# Ion Transport

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## PART 4: Models of Ion Permeation across Membranes

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# The Structure of the Skeletal Muscle Calcium Channel

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## 1 Introduction

Voltage-activated $Ca^{2+}$ channels are classified into three types (T, N and L), which differ in their pharmacological behaviour and functional significance (Nowycky *et al.*, 1985). L-type channels are sensitive to organic drugs the $Ca^{2+}$ channel blockers (CaCB) which include the dihydropyridines (1,4-DHPs), the phenylalkylamines (PAAs) and the benzothiazepines (BTZs) (Hofmann *et al.*, 1987). In cardiac muscle, β-adrenoceptor agonists increase the open state probability of this channel type either by phosphorylation of the channel or a channel associated protein via the catalytic subunit of cAMP-dependent protein kinase (Trautwein *et al.*, 1986), or by stabilizing the open state via the α-subunit of the GTP binding protein $G_s$ (Yatani *et al.*, 1987). L-type channels occur in peripheral and central neurons, smooth muscle, invertebrate skeletal muscle and heart. Their biological function in vertebrate skeletal muscle has not been established unequivocally, but probably the channel has the same function as in cardiac muscle, i.e. participating in the maintenance of an adequate amount of intracellular $Ca^{2+}$ for muscle contraction (Ildefonse *et al.*, 1985). However, the channel protein may participate in skeletal muscle excitation contraction-coupling not only as $Ca^{2+}$ channel but also as a voltage sensor (Berwe *et al.*, 1987; Lamb and Walsh, 1987; Rios and Brum, 1987). L-type $Ca^{2+}$ channels of different tissues probably are not identical and will be divided up further

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as our knowledge of their electrophysiological and molecular properties, their functional significance and their modulation by hormones, neurotransmitters and drugs expands.

## 2 Structural composition of the purified skeletal muscle $Ca^{2+}$ channel

Most of the information on the structural composition of the L-type $Ca^{2+}$ channel originates from studies with rabbit skeletal muscle. SDS-gel analysis of the purified skeletal CaCB-receptor yields several stained bands with apparent molecular weights of 165 kDa ($\alpha_1$), 55 kDa ($\beta$) and 32 kDa ($\gamma$) in a constant ratio of 1:1.7:1.4 (Sieber *et al.*, 1987). A further protein containing a 130-($\alpha_2$) and 28-kDa ($\delta$) disulfide-linked peptide co-purifies in variable amounts with the $\alpha_1$-, $\beta$- and $\gamma$-subunits of the CaCB-receptor. The $\alpha_2$- and the $\gamma$-peptides are heavily glycosylated, whereas the $\alpha_1$- and $\beta$-subunit contain none or a low amount of carbohydrates (Takahashi *et al.*, 1987). The purified receptor binds all three major classes of $Ca^{2+}$ channel blockers (i.e. DHPs, PAAs and BTZs) in a stereospecific manner. The photo-affinity analogues of the 1,4-DHPs and PAAs, azidopine and LU 49888, label only the 165-kDa ($\alpha_1$) subunit, indicating that this protein carries the drug receptor sites for 1,4-DHPs and PAAs (Galizzi *et al.*, 1986; Sieber *et al.*, 1987; Tanabe *et al.*, 1987; Striessnig *et al.*, 1986, 1987). The constant stoichiometry of the 165-, 55- and 32-kDa proteins suggests that these proteins are constituents of the $Ca^{2+}$ channel. Further evidence for the existence of a functional complex with this composition comes from studies with antibodies against the $\alpha_1$-subunit which precipitate the $\alpha_1$-, $\beta$- and $\gamma$-subunits (Takahashi *et al.*, 1987). Antibodies specific against the $\alpha_1$- or $\beta$-subunits immunoprecipitates the $\alpha_1$- or $\beta$-subunits (Leung *et al.*, 1988). Furthermore, antibodies specific against the $\alpha_1$-, $\beta$- and $\gamma$-subunits modulate the $Ca^{2+}$ current *in vivo* (Campbell *et al.*, 1988; Morton *et al.*, 1988; Vilven *et al.*, 1988). Attempts to isolate only the $\alpha_1$-subunit under non-denaturing conditions have not been successful so far, suggesting that the $\beta$- and $\gamma$-subunits stabilize the channel in a high-affinity CaCB binding conformation. At present, it is not known whether or not the 130/28-kDa protein belongs also to this structure (Leung *et al.*, 1988) or is only a contaminant.

## 3 Phosphorylation of the purified CaCB-receptor

The 165- and 55-kDa subunits of the purified CaCB-receptor are phosphorylated readily by cAMP-dependent protein kinase (Curtis and

**Table 1** Kinase-specific phosphopeptides of the skeletal muscle CaCB-receptor

| Protein kinase | Phosphopeptide number | |
|---|---|---|
| | 165 kDa | 55 kDa |
| cAMP-kinase | 1,10 | 7 |
| cGMP-kinase | 1,2 | 1,3 |
| Protein kinase C | none | 7/8 |
| Casein kinase II | 7,11 | 9,10 |

The purified receptor was phosphorylated by each kinase as described by Jahn *et al.*, (1988). The phosphorylated subunits were separated by SDS-gel electrophoresis. Individual gel pieces containing the phosphorylated $\alpha_1$- or β-subunit were digested by trypsin over night. The phosphopeptides were then separated by 2-D thin-layer chromatography. The number of the kinase-specific phosphopeptide(s) is shown. See also Jahn *et al.* (1988).

Catterall, 1985; Flockerzi *et al.*, 1986a,b) and other kinases *in vitro* (Nastainczyk *et al.*, 1987; O'Callahan *et al.*, 1988; Jahn *et al.*, 1988). The $\alpha_1$-subunit is a good substrate for cAMP-kinase and casein kinase II, whereas the 55-kDa subunit is preferentially phosphorylated by protein kinase C and cGMP-dependent protein kinase. Two-dimensional peptide maps yield 11 phosphopeptides from the 165-kDa subunit and 11 from the 55-kDa subunit using these kinases (Jahn *et al.*, 1988). With the exception of protein kinase C, each kinase apparently phosphorylates one or two peptides specifically in each subunit (Table 1). Protein kinase C does not phosphorylate specifically a peptide in the 165-kDa peptide, but modifies rapidly peptide 7/8 of the 55-kDa subunit. Neither the 32-kDa nor the 130/28-kDa peptides are phosphorylated by the above-mentioned kinases. At physiological concentrations cAMP-dependent protein kinase incorporates 1 mole phosphate per mole 165-kDa subunit within 10 min (Curtis and Catterall, 1985; Nastainczyk *et al.*, 1987). This suggests that the phosphorylation of this site may be functionally important. A second site is phosphorylated during prolonged incubation. The rapidly phosphorylated peptide was isolated and sequenced. The phosphorylated amino acid was identified as Ser 687 of the deduced amino acid sequence of the $\alpha_1$-subunit (Röhrkasten *et al.*, 1988). This serine is located in the cytoplasmic loop between transmembrane regions II and III (see Fig. 1). It is possible that *in vivo* cAMP-dependent phosphorylation of this serine increases the open state probability of the $Ca^{2+}$ channel.

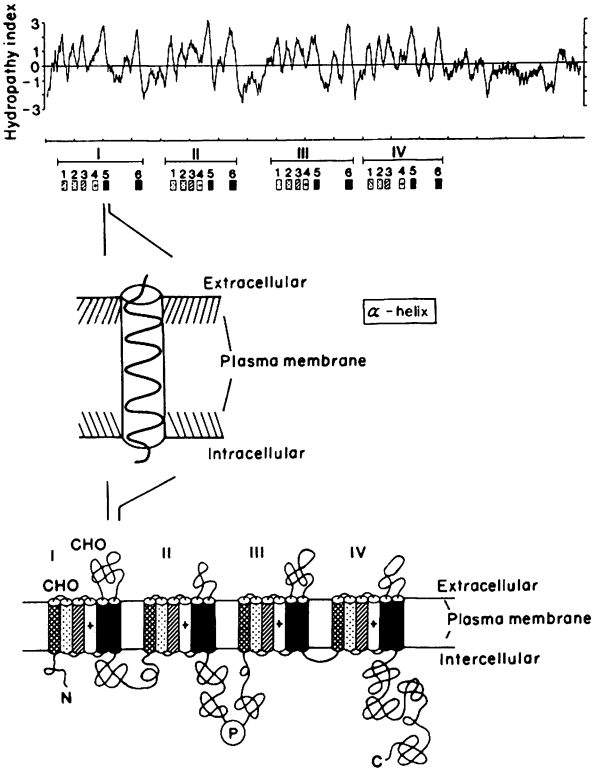


**Fig. 1** Hydrophobicity profile and transmembrane topology of the skeletal muscle $Ca^{2+}$ channel $\alpha_1$-subunit. The hydrophobicity profile of the $\alpha_1$-subunit according to Tanabe *et al.* (1987). Positive indices represent hydrophobic amino acid regions. The protein consists of four homologous domains (I, II, III, IV) each composed of six membrane spanning helices (1, 2, 3, 4, 5, 6). Transmembrane regions are based on their hydropathy value, polarity index and hydrophobic moment analysis according to Chou and Fasman (1978). The homologous regions (I, II, III, IV) each containing six transmembrane spirals are shown linearly. They are supposed to form the ionic pore. The carboxy- and amino-termini are located at the cytoplasmic site of the plasma membrane. The phosphorylation site of cAMP-dependent protein kinase, serine residue 687, is indicated between domains II and III.

## 4 Structure of the $\alpha_1$- and $\beta$-subunits of the skeletal muscle $Ca^{2+}$ channel

Identification and cloning of the $\alpha_1$- subunit of the $Ca^{2+}$ channel from skeletal muscle was a major step in $Ca^{2+}$ channel research (Tanabe *et al.*,

1987). The cloned rabbit skeletal muscle $\alpha_1$-subunit has 29% homology with the voltage-dependent $Na^+$ channel. It is assumed that four homologous regions, each consisting of five hydrophobic $\alpha$-helices (S1, S2, S3, S5 and S6) and one hydrophilic $\alpha$-helix (S4), span the membrane and form the $Ca^{2+}$ channel pore (Fig. 1). S4, which is present in each transmembrane region, is a positively charged helix that could act as a voltage sensor. A homologous helix is found in other voltage-activated ion channels, i.e the $Na^+$ channel of eel, fly and rat and the $K^+$ channel of various tissues. The positive charges of the S4 segment could respond to a change in the membrane potential by a transmembrane shift of its positive charges, and thereby affect the open/closed state of the channel.

The primary structure of the rabbit skeletal muscle $\beta$-subunit has been deduced from the cloned cDNA. The cDNA has a length of 1.85 kilobase. The deduced peptide consists of 524 amino acids with a $M_r$ of 58 kDa. The primary structure of the $\beta$-subunit agrees with that of a peripheral membrane protein. It contains four homologous $\alpha$-helices (Fig. 2). Analysis of the primary structure reveals two further apparently specific protein kinase C phosphorylation sites. This is interesting since the $\beta$-subunit is preferentially phosphorylated by protein kinase C *in vitro*. Furthermore,

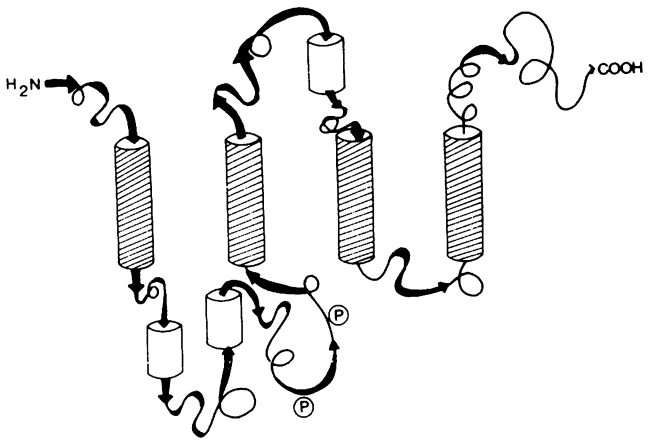


**Fig. 2** Predicted secondary structure of the $\beta$-subunit of the skeletal muscle $Ca^{2+}$ channel. The secondary structure of the deduced amino acid sequence was predicted by the method of Garnier *et al.* (1978). The $\beta$-subunit contains four hydrophilic helical regions (shaded rods), each composed of 26–33 amino acids. The helices are joined by $\beta$-sheets (arrows) and coils. These secondary structures are interrupted at three positions by helical structures with a length of 10–15 amino acids (open rods). The *in vitro* phosphorylation sites are indicated by P. The site closer to the amino terminus is the major *in vitro* phosphorylation site of cAMP-dependent protein kinase.

there is ample evidence that protein kinase C alters L-type channel function *in vivo* (Kaczmarek, 1987). In addition, the primary sequence contains a site specific for cGMP-dependent protein kinase. This was expected from the *in vitro* phosphorylation experiments (see Table 1).

The biological significance of these primary structures remains to be elucidated. Antibodies against the $\beta$-subunit enhance $Ca^{2+}$ currents through L-type channels and prevent the blocking action of nitrendipine (Vilven *et al.*, 1988), whereas antibodies against the $\gamma$-subunit inhibit $Ca^{2+}$ current (Campbell *et al.*, 1988). This suggests that the two smaller subunits of the skeletal muscle $Ca^{2+}$ channel are necessary for proper channel function.

The primary structure of the $\gamma$-subunit is not known. However, the primary structure of the 130-kDa ($\alpha_2$) protein has been deduced from cloned rabbit skeletal muscle cDNA (Ellis *et al.*, 1988). The $\alpha_2$ protein has the sequence of a hydrophilic protein and may contain up to three transmembrane helices and eight extracellular N-glycosylation sites. This predicted topography is in accordance with the finding that the purified protein is heavily glycosylated. Hybridization studies show that the mRNA for the $\alpha_2$ protein is expressed in many tissues, whereas the cDNA for the skeletal muscle $\alpha_1$-subunit hybridizes only weakly or not at all to the messenger RNA from other tissues (Ellis *et al.*, 1988). Although these data are not conclusive, they support the notion that the 130/28-kDa ($\alpha_2/\delta$) protein may not be an essential part of the calcium channel.

## 5 Identification of L-type $Ca^{2+}$ channel proteins in other tissues

A $Ca^{2+}$ channel $\alpha_1$-subunit of a slightly larger size than that of rabbit skeletal muscle has been identified in brain, smooth muscle and heart by northern blot analysis of the respective mRNA (Table 2). This difference in size is supported by photo-affinity labelling of the high-affinity receptor for CaCBs. Azidopine and LU 49888 identify a 195-kDa protein in a partially purified preparation of the bovine cardiac muscle CaCB-receptor (Schneider and Hofmann, 1988). An identically sized $\alpha$-subunit is labelled in hippocampus (Striessnig *et al.*, 1988). These differences are not caused by species differences, since a monoclonal antibody to the $\alpha_1$-subunit of rabbit skeletal muscle recognizes the same subunit in the skeletal muscle of guinea pig, hamster, rat, cow and pig, but does not bind to the bovine heart subunit. This suggests that the differences are real and reside in the primary sequence of the $\alpha$-subunits from different tissues.

**Table 2** Size of the putative α-subunit of the $Ca^{2+}$ channel in other tissues

| | | Size of putative $\alpha_1$-subunit | |
|---|---|---|---|
| Tissue | Species | Protein (kDa) | mRNA (kB) |
| Skeletal muscle | rabbit | 165 | 6.2 |
| Heart | cow | 195 | 8.3 |
| Lung | cow | — | 8.2 |
| Brain | guinea pig | 195 | 8.1 |

Membranes or a partially purified preparation of the CaCB-receptor were affinity-labelled with azidopine or LU 49888. The molecular weight was determined by SDS-gel electrophoresis. The size of the mRNA was determined by hybridization with a probe derived from the cloned $\alpha_1$-subunit of the rabbit skeletal CaCB-receptor. For further detail see Sieber *et al.* (1987), Schneider and Hofmann (1988) and Striessnig *et al.* (1988).

## 6 Reconstitution of an L-type $Ca^{2+}$ channel from the skeletal muscle CaCB-receptor

The purified dihydropyridine receptor from skeletal muscle has been reconstituted into phospholipid vesicles which were fused with a phospholipid bilayer (Flockerzi *et al.*, 1986a; Hymel *et al.*, 1988; Ma and Coronado, 1988; Pelzer *et al.*, 1988; Talvenheimo *et al.*, 1987). In agreement with whole-cell recording data, the reconstituted protein channel has a single-channel conductance of about 20 pS (Flockerzi *et al.*, 1986a; Pelzer *et al.*, 1988; Talvenheimo *et al.*, 1987). Its open state probability ($p_O$) is reduced by the $Ca^{2+}$ channel blockers gallopamil and PN 200-110, and increases in the presence of the $Ca^{2+}$ channel agonist Bay K 8644. The $p_O$ is also increased several fold by the addition of ATP·Mg and the catalytic subunit of cAMP-dependent protein kinase (Flockerzi *et al.*, 1986a; Hymel *et al.*, 1988; Pelzer *et al.*, 1988). These results suggest that the reconstituted channel has some properties of the cardiac L-type $Ca^{2+}$ channel. Further analysis of the single-channel kinetics showed that open and closed times of the reconstituted channel are about 10 times longer than that of an *in vivo* cardiac muscle L-type channel (Pelzer *et al.*, 1988), indicating that the reconstituted channel has properties which differ from that of the cardiac muscle channel. The slower channel kinetics of the purified receptor are not caused by proteolysis of the receptor during purification. The same kinetics were obtained when solubilized microsomal membranes were reconstituted.

Both preparations, the solubilized membranes and the purified receptor, contain a second channel with a conductance around 10 pS (Ma and Coronado, 1988; Pelzer *et al.*, 1988; Talvenheimo *et al.*, 1987). The $p_O$ of the channel showing the smaller conductance was not affected by phosphorylation, $Ca^{2+}$ channel blockers or agonists (Pelzer *et al.*, 1988). So far, an interconversion of a small non-regulated into a large regulated conductance has not been observed (Pelzer *et al.*, 1988). The two conductances could be distinguished further by the voltage-dependence of $p_O$. The small conductance showed a regular voltage dependence of its open state probability, whereas the large conductance yielded a bell-shaped dependency. $p_O$ was greatest at a membrane potential around 0 mV (Pelzer *et al.*, 1988). These differences in electrophysiological parameters clearly distinguish the two conductances.

## 7 Conclusions

The biochemical identity of these conductances is not clear at present. Recent reconstitution experiments of the isolated $\alpha_1$-subunit suggest that the 165-kDa subunit is the $Ca^{2+}$-conducting unit (Pelzer *et al.*, 1988). This conclusion is supported by experiments in which the cloned cDNA of the $\alpha_1$-subunit was expressed in embryonic muscle cells from dysgenic mice (Tanabe *et al.*, 1988). The dysgenic myotubes do not express the 6.5-kB mRNA of the $\alpha_1$-subunit and are defective in EC-coupling. The intranuclear injection and the expression of the cloned $\alpha_1$-subunit mRNA in myotubes of dysgenic mice restores EC-coupling and a slow L-type $Ca^{2+}$ channel in these cells. Interestingly, the T-tubular voltage sensor and the $Ca^{2+}$ channel is regained together with the expression of the $\alpha_1$-subunit, suggesting that both functions require the $\alpha_1$-subunit. These two functions may not depend alone on the presence of the 165-kDa ($\alpha_1$)-subunit, but correct functioning may require the presence of the other subunits. This conclusion is supported also by the modulatory effect of $\beta$- (Vilven *et al.*, 1988) and $\gamma$-(Campbell *et al.*, 1988) subunit-specific antibodies on $Ca^{2+}$ current (see above). Furthermore, the survival of a $Ca^{2+}$ channel reconstituted from T-tubular membranes increases dramatically in the presence of activated $G_s$ (Yatani *et al.*, 1988). It is likely, therefore, that the 165-kDa subunit contains the $Ca^{2+}$-conducting part of a L-type $Ca^{2+}$ channel, which requires *in vivo* the presence of other smaller subunits and G proteins for proper function.

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