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High-Voltage Activated Ca^{2+} Channel

F. HOFMANN, M. BIEL, E. BOSSE, R. HULLIN, P. RUTH, A. WELLING, and V. FLOCKERZI

A. Introduction

Calcium channels are part of the signal system which is vital for intercellular communication in higher multicellular organisms. They transduce electrical or hormonal signals into a chemical second messenger, namely calcium. The cytosolic calcium concentration controls numerous cellular functions by binding to distinct calcium receptor binding proteins such as calmodulin, troponin, or calcium-activated potassium channels. Voltage-dependent calcium channels are of particular interest since their opening or closing determinates the cellular calcium concentration of many cells. In the normal heart they are essential to the generation of normal cardiac rhythm, to impulse propagation through the atrioventricular node, and to contraction in atrial and ventricular muscle. In vascular smooth muscle calcium channels provide part of the calcium that controls smooth muscle contraction and vascular tone. In skeletal muscle they are an essential part of the tubular excitation-contraction coupling mechanism. In neuronal and neuroendocrine cells they are essential for neurotransmitter release (for recent reviews see BERTOLINO and LLINAS 1992; BROWN and BIRNBAUMER 1990; MILLER 1992; RIOS et al. 1992; TRAUTWEIN and HESCHELER 1990).

B. Identified cDNAs of High-Voltage Activated Calcium Channels

High-voltage activated calcium channels are present in many tissues and are the major pathway for voltage-dependent calcium entry in excitable cells. They are activated at a high membrane potential, inactivate slowly (long lasting) and are readily blocked by different compounds. L-type calcium channels are blocked by the organic calcium channel blockers (CaCB) such as nifedipine and verapamil, N-type by ω-conotoxin, and P-type by the funnel web spider toxin ω-Age IVA (MINTZ et al. 1992). The principal channel-forming subunit of a high-voltage activated calcium channel is the α_1 subunit. When purified from rabbit skeletal muscle, this protein (apparent molecular mass 165 kDa) is associated with a 55-kDa protein (β), a 32-kDa protein (γ), and a disulfide-linked dimer of 130/28 kDa (α_2/δ) (see HORMANN et al. 1990 and references cited there). The primary structure of

Table 1. Classification of cloned and expressed mammalian calcium channel cDNA's

Gene	Snutch class	Source	Species	Functionally expressed	Sensitive to	Reference
α_1 subunit						
CaCh1	–	Skeletal muscle	Rabbit	Yes	DHP	TANABE et al. 1987
CaCh2a	C	Heart	Rabbit	Yes	DHP	MIKAMI et al. 1989
		Brain	Rat	–		SNUTCH et al. 1991
CaCh2b	C	Lung, smooth muscle	Rabbit	Yes	DHP	BIEL et al. 1990
		Brain	Rat	–		SNUTCH et al. 1991
		Aorta	Rat	Yes	DHP	KOCH et al. 1990
CaCh3	D	Brain	Human	Yes	DHP, ω-conotoxin	WILLIAMS et al. 1992a
		Brain	Rat	–		HUI et al. 1991
		Pancreatic islet	Human	–		SEINO et al. 1992
CaCh4	A	Brain	Rabbit	Yes	Spider venom	MORI et al. 1991
		Brain	Rat	–		STARR et al. 1991
CaCh5	B	Brain	Human	Yes	ω-Conotoxin	WILLIAMS et al. 1992b
		Brain	Rat	–	ω-Conotoxin	BUBEL et al. 1992
α_2/δ subunit						
$CaA_2$1a	–	Skeletal muscle	Rabbit	Yes	–	ELLIS et al. 1988; MIKAMI et al. 1989
		Brain	Human	Yes		WILLIAMS et al. 1992a
$CaA_2$1b	–	Brain	Rat	–		KIM et al. 1992
β subunit						
CaB1	–	Skeletal muscle	Rabbit	Yes	–	RUTH et al. 1989
		Brain	Rat	–		PRAGNELL et al. 1991
		Brain	Human	Yes	–	WILLIAMS et al. 1992a
CaB2*	–	Heart	Rabbit	Yes	–	HULLIN et al. 1992
		Brain	Rat	Yes	–	PEREZ-REYES et al. 1992
CaB3	–	Heart	Rabbit	Yes	–	HULLIN et al. 1992
γ subunit						
CaG1	–	Skeletal muscle	Rabbit	Yes	–	JAY et al. 1990; BOSSE et al. 1990

Only full length clones have been included in this table. The nomenclature used for the α_1 subunit is adapted from PEREZ-REYES et al. (1990). For the Snutch classes see SNUTCH et al. (1990). The references in the table refer to the first published sequence. In some cases functional expression of the particular clone has been reported in a different publication.
–, not reported; DHP, dihydropyridine; *, at least three different variants (a–c) of the same gene have been identified.

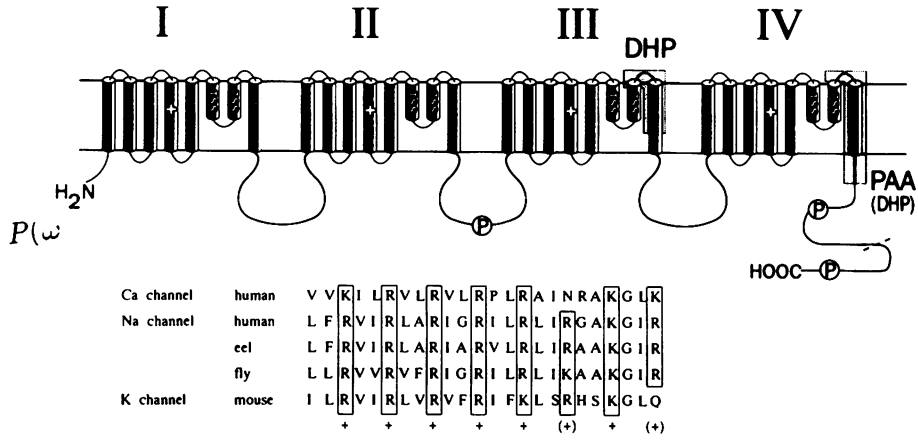

Fig. 1. Proposed topography of the α_1 subunit of the skeletal muscle calcium channel gene CaCh1. *Shaded areas*, proposed binding site for dihydropyridines (*DHP*) and phenylalkylamines (*PAA*). *P*, the in vitro identified cAMP kinase phosphorylation sites, *dashes*, the proposed truncation of the carboxy terminus; +, the amphipathic helix S4. *Below*, the amphipathic helix S4 sequence is compared with the S4 helices of other voltage-dependent ion channels

these proteins has been deduced by cloning their cDNAs (see Table 1 for references).

I. The α_1 Subunit

Complete cDNA clones of four different α_1 subunit gene products (CaCh 1–4) have been sequenced and shown to direct the synthesis of functional calcium channel after expression of their cRNA in *Xenopus oocytes* or cell culture cells (Table 1). The primary sequences of these different gene products are homologous to each other and predict a transmembrane topology which is similar to that of other voltage-dependent ion channels (Fig. 1). The primary sequences of the α_1 subunits predict proteins of 212–273 kDa containing four homologous repeats, each of which is composed of five hydrophobic putative transmembrane α helices and one amphipathic segment (S4) (TANABE et al. 1987; Fig. 1). The "extracellular" loop between the transmembrane helices 5 and 6 (SS1–SS2 region) is predicted to fold into the membrane and to form part of the pore of the channel (GUY and CONTI 1990). The skeletal and the cardiac/smooth muscle calcium channels are encoded by two different genes, CaCh1 and CaCh2. Several splice variants of the CaCh2 gene have been identified (BIEL et al. 1991). One major difference is the presence of two different exons at the transmembrane region IV S3 which alternate between the cardiac (CaCh2a) and smooth muscle (CaCh2b) isochannels. Polymerase chain reaction (PCR) amplification of the sequences around IV S3 suggests that a deletion within

each exon results in two additional splice variants (PEREZ-REYES et al. 1990). The two alternative channels CaCh2a and CaCh2b have been expressed stably in CHO-cells.

No major differences in basic electrophysiological characteristics have been observed, including the amplitude and voltage dependence of inward current and time of activation and inactivation (WELLING et al. 1992b). However, the two splice variants are expressed differentially in heart and smooth muscle (BIEL et al. 1991) and during cardiac development (DIEBOLD et al. 1992). The third gene is expressed in neuroendocrine tissues, whereas the forth gene appears to be brain specific. The currents induced by the expression of the CaCh1, CaCh2, and CaCh3 genes are inhibited by low concentrations of dihydropyridines and therefore are classified as L-type calcium channels. The neuroendocrine channel CaCh3 is inhibited only at rather high concentration by ω-conotoxin and is not an N-type calcium channel. The current through the CaCh4 gene product is not affected by dihydropyridines but is inhibited by low concentrations of a mixture of the funnel web spider toxines and has been classified as a neuronal P-type channel. The α_1 subunit of CaCh5 has a high-affinity ω-conotoxin binding site (DUBEL et al. 1992; WILLIAMS et al. 1992b) and is expressed as calcium channel only in the presence of the β and α_2 subunits. The current is inhibited at picomolar concentrations of ω-conotoxin (WILLIAMS et al. 1992b), identifying the CaCh5 protein as a neuronal N-type channel.

II. The α_2/δ Subunit

The deduced amino acid sequence of the α_2/δ protein is that of a membrane protein of 125 018 Da (ELLIS et al. 1988; Table 1). It contains three putative transmembrane segments and a large extracellular domain with several consensus sequences for glycosylation. The δ subunit sequence is identical with the carboxy terminal part of the deduced primary structure of the α_2 cDNA starting at amino acid 935 of the predicted sequence (DE JONGH et al. 1990). Presumably, the mature α_2 and δ proteins are the product of the same gene and arise by posttranslational processing. The mature α_2 protein may be located completely extracellularly, linked by a disulfide bridge to the transmembrane δ protein (JAY et al. 1991). Immuno- (NORMAN et al. 1987) and northern blots (ELLIS et al. 1990; BIEL et al. 1991) show that the α_2/δ protein is expressed in skeletal muscle, heart, brain, airway, vascular, and intestinal smooth muscle. Northern blots have identified a predominant 8-kB and a low-abundance 7-kB transkript with a skeletal muscle α_2 probe at high and low stringency (BIEL et al. 1991). Recently an α_2/δ cDNA has been cloned from human brain which is identical to the skeletal muscle α_2/δ cDNA (WILLIAMS et al. 1992a). A splice variant of this α_2/δ gene has been cloned from rat brain, which differs in part from the putative α_2 proteins but contains an identical δ protein (KIM et al. 1992). These results suggest that heart, brain, and smooth muscle express a conserved α_2 protein whereas brain contains an additional α_2/δ protein.

III. The β Subunit

The deduced primary sequence of the skeletal muscle β subunit is compatible with that of a peripheral membrane protein of 57868 Da (Table 1; RUTH et al. 1989). It contains four α-helical domains, each of which contains a homologous stretch of eight amino acids. Domains II, IIII, and IV contain a heptad repeat structure. Heptad repeats have been found in cytoskeletal proteins. This suggests that the β subunit may be a cytoskeletal protein which anchors the α_1 subunit to the cytoskeleton. The β-like proteins which are different from the skeletal muscle β subunit exist in heart, aorta, and brain and are derived from two different genes (CaB2 and CaB3; HULLIN et al. 1992). The primary transcript of CaB2 is differentially spliced and leads to the expression of at least three different isoforms (CaB2a, CaB2b, and CaB2c). The overall homology between the novel β subunits found in heart, aorta, and brain and the skeletal muscle β subunit (CaB1) is 71% for CaB2a, 71.5% for CaB2b, and 66.6% for CaB3. Northern blot and PCR analyses show that CaB1 is present in large amounts in skeletal muscle and brain, CaB2 in heart and aorta, and CaB3 in brain and tissues which are rich in smooth muscle such as aorta, lung, and trachea.

IV. The γ Subunit

The deduced primary sequence of the skeletal muscle γ subunit is in agreement with that of an integral membrane protein of 25058 Da (Table 1; BOSSE et al. 1990; JAY et al. 1990). The deduced sequence contains four putative transmembrane domains and two glycosylation sites which are located extracellularly. A complete cDNA for the γ subunit has been detected only in skeletal muscle. Northern and PCR analyses have not indicated that the same mRNA is present in higher concentrations in other tissues.

C. Structure-Function of the Cloned Calcium Channel Proteins

I. Expression and Function of the Channel Subunits

The cloned cDNA of the four calcium channel genes has been expressed in different cells (Table 2). The skeletal muscle α_1 subunit (CaCh1) has been expressed in L cells (PEREZ-REYES et al. 1989; LACERDA et al. 1991; VARADI et al. 1991) and skeletal muscle myotubes from mice with the muscular dysgenesis mutation (TANABE et al. 1988). Neither cell type has a functional α_1 subunit. The mice myotubes contained the α_2 and the other subunits may also be present. Expression of the skeletal muscle α_1 subunit (CaCh1) in L cells induces a barium current which activates extremely slowly ($\tau_{act.}$ $\approx$665 ms; PEREZ-REYES et al. 1989). Channel activation is accelerated 75-fold

Table 2. Functional effects of calcium channel subunits on currents induced by different α_1 subunits

α_1 Gene	Subunits expressed	Cell line	DHP sites[a]	I_{Ba}[b]	Activation time	Voltage dependence[c]	Reference
Heterologous subunits							
CaCh2a	α_2	Oocyte	–	(↑)	–	–	Mikami et al. 1989
CaCh2a	α_2	Oocyte	–	(↑)	↓	(↑)	Singer et al. 1991
CaCh2a	β	Ooctye	–	(↑)	(↓)	(↑)	Singer et al. 1991
CaCh2a	γ	Oocyte	–	(↑)	(↓)	↑ ↑	Singer et al. 1991
CaCh2a	$\alpha_2\beta$	Oocyte	–	↑ ↑	↓	↑	Singer et al. 1991
CaCh2a	$\alpha_2\beta\gamma$	Oocyte	–	↑ ↑	↓	↑ ↑	Singer et al. 1991
CaCh2a	β	Oocyte	–	↑	↓	≈	Wei et al. 1991
CaCh2a	$\beta\gamma$	Oocyte	–	↑	(↓)	(↑)	Wei et al. 1991
CaCh2b	β	CHO	↑	↑	(↓)	(↑)	Welling et al. 1992
CaCh2a	β	Oocyte	–	(↑)	–	–	Itagaki et al. 1992
CaCh3	β	Ooctye	–	↑	–	–	Williams et al. 1992a
CaCh4	$\alpha_2\beta$	Ooctye	–	↑ ↑	–	–	Mori et al. 1991
Homologous subunits							
CaCh1	β	L cell	↑	≈	↓	–	Lacerda et al. 1991
CaCh1	α_2	L cell	(↑)	≈	≈	–	Varadi et al. 1991
CaCh1	β	L cell	↑	↓*	≈	–	Varadi et al. 1991
CaCh1	γ	L cell	↓	↓*	≈	–	Varadi et al. 1991
CaCh1	$\beta\gamma$	L cell	↓	↓*	(↓)	–	Varadi et al. 1991
CaCh1	$\alpha_2\beta\gamma$	L cell	(↑)	(↓)	–	–	Varadi et al. 1991
CaCh2a	β_2	Oocyte	–	↑	↓	–	Hullin et al. 1992
CaCh2a	β_3	Oocyte	–	↑	↓	–	Hullin et al. 1992
CaCh2a	β_2	Oocyte	–	↑	↓	(↑)	Perez-Reyes et al. 1992
CaCh2a	β_2	COS	↑	–	–	–	Perez-Reyes et al. 1992
CaCh5	$\alpha_2\beta$	HEK293	↑ ↑§	↑ ↑	–	–	Williams et al. 1992b

All effects are compared with that of cells expressing only the α_1 subunit.
[a] The number of dihydropyridine binding sites per mg protein.
[b] Barium inward current.
[c] A shift in voltage dependence of the I/V curve or steady state activation or inactivation to more negative values.
α_2, β and γ are identical with CaA1a, CaB1 and CaG1. –, not reported; ≈, similar to cells expressing α_1 alone; *, I_{Ba} not sensitive to BayK 8644. (↑), ↑, and ↑ ↑ small, moderate and large increase or shift; (↓) and ↓, small and moderate decrease; §, ω-conotoxin binding sites.

($\tau_{act.} \approx 8$ ms) by the coexpression of the skeletal muscle β subunit (CaB1; LACERDA et al. 1991). Expression of the skeletal muscle α_1 subunit in the dysgenic myotubes generates cells with a slowly activating calcium current and normal skeletal muscle excitation-contraction coupling, which does not depend on the influx of calcium (TANABE et al. 1988). Expression of the cardiac muscle α_1 subunit (CaCh2a) produces myotubes with Ca^{2+} currents and excitation contraction coupling as in cardiac muscle (TANABE et al. 1990). TANABE et al. (1990) constructed several chimeras by starting with the cardiac muscle α_1 subunit and introducing skeletal musclelike intracellular loops. Changing the large intracellular loop that connects repeats II and III switched the mode of excitation-contraction coupling to that characteristic of skeletal muscle. Interestingly, however, the Ca^{2+} current produced by this chimera remained characteristic of cardiac muscle, i.e., rapidly activating. Chimeras in which the four homologous repeats of the cardiac muscle protein were each switched to the equivalent skeletal muscle sequence showed that changing merely the first homologous repeat switched the characteristics of the Ca^{2+} current from fast activating (cardiac type) to slowly activating (skeletal muscle type) whereas switching the other three repeats did not have this effect (TANABE et al. 1991).

Stable expression of the α_1 subunits from smooth muscle (CaCh2b) in CHO cells induces dihydropyridine-sensitive barium currents, which have the physiological characteristics as a smooth muscle calcium channel (BOSSE et al. 1992). The single-channel conductance is 26 pSi in the presence of 80 m*M* Ba^{2+}. The channel has the same voltage dependence of activation and inactivation as reported for the naturally occurring smooth muscle calcium channel. The cardiac α_1 subunit (CaCh2a) cDNA directs the expression of a channel with electrophysiological properties which are indistinguishable from those of the smooth muscle α_1 subunit (WELLING et al. 1992b). Stable coexpression of the CaCh2b protein with the skeletal muscle β gene (CaB1) increases in parallel the number of dihydropyridine binding sites and the amplitude of whole cell barium current, suggesting that the amplitude of inward current is directly related to the number of expressed α_1 subunits of the protein (WELLING et al. 1993). In addition, the coexpression of the β subunit decreases the activation time of the channel by a factor of two and shifts the voltage dependence of steady state inactivation by 18 mV to −13 mV (WELLING et al. 1993). Coexpression of the β subunit does not influence the sensitivity of the expressed channel toward the dihydropyridine agonist Bay K 8644. Similar results were obtained by coexpression of the cardiac (CaCh2a), smooth muscle (CaCh2b), neuroendocrine (CaCh3), and neuronal (CaCh4) α_1 subunit with the skeletal muscle β subunit (CaB1) in *Xenopus* oocytes (Table 2). In each case the current density increased with coexpression of the β subunit (CaB1). Expression of the N type α_1 subunit CaCh5 in HEK239 cells requires the presence of a neuronal β (CaB1) and α_2 subunit to induce ω-conotoxin binding sites and calcium current (WILLIAMS et al. 1992b).

The coexpression of the α_1 (CaCH2a) and β (CaB1–3) subunit together with the α_2 subunit cRNA enhanced also the barium current in *Xenopus* oocytes. In *Xenopus* oocytes the α_2 subunit (SINGER et al. 1991) and the β subunit (WEI et al. 1991) decreased the activation time of the channel (CaCh2a). Identical results were observed when the cardiac α_1 subunit (CaCh2a) was coexpressed with the cardiac (CaB2) or the smooth muscle/ neuronal (CaB3) β subunit and the α_2 subunit (HULLIN et al. 1992) in *Xenopus* oocytes. The skeletal muscle γ subunit (CaG1) shifted the voltage dependence of steady-state inactivation of the cardiac α_1 subunit (CaCh2a) by 40 mV to a negative membrane potential as is observed in skeletal muscle. These results suggest that (a) the skeletal muscle β subunit interacts with different α_1 subunits, (b) the β subunits increase barium currents by increasing the number of functional calcium channel proteins, and (c) the β subunits affect the activation time of the channel and the voltage dependence of steady-state inactivation. These conclusions are not supported by the experiments of VARADI et al. (1991), who reported that homologous coexpression of skeletal muscle α_1 and β, α_1 and γ, α_1, β and γ, α_1, α_2, β and γ in L cells decreases the inward current and the stimulatory effect of the calcium channel agonist Bay K 8644. The latter results are difficult to reconcile with those from other laboratories. They could be caused by a nonstoichiometric expression of the channel subunits, i.e., a higher expression of the β subunit than the α_1 subunit (LORY et al. 1992).

II. The Binding Sites for Calcium Channel Blockers

Photoaffinity labeling of the skeletal muscle α_1 subunit and expression of CaCh1 and CaCH2b gene in L cells (KIM et al. 1990) or CHO cells (BOSSE et al. 1992) shows that the α_1 subunit itself contains the binding sites for the known organic calcium channel blockers, the dihydropyridines, phenylalkylamines, and benzothiazepines. Binding of these drugs requires the binding of calcium to a high-affinity binding site (SCHNEIDER et al. 1991; STAUDINGER et al. 1991). The allosteric modulation of the dihydropyridine binding site by phenylalkylamine and benzothiazepine is preserved within each α_1 subunit (KIM et al. 1990; BOSSE et al. 1992). The current induced in cell culture cells or *Xenopus* oocytes by the CaCh1, CaCh2a, and CaCh2b proteins is increased by Bay K 8644, a calcium channel agonist, and is inhibited by the known calcium channel blockers.

Photoaffinity labeling of the purified skeletal muscle α_1 subunit by dihydrophyridines and phenylalkylamines suggests that the dihydropyridines bind to the SS1–SS2 region of repeat III (STRIESSNIG et al. 1991; NAKAYAMA et al. 1991) and apparently to a sequence following IVS6 (REGULLA et al. 1991) (Fig. 1). The extracellular location of the binding site at the SS1–SS2 region of repeat III is supported by the finding that dihydropyridines block the calcium channel from the extracellular space (KASS et al. 1991). The

phenylalkylamines label a second putative intracellular site located directly after the IVS6 (STRIESSNIG et al. 1990).

III. Phosphorylation of the Channel Proteins

The L-type current of cardiac, smooth and skeletal muscle, neuroendocrine, and neuronal calcium channels is modulated by hormones through the α subunits of different G-proteins (BROWN and BIRNBAUMER 1990). The open probability of the cardiac and skeletal muscle and of some neuroendocrine cells is increased by cAMP-dependent phosphorylation, suggesting that phosphorylation of the α_1 subunit or a different subunit of the calcium channel is important for its hormonal control. In skeletal muscle, about 90% of the full-length α_1 subunit (CaCh1) is apparently processed to a smaller protein with the carboxy terminus being close to amino acid residue 1690 (DE JONGH et al. 1991). cAMP-kinase phosphorylates in vitro rapidly Ser-687 (RÖHRKASTEN et al. 1988), which is located at the cytosolic loop between repeat II and III, and Ser-1854 (ROTMAN et al. 1992), which is present only in the full-length skeletal muscle α_1 subunit, and slowly Ser-1617 (RÖHRKASTEN et al. 1988). Phosphorylation of these sites may be significant, since the open probability of the reconstituted skeletal muscle CaCB-receptor/calcium channel is increased several-fold by cAMP-dependent phosphorylation (FLOCKERZI et al. 1986; HYMEL et al. 1988; NUNOKI et al. 1989; MUNDINA-WEILENMANN et al. 1991). The α_1 subunit is phosphorylated also in vivo at least at two sites in response to isoproterenol in isolated rat myocytes (LAI et al. 1990; MUNDINA-WEILENMANN et al. 1991).

These in vivo phosphorylation sites may be identical with Ser-687 and Ser-1854. However, it is not clear which of these phosphorylation sites – one of which is present only in the unprocessed α_1 subunit – affect the open probability of the skeletal muscle calcium channel. The in vitro identified phosphorylation sites of the CaCh1 gene are not conserved in the sequences of the other calcium channel genes and therefore are not important for the hormonal regulation of the calcium channel in heart and neuroendocrine cells. The hormonal control of the calcium channels may be exerted by tissue specific β subunits. The deduced amino acid sequence of the skeletal β subunit (CaB1) contains several potential phosphorylation sites. Two of these sites, Ser-182 and Thr-205, are phosphorylated in vitro by cAMP kinase (RUTH et al. 1988; DE JONGH et al. 1989). The products of the CaB2 gene contain a cAMP-kinase phosphorylation site equivalent to Thr-205 of CaB1. This phosphorylation site is not present in the product of CaB3, which is expressed mainly in brain and smooth muscle (HULLIN et al. 1992). This is interesting since in vivo whole cell calcium current is increased in heart (KAMEYAMA et al. 1986) and skeletal muscle (GARCIA et al. 1990) but not smooth muscle (WELLING et al. 1992a) by cAMP-dependent phosphorylation. Expression of the cardiac α_1 subunit with the α_2 and β subunit in *Xenopus* oocytes indicates that cAMP-dependent regulation of

the cardiac calcium channel is mediated by phosphorylation of the β subunit (KLÖCKNER et al. 1992; DASCAL et al. unpublished observation).

D. Conclusion

High-voltage activated calcium channels are encoded by different genes. Their electrophysiological and hormonal regulation may depend on the coexpression of different subunits. The interaction of these channel subunits with additional proteins such as the α subunit of trimeric G-proteins may be required for basic and hormonal regulation of the channel (HAMILTON et al. 1991; CAVALIE et al. 1991; KLEUSS et al. 1991). The availability of the cloned cDNA of several channel proteins and channel regulators will facilitate understanding of the complexities of voltage-gated calcium channels.

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