several deletion and chimeric mutants between β_{1a} and β_{2a} and found that the C-terminus of the β_{1a} subunit, when transferred to the β_{2a} subunit normalized the charge movement and Ca^{2+} transient to the β_{1a} wild type level. These constructs also carried a T7 tag for localization studies. The authors found that all constructs were expressed abundantly throughout the length of the myotubes, with high concentrations at the cell periphery. The authors suggested that this distribution is consistent with the distribution of L-type channels in cultured myotubes. The periphery is the site where the plasma membrane is coupled to the sarcoplasmic reticulum. The clustering of the β subunit and/or $\alpha_1$1.1 subunit and colocalization with the RyR was not analyzed.

The C-Terminus of $\alpha_1$1.1 Subunits Mediates Targeting and Clustering in Muscle Cells

To identify the region within the $\alpha_1$1.1 subunit responsible for localization and targeting of the skeletal muscle Ca^{2+} channel, Proenza *et al.* (2000) performed

a yeast two hybrid screen with intracellular protein domains from the $\alpha_1$1.1 subunit (bait) against a human skeletal muscle library. Here, the C-terminus interacted with many other proteins as indicated in the number of identified yeast colonies (>19,000), while the other intracellular protein domains (N-terminus, loopI-II, II-III, and III-IV) gave fewer than 12 colonies. In order to determine which region of the C-terminus was responsible for the interaction, the group used a deletion strategy to identify a 27 amino acid long sequence (1621–1647) containing a consensus PDZ binding motif. A point mutation within this motif (V1642D) disrupted the interaction with proteins of the skeletal muscle. To further test the physiological significance of this sequence motif in excitation contraction coupling and targeting, deletion constructs of the full length GFP-$\alpha_1$1.1 subunits were molecularly engineered and transfected into dysgenic myotubes. Deletion of the PDZ domain and/or disruption of the binding motif via point mutations caused a reduction in contraction after electrical stimulation in comparison to the full length GFP-$\alpha_1$1.1 subunit. While this effect could be due to nonlocalization of the construct or nonfunctional channels the group analyzed both parameters. While the full length GFP-$\alpha_1$1.1 distributed along the length of the myotube, the C-terminal deletion as well as the point mutation revealed a restricted localization close to the region of the injected nucleus. In addition, for both constructs charge movement was diminished but channels were functional. In contrast, a second deletion mutant, where most of the C-terminus was truncated had more drastic effects on all analyzed physiological parameters and fluorescence was largely reduced after injection.

To identify the structure motifs within the $\alpha_1$1.1 subunit, which are responsible and sufficient for L-type channel targeting to the triads Flucher *et al.* (2000) constructed chimeras between P/Q-type $\alpha_1$2.1 and skeletal muscle $\alpha_1$1.1 subunits. A GFP-tagged $\alpha_1$2.1 subunits (N-terminal tagging) was retained in a reticular sarcoplasmic membrane fraction (ER/SR) and was not colocalized with RyR, while N-terminally tagged $\alpha_1$1.1 subunits clustered and colocalized with RyR. Exchange of the intracellular loops between $\alpha_1$1.1 and $\alpha_1$2.1 revealed that only the C-terminus but not the other intracellular loops had effects on localization. Exchange of the $\alpha_1$1.1 C-terminus against the $\alpha_1$2.1 disrupted localization, i.e. this construct was localized in the ER/SR system, while introduction of the $\alpha_1$1.1 C-terminus into $\alpha_1$2.1 transferred triad targeting and colocalization with the RyR to the P/Q-type channel subunit. Again using a chimeric approach a region of 55 amino acids (1607–1661) within the C-terminus could be identified, which is responsible for channel targeting. Correct targeting of the P/Q-type channel carrying the targeting sequence of the skeletal muscle channels restored EC coupling in the dysgenic myotubes.

The Distribution of β Subunits in Heart Cells

L-type Ca^{2+} channels in the heart are important for cardiac excitability as well as excitation contraction coupling. The cardiac $\alpha_1$1.2/β_2 containing L-type channels are localized in the T-tubules in close proximity to the Ca^{2+} release channels in the junctions of the sarcoplasmic reticulum. Activation of these L-type channels lead to a Ca^{2+}-activated Ca^{2+} release and subsequent contraction. The targeting mechanisms of the cardiac L-type channel complex is unknown. The colocalization of $\alpha_1$1.2 and β_2 subunit in T-tubules was demonstrated by using specific antibodies against both subunits (Gao *et al.*, 1997a,b) and the distribution of GFP-tagged β subunits in heart cells has recently been exploited (Colecraft *et al.*, 2002; Wei *et al.*, 2000). The most prominent subunit in heart is the β_2 subunit (Hullin *et al.*, 1992; Ludwig *et al.*, 1997; Perez-Reyes *et al.*, 1992; Reimer *et al.*, 2000). Exogenous expression of GFP tagged β subunits in adult rat heart cells via adenovirus-mediated gene transfer revealed that β_{1b} and β_3 subunits distribute with similar intensities in the nuclear and sarcoplasmic compartments as GFP. In contrast β_4 subunits which are described to be temporally expressed in developing heart (Haase *et al.*, 2000) revealed a strong nuclear staining and also a regularly spaced transverse striation. β_2 subunits as expected revealed a predominant localization at the surface sarcolemma with no representation in the sarcoplasmic or nuclear compartments. All four β subunits induced subunit specific effects on the single channel gating of the endogenous cardiac L-type channels. This result is in agreement with the above-described finding that β_{1a} and β_{2a} subunits restore L-type Ca^{2+} channels in skeletal mouse myotubes lacking β_1 subunits (Beurg *et al.*, 1999a,b).

TARGETING OF CA^{2+} CHANNELS IN NEURONS

Subcellular localization determined with peptide specific antibodies against the α_1 subunits reveals a complex pattern of localization in neurons. Presynaptic terminals of various brain cells contain high densities of P/Q-, N-, and R-type channels. In contrast the $\alpha_1$1.2 and $\alpha_1$1.3 containing L-type channels are localized primarily on cell bodies and proximal dendrites, with $\alpha_1$1.2 also on distal dendrites. The expression of the four mammalian β subunits and three $\alpha_2\delta$ subunits has been described for various brain regions (Ellis *et al.*, 1988; Klugbauer *et al.*,

1999), with β subunit immunoreactivity being observed in neuronal cell bodies, dendrites, and neuropil (Birnbaumer *et al.*, 1998; Day *et al.*, 1998; Lie *et al.*, 1999; Ludwig *et al.*, 1997; Volsen *et al.*, 1997). A pre- and postsynaptic localization has been suggested for β_1, β_3 and β_4 subunits. Mutations in the ancillary subunits also cause severe malfunctions. A mutation in β_4 which results in a loss of β_4 subunit in the mouse, leads to an epileptic phenotype (lethargic; lh/lh; Burgess *et al.*, 1997). Excitatory synaptic transmission in the lethargic mouse is reduced, suggesting a presynaptic function for β_4 subunits in transmitter release (Caddick *et al.*, 1999). Ca^{2+} channels have been detected in vesicle-like structures (Ahmari *et al.*, 2000; Leitner *et al.*, 1999; Passafaro *et al.*, 1996; Sher, 1997; Sher *et al.*, 1998; Shapira *et al.*, 2003), indicating that vesicle-mediated transport plays a role for axonal and dendritic delivery of Ca^{2+} channel complexes. However, very little information is available about the mechanisms of Ca^{2+} channel targeting in neurons.

Ca^{2+} Channel β_4 Subunits Target to Synaptic Sites

Our own studies concerning the localization of Ca^{2+} channel β subunits in hippocampal neurons revealed a synaptic staining pattern and presynaptic function of Ca^{2+} channel β_4 subunits (Wittemann *et al.*, 2000). In these studies GFP was tagged to the N-terminus of β_4 and cloned into the semliki forest virus expression system for sufficient infection of hippocampal neurons. Low density hippocampal neurons were transfected with semliki forest virus carrying GFP-β_4. GFP-β_4 distributed in a punctate staining pattern indicating clustering of this subunit at synaptic sites. GFP-β_4 colocalized with endogenous synaptobrevin II and endogenous P/Q-type Ca^{2+} channel α_1 subunits. To analyze whether the GFP-β_4 was transported into the axons a costaining between the dendritic marker MAP2 and GFP-β_4 was performed indicating that GFP-β_4 targets to the axon as well as dendrites in the hippocampal culture system. Thus, these data suggested that GFP-β_4 subunits are transported to the presynaptic terminal and clustered at synaptic sites.

Targeting signals to specific organelles have been identified in the extracellular and transmembrane domains of various proteins (Bradke and Dotti, 1998; Keller and Simons, 1997). While no conserved axonal sorting signals have been described to date (Winckler and Mellman, 1999), infection of fully polarized cultured hippocampal neurons with viral vectors has been used to identify specific axonal sorting signals. For example, synaptobrevin contains a 92 amino acids long N-terminal region, which is responsible for axonal targeting (West *et al.*, 1997), while the mGluR7 contains a 60 amino acids long cytoplasmic domain, which mediates both axonal and dendritic targeting (Stowell and Craig, 1999). A dileucine motif in a neuronal glycine transporter (Poyatos *et al.*, 2000) and a tyrosine-based sequence motif within the transferrin receptor (West *et al.*, 1997) are essential for targeting. A dileucine motif is present in all Ca^{2+} channel β subunits, while a conserved tyrosine motif (EEDY) is only present in the C-termini of β_1, β_3, and β_4 but not β_2. It seems a general strategy that the targeting sequences are localized in the nonconserved regions among the protein families. To identify the region within the β subunits responsible for clustering of β_4, the low homology domains of β_4 (i.e. the N- and C-terminus (Fig. 1)) and the highly conserved middle part of the β_4 subunit was tagged to GFP and exogenously expressed in the same culture system. Synaptic clustering and colocalization with synaptobrevin II was observed for the N- and the C-terminus but not for the conserved core region of β_4 (Fig. 1). The β_4 core region was located primarily in the soma of the cell.

Presynaptically targeted β_4 subunits should assemble with α_1 subunits to form functional channels in the presynaptic terminal. Exogenously expressed β_4 subunits may therefore create a certain amount of presynaptic Ca^{2+} channels containing the β_4 subunits. β_4 assembled P/Q-type channels have different biophysical properties than β_{1b} assembled channels. This becomes obvious in particular in their inactivation properties during depolarization trains. During a 20-Hz stimulation protocol β_4 assembled channels, channels inactivate slower than β_{1b} assembled channels allowing a larger ion influx during repetitive stimulation. Since the influx of Ca^{2+} into the presynaptic terminal determines the synaptic transmitter release and short term synaptic plasticity like pulse facilitation, (Fisher *et al.*, 1997; Zucker, 1999) assembly of presynaptic Ca^{2+} channels with different β subunits may determine the plasticity of the neuronal circuit (Fig. 2). In order to show that Ca^{2+} channel β_4 subunits change presynaptic parameters of transmitter release, we analyzed the EPSC amplitude and paired pulse facilitation properties of autaptic hippocampal neurons in the presence and absence of exogenously expressed β subunit constructs. As a control construct we used the non synaptically targeted β subunit, where the N- and C-termini were deleted ($\beta_{4\Delta 51\text{-}407}$). GFP-$\beta_4$ and GFP-$\beta_{4\Delta 51\text{-}407}$ assembled with $\alpha_1 2.1$ subunits and $\alpha_2\delta$ to form functional P/Q-type channels. We observed that in the presence of GFP-β_4 EPSC amplitude was largely increased in comparison to GFP-infected neurons or in neurons infected with GFP-$\beta_{4\Delta 51\text{-}407}$. More importantly during a 20-Hz stimulation protocol, pulse facilitation was increased for GFP-β_4 but not GFP-$\beta_{4\Delta\text{-}51\text{-}407}$ indicating that Ca^{2+} channel β_4

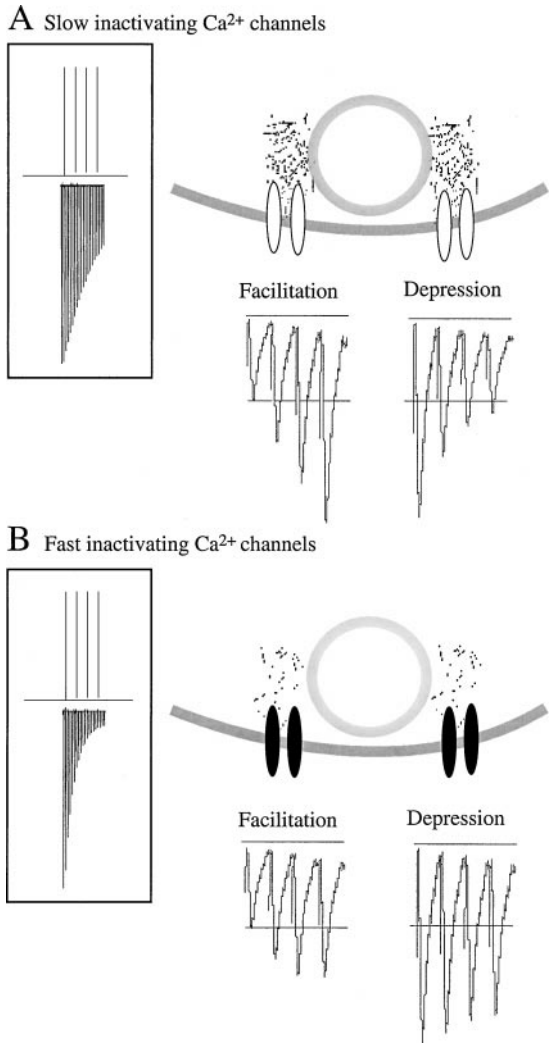

Fig. 2. Ca^{2+} channel targeting and synaptic plasticity. The influx of Ca^{2+} into the presynaptic terminal determines the synaptic transmitter release. Modulation of the presynaptic Ca^{2+} influx may change short term synaptic plasticity of the neuronal circuit (Fisher *et al.*, 1997; Zucker, 1999). For example the Ca^{2+} influx through (A) slow inactivating Ca^{2+} channels (e.g. α_1/β_4 assembled channels) is larger than through (B) fast inactivating Ca^{2+} channels (e.g. α_1/β_1 assembled channels) during high frequency stimulations (high firing rate of action potentials). Depending on the synapse type (i.e. low release of probability or high release of probability) short term synaptic plasticity (i.e. facilitation or depression, respectively) will change according to the biophysical properties of the Ca^{2+} channels. Thus, targeting of Ca^{2+} channels complexes to presynaptic terminals with defined subunit compositions may influence short-term synaptic plasticity.

containing clusters are correlated with changes in presynaptic function. Thus, targeting of Ca^{2+} channel β subunits to specific terminals may define short term plasticity in the brain.

The Importance of the C-Terminus of the α_1 Subunit in Ca^{2+} Channel Complex Clustering

The muscle-type Ca^{2+} channel $\alpha_1 1.1$ and $\alpha_1 1.2$ subunits contain the targeting signal in its nonconserved C-terminus (Flucher *et al.*, 2000; Gao *et al.*, 2000). Several C-terminal truncated and expanded isoforms of L-type channel α_1 subunits have been described (De Jongh *et al.*, 1991, 1996; Gao *et al.*, 1997a,b; Hell *et al.*, 1993a,b; Ihara *et al.*, 1995). A truncated form of $\alpha_1 2.1$ that stretches from the start of the protein through the middle of the intracellular loop connecting domain 2 and 3 was purified (Scott *et al.*, 1998) and C-terminal splice variants have been detected in pancreatic islets (Ligon *et al.*, 1998). The presence of various C-terminal spliced isoforms of $\alpha_1 2.1$ in human brain have been linked to a severe form of human ataxia (Zhuchenko *et al.*, 1997). In addition, a C-terminal truncation in the leaner mouse displays seizures that resemble those observed in human absence (petit mal) epilepsy (Fletcher *et al.*, 1996). Using a biochemical approach Maximov *et al.* (1999) showed the interaction of the α_1 C-terminus of the presynaptic N- and P/Q-type Ca^{2+} channels with modular adaptor proteins. The modular adaptor proteins Mint-1, CASK, and Veli form a tripartite complex in the presynaptic terminal and interact with other signaling proteins like β neurexins. Thus, the putative interaction of Ca^{2+} channels with this protein-signaling complex may be involved in clustering and precise positioning of channels within the terminal (Bezprozvanny and Maximov, 2001; Jarvis and Zamponi, 2001; Maximov *et al.*, 1999). Using yeast two hybrid and immunoprecipitation assays Maximov *et al.* (1999) first demonstrated that Mint1-1 and CASK bind to the C-terminus of N-type channel subunit $\alpha_1 2.2$. The interaction involved the first PDZ domain of Mint-1 and the SH3 domain of CASK. The authors also identified sequence motifs in the N-type channel C-terminus which were responsible for the interaction. The interaction with Mint1 involved a new class of PDZ domain (E/D-X-W-C/S-COOH). By using the yeast two hybrid system for detection of protein interactions the authors showed that only the long-splice variants of P/Q- and N-type channels, which contain the sequence motif, but not R- or L-type channels interact with Mint-1. In addition, they demonstrated that CASK interaction involved a proline rich region in the middle of the C-terminus. In a further study Maximov and Bezprozvanny (2002) studied the synaptic targeting of N-type channels in hippocampal neurons. By cotransfecting HA-tagged $\alpha_1 2.2$ subunits together with β_3 and $\alpha_2\delta$-1 subunits the authors demonstrated that in mature cultured hippocampal neurons N-type channels were absent from dendrites but were highly concentrated and

clustered in axonal processes. The clustering of N-type channels was dependent on synapse formation. To characterize the structural domains involved in channel clustering, deletion and point mutations were introduced into the C-terminus of $\alpha_1$2.2a (long form). Truncation of 300 aa of the long form of $\alpha_1$2.2 abolished synaptic clustering. Mutations within the SH3 domain and the PDZ domain of the C-terminus (Fig. 1) reduced clustering, while combining these mutations within one construct abolished clustering. The experiments suggested that the long form of the N-type channels is responsible for presynaptic transport and clustering in mature hippocampal neurons. To further verify this hypothesis the authors immediately analyzed the distribution of the short N-type channel form ($\alpha_1$2.2b). As expected, $\alpha_1$2.2b did not localize to axons and clustered in mature neurons only at the soma and proximal dendrites. By using Western blot and immunoprecipitation experiments the authors also demonstrated that the long form but not the short form of the native N-type channels were concentrated in synaptic locations. Impairment of synaptic function and channel clustering was observed when the GFP tagged C-terminus was overexpressed in the neurons. Depolarization-induced antibody uptake representing exocytosis/endocytosis events was diminished in the presence of the C-terminus, indicating that the C-terminus acts as a dominant negative mutant. Fusion of the C-terminus of the N-type channel (lacking the proline rich region) and the P/Q-type channel to the extracellular and transmembrane domain of the CD4 receptor was sufficient for synaptic targeting. The PDZ domain binding motif was necessary for the clustering, since deletion of this binding motif resulted in diffuse distribution of the constructs within the neurons. Coexpression of GFP-tagged Mint1, CASK, and HA-$\alpha_1$2.2 indicated that all three proteins are colocalized within the synaptic clusters. (Maximov and Bezprozvanny, 2002). Thus, the data implicate an important function of the C-terminus of presynaptic Ca^{2+} channels in synaptic targeting, and clustering, which involves the interaction of the channel with modular adaptor proteins. In a more recent study Mochida's and Catterall's groups (Mochida *et al.*, 2003a,b) suggested a role of the loopII-III, in particular the synprint binding site (Sheng *et al.*, 1994), in synaptic targeting and function. By overexpressing $\alpha_1$2.1 subunit containing P/Q-type channels in superior cervical ganglion neurons the groups were able to rescue synaptic transmission when the endogenous N-type channels were blocked (note that synaptic transmission in these neurons completely relies on N-type channels). The data suggests the precise targeting and localization of P/Q-type channels in these neurons to synaptic release sites. Deletion of the synprint site within the P/Q-type channel loopII-III resulted in impairment of rescuing synaptic transmission and a loss of presynaptic localization of these channels suggesting an important role of this protein domain in synaptic targeting.

Thus, the C-terminus as well as the loopII-III with its interaction domains for modular adaptor proteins or proteins of the synaptic release machinery are important for synaptic targeting and clustering of voltage-gated Ca^{2+} channels.

PERSPECTIVE

The understanding of Ca^{2+} channel trafficking and its subcellular localization in particular in neurons is at its infancy. Since Ca^{2+} channels subtypes are involved in very specific, regulated functions within their physiological environment, mechanisms for subcellular targeting have to exist. In general we can distinguish three levels involved in the specificity of localization. First biosynthesis within the ER may allow certain channel combinations to exit the biosynthetic pathway, while others are retrieved. As described in this review Ca^{2+} channel subunits are localized within the ER and can be transported to the plasma membrane. This involves the interaction between α_1 and β subunits, where β subunits shield an ER retention signal of the α_1 subunit. Further analysis will reveal if this mechanism is true for the skeletal muscle L-type channel and in particular for T-type channels. No interaction between T-type α_1 subunits and β subunits could be observed. The second decision has to be made regarding the transport of channel to its specific subcellular location, e.g., soma, dendrite, axon, or T-tubule. It can be expected that certain domains of the Ca^{2+} channel subunits, in particular those underlying splicing may interact with specific transport and adaptor proteins, which help to sort the different channel complexes. Considering specific mRNA transport to dendrites and at least in invertebrates into axons, sorting mechanisms could take place also at the mRNA level. The third step is the anchoring of Ca^{2+} channel complexes within the subcellular structures. The C-terminus and the loopII-III of α_1 subunits seem to be the targets of some of these clustering and anchoring proteins. We can expect that future work will identify sequence motifs and their interacting partners, which dynamically target the channel complex to its destiny.

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