Ion Transport

Edited by

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The Structure of the Skeletal Muscle Calcium Channel

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1 Introduction

Voltage-activated Ca2+ 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 Ca²⁺ channels of different tissues probably are not identical and will be divided up further

ION TRANSPORT ISBN 0.12.403985.5 Copyright © 1989 Academic Press Limited All rights of reproduction in any form reserved 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 Ca²⁺ channel

Most of the information on the structural composition of the L-type Ca²⁺ 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 (α_1), 55 kDa (β) and 32 kDa (γ) in a constant ratio of 1:1.7:1.4 (Sieber et al., 1987). A further protein containing a 130- (α_2) and 28-kDa (δ) disulfide-linked peptide co-purifies in variable amounts with the α_1 -, β - and γ -subunits of the CaCB-receptor. The α_{2} - and the γ -peptides are heavily glycosylated, whereas the α_{1} - and β -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 (α_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 Ca²⁺ channel. Further evidence for the existence of a functional complex with this composition comes from studies with antibodies against the α_1 -subunit which precipitate the α_1 -, β - and γ -subunits (Takahashi et al., 1987). Antibodies specific against the α_1 - or β -subunits immunoprecipitates the α_1 - or β -subunits (Leung *et al.*, 1988). Furthermore, antibodies specific against the α_1 -, β - and γ -subunits modulate the Ca²⁺ current in vivo (Campbell et al., 1988; Morton et al., 1988; Vilven et al., 1988). Attempts to isolate only the α_1 -subunit under non-denaturing conditions have not been successful so far, suggesting that the β - and γ -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

	Phosphopeptide number					
Protein kinase	l65 kDa	55 kDa				
cAMP-kinase	1,10	7				
cGMP-kinase	1,2	1,3				
Protein kinase C	none	7/8				
Casein kinase H	7,11	9,10				

Table 1 Kinase-specific phosphopeptides of the skeletal muscle CaCB-red	eptor
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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 α_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 α_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 α_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 Ca²⁺ channel.



Fig. 1 Hydrophobicity profile and transmembrane topology of the skeletal muscle Ca^{2+} channel α_1 -subunit. The hydrophobicity profile of the α_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, scrine residue 687, is indicated between domains II and III.

4 Structure of the α_1 - and β -subunits of the skeletal muscle Ca²⁺ channel

Identification and cloning of the α_1 - subunit of the Ca²⁺ channel from skeletal muscle was a major step in Ca²⁺ channel research (Tanabe *et al.*,

1987). The cloned rabbit skeletal muscle α_1 -subunit has 29% homology with the voltage-dependent Na⁺ channel. It is assumed that four homologous regions, each consisting of five hydrophobic α -helices (S1, S2, S3, S5 and S6) and one hydrophilic α -helix (S4), span the membrane and form the Ca²⁺ 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 cel, 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 β -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 β -subunit agrees with that of a peripheral membrane protein. It contains four homologous α -helices (Fig. 2). Analysis of the primary structure reveals two further apparently specific protein kinase C phosphorylation sites. This is interesting since the β -subunit is preferentially phosphorylated by protein kinase C in vitro. Furthermore,



Fig. 2 Predicted secondary structure of the β -subunit of the skeletal muscle Ca²⁺ channel. The secondary structure of the deduced amino acid sequence was predicted by the method of Garnier *et al.* (1978). The β -subunit contains four hydrophilic helical regions (shaded rods), each composed of 26–33 amino acids. The helices are joined by β -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 β -subunit enhance Ca²⁺ currents through L-type channels and prevent the blocking action of nitrendipine (Vilven *et al.*, 1988), whereas antibodies against the γ -subunit inhibit Ca²⁺ current (Campbell *et al.*, 1988). This suggests that the two smaller subunits of the skeletal muscle Ca²⁺ channel are necessary for proper channel function.

The primary structure of the γ -subunit is not known. However, the primary structure of the 130-kDa (α_2) protein has been deduced from cloned rabbit skeletal muscle cDNA (Ellis *et al.*, 1988). The α_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 α_2 protein is expressed in many tissues, whereas the cDNA for the skeletal muscle α_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 (α_2/δ) protein may not be an essential part of the calcium channel.

5 Identification of L-type Ca²⁺ channel proteins in other tissues

A Ca^{2+} channel α_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 α -subunit is labelled in hippocampus (Striessnig *et al.*, 1988). These differences are not caused by species differences, since a monoclonal antibody to the α_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 α -subunits from different tissues.

		Size of putative α_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				

Table 2 Size of the putative α -subunit of the Ca²⁺ channel in other tissues

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 α_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 Ca²⁺ 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 singlechannel conductance of about 20 pS (Flockerzi et al., 1986a; Pelzer et al., 1988; Talvenheimo *et al.*, 1987). Its open state probability (p_{Ω}) is reduced by the Ca²⁺ channel blockers gallopamil and PN 200-110, and increases in the presence of the Ca²⁺ channel agonist Bay K 8644. The $p_{\rm O}$ 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 Ca²⁺ 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 $p_{\rm O}$ of the channel showing the smaller conductance was not affected by phosphorylation, Ca²⁺ 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 $p_{\rm O}$. The small conductance showed a regular voltage dependence of its open state probability, whereas the large conductance yielded a bell-shaped dependency. $p_{\rm O}$ 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 α_1 -subunit suggest that the 165-kDa subunit is the Ca²⁺-conducting unit (Pelzer *et al.*, 1988). This conclusion is supported by experiments in which the cloned cDNA of the α_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 α_1 -subunit and are defective in EC-coupling. The intranuclear injection and the expression of the cloned α_1 -subunit mRNA in myotubes of dysgenic mice restores EC-coupling and a slow L-type Ca²⁺ channel in these cells. Interestingly, the T-tubular voltage sensor and the Ca²⁺ channel is regained together with the expression of the α_1 -subunit, suggesting that both functions require the α_1 -subunit. These two functions may not depend alone on the presence of the 165-kDa (α_1)-subunit, but correct functioning may require the presence of the other subunits. This conclusion is supported also by the modulatory effect of β - (Vilven et al., 1988) and γ -(Campbell et al., 1988) subunit-specific antibodies on Ca^{2+} current (see above). Furthermore, the survival of a Ca²⁺ 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 Ca²⁺-conducting part of a L-type Ca²⁺ channel, which requires in vivo the presence of other smaller subunits and G proteins for proper function.

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