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MOLECULAR BASIS FOR CA^{2+} CHANNEL DIVERSITY

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TYPES OF HIGH VOLTAGE–ACTIVATED CALCIUM CHANNELS

Neurotransmitter release, neurosecretion, neuronal excitation, survival of neurons, and many other neuronal functions are controlled by the cellular calcium concentration. Calcium entry across the plasma membrane in response to membrane depolarization or activation of neurotransmitter receptors represents a major pathway for the cellular control of calcium. The voltage-dependent calcium channels, activated and inactivated at a low or high membrane potential, are the best characterized plasmalemmal calcium entry pathway, primarily because powerful and specific channel blocking agents are available. The T (tiny)-type calcium channel is activated and inactivated at a low membrane potential and is present in a wide variety of excitable and non-excitable cells. T-type calcium channels are not considered in detail because the lack of specific blockers renders identification of cloned and expressed channels as T-type channels difficult.

In contrast, the high voltage activated and inactivated calcium channels have been subdivided into four distinct classes, using the organic calcium channel blockers (CaCB) (originally introduced by Fleckenstein and colleagues 1967) and several neurotoxins. They have been separated into the B (B stands for brain; B channels may include T-type channels)-, L (long lasting)-, N (neither L nor T channel)-, and P (Purkinje)-type calcium channels (Table 1). B-, L-, N- and P-type calcium channels are activated at a high membrane potential (around −30 mV), inactivate slowly (long lasting), and are expressed in neuronal and nonneuronal cells (Tsien et al 1991, Bertolini & Llinás 1992). N- and P-type calcium channels are blocked specifically by

Table 1 Cloned and expressed mammalian calcium channel subunit cDNAs[a]

Gene	Type	Numa	Agreed[b] nomenclature	Source	Species	Functionally expressed	Sensitive to	Reference
α_1 subunits								
CaCh1	L	Sk	S	Skeletal m	Rabbit	Yes	DHP	Tanabe et al 1987
CaCh2a	L	C	Ca	Heart	Rabbit	Yes	DHP	Mikami et al 1989
				Brain	Rat	—		Snutch et al 1991
				Heart	Rat	—		Diebold et al 1992
				Brain	Mouse	Yes		Ma et al 1992
CaCh2b	L	—	Cb	Lung, smooth m	Rabbit	Yes	DHP	Biel et al 1990
				Brain	Rat	—		Snutch et al 1991
				Aorta	Rat	Yes		Koch et al 1990
				Heart	Rat	—		Diebold et al 1992
				Fibroblast	Human	—		Soldatov 1992
				Brain	Mouse	Yes		Ma et al 1992
CaCh3	L	—	D	Brain	Human	Yes	DHP	Williams et al 1992b
				Pancreatic islet	Human	—		Seino et al 1992
CaCh4	P	BI	A	Brain	Rabbit	Yes	Spider venom	Mori et al 1991
				Brain	Rat	—	ω-Aga IVA	Starr et al 1991
CaCh5	N	BIII	B	Brain	Human	Yes	ω-CTX GVIA	Williams et al 1992a
				Brain	Rat	—		Dubel et al 1992
				Brain	Rabbit	Yes	ω-CTX GVIA	Fujita et al 1993
CaCh6	B	BII	E	Brain	Rabbit	—	—	Niidome et al 1992
				Brain	Rat	Yes	—	Soong et al 1993

α_2/δ subunits							
$CaA_2$1a	—	$\alpha2/\delta$a	Skeletal m	Rabbit	Yes	—	Ellis et al 1988
$CaA_2$1b	—	$\alpha2/\delta$b	Brain	Human	Yes	—	Williams et al 1992b
			Brain	Rat	—		Kim et al 1992
β subunits							
CaB1*	—	β_1	Skeletal m	Rabbit	Yes	—	Ruth et al 1989
			Brain	Rat	—		Pragnell et al 1991
			Brain	Human	Yes	—	Williams et al 1992b
			Brain	Human		—	Powers et al 1992
CaB2*	—	β_2	Heart	Rabbit	Yes	—	Hullin et al 1992
			Brain	Rat	Yes	—	Perez-Reyes et al 1992
CaB3*	—	β_3	Heart	Rabbit	Yes	—	Hullin et al 1992
CaB4	—	β_4	Brain	Rat	Yes	—	Castellano et al 1993
γ subunit							
CaG1	—	γ	Skeletal m	Rabbit	Yes	—	Bosse et al 1990 Jay et al 1990

[a] Only full-length clones have been included in this table. The nomenclature for the α_1 subunit is adapted from Perez-Reyes et al (1990). The Numa nomenclature is used in his laboratory for the brain calcium channels. The agreed nomenclature is based on that of Snutch et al (1990). The references are 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; ω-CTX GVIA, ω-conotoxin VIA; * at least three different variants (a–c) of the same gene have been identified.

[b] In August 1993 several laboratories agreed to use in the future following nomenclature for mammalian voltage-dependent calcium channels: α_1 subunits and their important splice variants are identified by the capital Roman letters A, B, . . . (S for skeletal muscle) and the small Roman letters a, b, . . ., respectively (example $\alpha 1_{Ca}$ = CaCh2a = α_1 subunit of the cardiac L-type calcium channel; α_2/δ subunits are identified by α_2/δ with, if necessary, small Roman letter a, b, . . . indicating the splice variant; β subunits are identified by Arabic numbers 1, 2, ... as index (example β_1); γ subunit is identified by γ; The complex of the skeletal muscle calcium channel will be $\alpha 1_S \alpha 2/\delta \beta_1 \gamma$.

w-conotoxin GVIA and the funnel web spider toxin ω-Aga-IVA, respectively (Mintz et al 1992b). Both channels have been identified in neurons and neuroendocrine cells. P-type channels are also present in the distal convoluted tubule of rat kidney (Yu et al 1992).

L-type channels are readily blocked by the classical CaCB's nifedipine (a 1,4-dihydropyridine, DHP), verapamil (a phenylalkylamine, PAA), and diltiazem (a benzothiazepine) (Catterall et al 1988, Glossmann & Striessnig 1988, Hofmann et al 1990). L-type calcium channels are expressed in neuronal and endocrine cells, in cardiac, smooth and skeletal muscle, in fibroblasts and kidney. In skeletal muscle they are essential for excitation-contraction coupling, which does not require calcium influx through the channel (Rios et al 1992). In the normal heart they are necessary for the generation and propagation of electrical impulses and for the initiation of contraction in atrial and ventricular muscle. In smooth muscle they are involved in tension development, for which process they provide part of the necessary calcium. L-type channels apparently also control the intracellular calcium concentration in other cells. However, they are not involved in neurotransmitter secretion, which process is linked in many neuronal cells to N-type channels. B-type channels have been identified by cloning as a major neuronal calcium channel (Niidome et al 1992, Soong et al 1993). These cDNA clones could be responsible for a neuronal calcium current that is not blocked by a combination of blockers for L-, N- and P-type calcium channels (nimodipine, ω-conotoxin GVIA and ω-Aga-IVA) (Mintz et al 1992a).

COMPOSITION OF THE CALCIUM CHANNEL

The basic insight into the composition and the functional domains of calcium channels has been derived mainly from work carried out with the channel purified from skeletal muscle. However, caution is needed when the results obtained with the skeletal muscle channel are applied to the other channels because they may reflect specific properties of the skeletal muscle calcium channel that are not retained in the other channels. The L-type calcium channel as purified from skeletal muscle contains four proteins (Figure 1): the α_1 subunit (212 kDa), which contains the binding sites for all known CaCBs and the calcium conducting pore; the intracellularly located β subunit (57 kDa); the transmembrane γ subunit (25 kDa); and the α_2/δ subunit, a disulfide-linked dimer of 125 kDa (see Catterall et al 1988, Glossmann & Striessnig 1988, Hofmann et al 1990, and references cited there). Reconstitution of the purified complex into phospholipid bilayers results in functional calcium channels that are reversibly blocked by CaCBs and are modulated by cAMP-dependent phosphorylation (Flockerzi et al 1986, Hymel et al 1988, Nunoki et al 1989, Mundiña-Weilenmann et al 1991). The primary sequences of these proteins

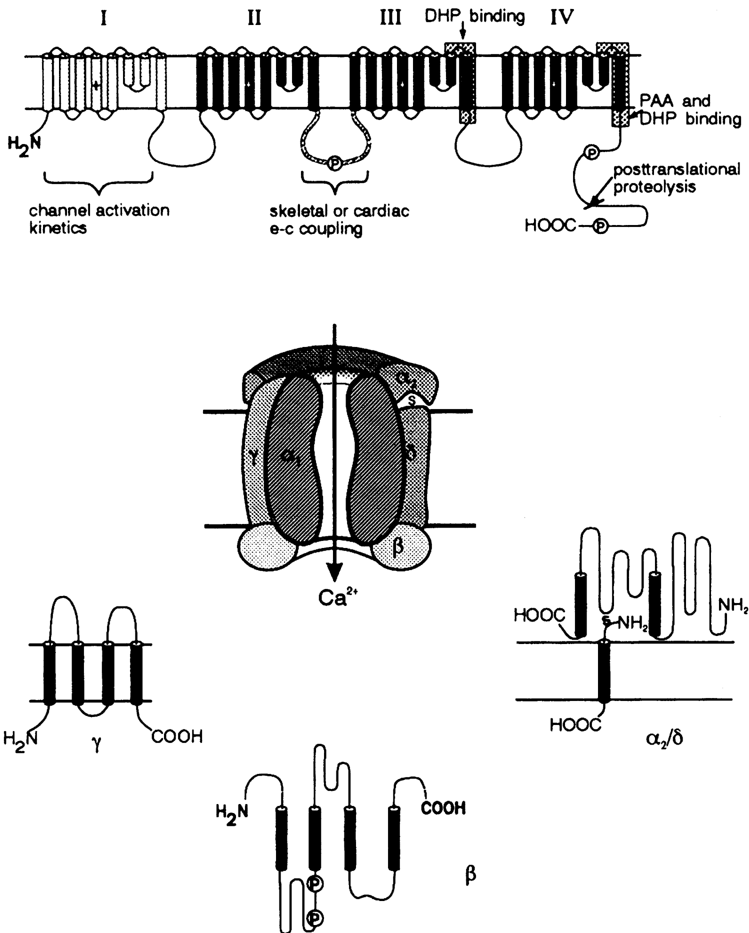

Figure 1 Proposed structure of the skeletal muscle calcium channel. The putative transmembrane configuration of individual subunits is taken from the hydropathicity analysis of the primary sequences. The suggested structure of the α_1 subunit is shown at the top. I, II, III, IV, proposed repeats of calcium channel α_1; +, proposed transmembrane amphipathic α helix, respectively the proposed voltage sensing helix of the channel; (P), sites phosphorylated in vitro by cAMP kinase; the SS1-SS2 region suggested to be part of the channel pore is shown by the short barrels; DHP and PAA, dihydropyridine and phenylalkylamine binding sites; e-c coupling, excitation contraction coupling. The brackets indicate parts of the protein that are responsible for the skeletal (CaCh1) or cardiac (CaCh2) properties of the channel. The dash at the carboxyterminal part indicates the area where the α_1 subunit is processed posttranslationally. The suggested structure of the γ, β, and α_2/δ subunit is shown on the bottom. s, disulfide bridge between the transmembrane δ and the extracellular α_2 subunit. The extracellular space is above the horizontal lines.

have been deduced by cloning their corresponding cDNAs from rabbit skeletal muscle (Tanabe et al 1987, Ellis et al 1988, Ruth et al 1989, Bosse et al 1990, Jay et al 1990). Using these cDNAs as probes, several laboratories have cloned different α_1 and β subunits from heart, smooth muscle, endocrine, and neuronal cells.

SUBUNITS OF THE CALCIUM CHANNEL

The α_1 Subunit

Complete cDNA clones of α_1 subunits that direct the expression of functional calcium channels in *Xenopus laevis* oocytes or cell culture cells have been isolated from skeletal, cardiac, smooth muscle, endocrine glands, and brain (Table 1). The primary sequences of these cDNAs are homologous to each other and encode proteins of predicted molecular masses of 212 to 273 kDa and exhibit homologies from 41% to 70%. Hydropathicity analysis of all α_1 subunits predicts a transmembrane topology similar to that of other voltage-dependent ion channels with four homologous repeats, each containing five hydrophobic putative transmembrane α helices and one amphiphathic segment (S4) (Figure 1). By functional expression of chimeras of the skeletal (CaCh1) and the cardiac (CaCh2a) muscle α_1 subunit, specific properties of the calcium channel were assigned to distinct parts of the ion conducting pore: Repeat I determines the activation time of the chimeric channel, i.e. slow activation upon membrane depolarization with the repeat from skeletal muscle and rapid activation with that from cardiac muscle (Tanabe et al 1991); the putative cytoplasmic loop between repeats II and III determines the type of excitation-contraction coupling. The loop from the skeletal muscle calcium channel α_1 subunit induces contraction in the absence of calcium influx, whereas the loop from the cardiac calcium channel α_1 subunit induces contraction only in the presence of calcium influx (Tanabe et al 1990). The "extracellular" loop between transmembrane helices 5 and 6 (SS1-SS2 region) is predicted to fold into the membrane, to form part of the pore of the channel (Guy & Conti 1990) and to take part in the control of ion selectivity (Heinemann et al 1992).

Photoaffinity labelling of skeletal muscle α_1 subunit followed by limited proteolysis and immunoprecipitation indicates that the DHP binding site is localized close to the SS1-SS2 region of repeat III (Striessnig et al 1991, Nakayama et al 1991) and to a sequence following the IVS6 segment (Regulla et al 1991), whereas the PAA binding site has been located directly after the IVS6 segment (Striessnig et al 1990) (Figure 1). Site directed mutagenesis of the α_1 subunit indicates that the loop between IVS5 and IVS6 is important for the inhibition of current by DHPs. Binding studies with radiolabelled DHPs demonstrate that the stably expressed α_1 subunits from skeletal and

smooth muscle alone contain the allosterically coupled binding sites for the known CaCBs (Kim et al 1990, Bosse et al 1992). The cDNAs cloned so far for the α_1 subunits are encoded by the six different genes, CaCh1 to CaCh6 (Table 1).

The CaCh1 Gene

The mRNA of the CaCh1 gene was originally cloned from rabbit skeletal muscle (Tanabe et al 1987). Carp skeletal muscle expresses a homologous α_1 subunit (Grabner et al 1991). The product of the CaCh1 gene occurs in rabbit skeletal muscle in two isoforms: a minor form (~ 5%) of 212 kDa containing the complete amino acid sequence encoded by α_1 mRNA, and a major form (~ 95%) of 190 kDa which is derived from the full-length product by posttranslational proteolysis close to amino acid residue 1690 (De Jongh et al 1991). Presumably, the shorter form is involved functionally at the triad in E-C coupling of the skeletal muscle, because the expression of a skeletal muscle CaCh1 mRNA truncated at amino acid residue 1662 fully restores in dysgenic myotubes both excitation-contraction coupling and calcium current (Beam et al 1992). The short and the long form are phosphorylated rapidly in vitro by cAMP-dependent protein kinase at 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 larger untruncated form, and slowly at Ser-1617 (Röhrkasten et al 1988). Additional forms of the CaCh1 gene may be present in skeletal muscle. Malouf and coworkers (1992) cloned an α_1 subunit cDNA that lacks repeats II and III and has an apparent molecular weight of 155 kDa. This form might be expressed in the skeletal muscle of newborn rabbits. Using peptide specific antibodies, Brawley & Hosey (1992) detected an α_1 subunit–like protein in rabbit skeletal muscle which contained an immunological identical amino and carboxyterminus but lacked part of the loop between repeats II and III. This protein was smaller (160 kDa) than the mature α_1 subunit (165 kDa) and was not retained by a wheat germ agglutinin column. The α_1 subunit–like protein was enriched in fractions containing mostly the sarcoplasmic cisternal membranes. Neither the identity of the proteins described by Malouf et al (1992) and Brawley & Hosey (1992) nor their functional significance has been established. Partial clones of the CaCh1 gene mRNA have been detected in other tissues including kidney and brain by the PCR technique, which suggests that the CaCh1 gene may be functionally expressed in skeletal muscle and other tissues.

The CaCh2 Gene

The α_1 subunits from cardiac (CaCh2a) (Mikami et al 1989) and smooth (CaCh2b) muscle (Biel et al 1990) are splice products of the second gene (CaCh2). This gene is expressed in most excitable and many nonexcitable

tissues including fibroblasts (see Table 1). The deduced amino acid sequence of CaCh2b is about 95% identical with that of CaCh2a. A major difference between the cardiac and the smooth muscle α_1 subunits is the use of alternative exons for the IVS3 segment (Perez-Reyes et al 1990). The alternative spliced primary transcripts of the CaCh2 gene have been identified in many tissues including kidney, heart, and brain (Perez-Reyes et al 1990, Biel et al 1991, Snutch et al 1991, Diebold et al 1992, Ma et al 1992, Yu et al 1992). Additional splice variations of the CaCh2 gene product have been noted (Perez-Reyes et al 1990, Biel et al 1990, Snutch et al 1991, Diebold et al 1992, Yu et al 1992, Schultz et al 1993). With one exception the functional significance of these splice variations is not clear. The two alternative splice variants CaCh2a and CaCh2b have been expressed transiently and stably in *Xenopus laevis* oocytes and CHO cells (Biel et al 1990, Biel et al 1991, Singer et al 1991, Bosse et al 1992, Lory et al 1993, Schultz et al 1993). No major differences have been observed in basic electrophysiological and pharmacological properties of the two isoforms including the amplitude of inward current, steady state activation and inactivation (Welling et al 1992a). However, recent experiments show that the DHP nisoldipine inhibits barium currents through the smooth muscle α_1 subunit (CaCh2b) at over 10-fold lower concentrations than that through the cardiac muscle α_1 subunit (CaCh2a) (Welling et al 1993b). The putative DHP binding sites, the loops between IIIS5 and IIIS6 and IVS5 and IVS6, are identical in both α_1 subunits and therefore not responsible for this remarkable difference. Northern blots and PCR analysis show that both splice variants are differentially expressed in heart and smooth muscle (Biel et al 1991) and during cardiac development (Diebold et al 1992). The vascular and cardiac muscle calcium channels exhibit in vivo a difference in their DHP sensitivity similar to that of the cloned and stably expressed channels, suggesting that the distinct pharmacology of the vascular and cardiac calcium channels is based on the expression of the alternatively spliced CaCh2 mRNA.

The CaCh3 and CaCh4 Genes

The cDNA of the third gene (CaCh3) was isolated from neural and endocrine tissues and represents a neuroendocrine specific L-type calcium channel (Williams et al 1992a, Seino et al 1992), whereas the gene products of the fourth and fifth gene (CaCh4 and CaCh5) were cloned from neuronal cDNA libraries. Calcium channels transiently expressed from cRNA of CaCh4 induce high voltage–activated calcium currents that are insensitive to nifedipine and ω-conotoxin but are inhibited by a mixture of toxins from the funnel web spider, thus characterizing this channel as a P-type calcium channel (Mori et al 1991). Transcripts of the CaCh3 and CaCh4 genes have been detected in both the brain and the kidney by PCR and Northern blots, suggesting again

a widespread occurence of the channels. The P-type channel (CaCh4) mRNA was predominantly present in the distal convolute tubule of renal cortex (Yu et al 1992).

The CaCh5 and CaCh6 Genes

The gene product of the CaCh5 has been cloned exclusively from brain. Coexpression of the CaCh5 α_1 subunit in myotubes of dysgenic mice (Fujita et al 1993) or together with the α_2/δ and β subunit (see below) in *Xenopus laevis* oocytes induces a barium current that is inhibited by picomolar concentrations of ω-conotoxin GVIA (Williams et al 1992a). In addition, the cloned α_1 subunits bind ω-conotoxin GVIA with high affinity, (Dubel et al 1992) thus identifying the CaCh5 protein as a neuronal N-type calcium channel. A B-type calcium channel α_1 subunit (CaCh6) has been cloned from a brain library (Niidome et al 1992, Soong et al 1993). The mRNA of this channel is abundant in brain and has been expressed functionally in *Xenopus laevis* oocytes or other cells. The expressed channel has properties of a low voltage-activated T-type channel, i.e. activation occurs at membrane potentials below 0 mV; the inward current is not affected by DHPs or ω-conotoxin and only partially inhibited by ω-Aga IVA. In contrast to T-type channels, the expressed channel inactivated relatively slowly. Therefore, its identity with T-type channels remains to be established.

Other α_1 Subunits

Two additional α_1 (Doe 1 and Doe 4) have been cloned from the marine ray *Discopyge omnata* (Horne et al 1993). The Doe 4 sequence most closely resembles the mammalian CaCh5 gene product, a N-type channel. Doe 1 is related to the gene product of CaCh6. In contrast to the expressed CaCh6 cDNA (Soong et al 1993) expression of the Doe 1, cDNA yields a rapidly inactivating, high voltage activated channel, which is blocked by micromolar ω-conotoxin GVIA but not by DHPs or ω-Aga IVA (Ellinor et al 1993)

The α_2/δ Subunit

The skeletal muscle α_2/δ subunit (CaA1) is a glycosylated membrane protein of 125 kDa (Ellis et al 1988) which is apparently highly conserved in most tissues. In the skeletal muscle the primary protein product of the α_2/δ gene is processed posttranslationally by proteolysis, resulting in an α_2 protein containing amino acid 1 through 934 and a δ protein containing the amino acid 935 through 1080 (DeJongh et al 1990). The transmembrane δ subunit anchors the α_2 protein located extracellularly by disulfide bridges to the plasma membrane (Jay et al 1991). Two identical α_2/δ cDNAs isolated from human (Williams et al 1992b) and rat brain (Kim et al 1992) are splice variants of the skeletal muscle α_2/δ cDNA. The α_2/δ cDNA isolated from rat brain

predicts an identical δ protein and a splice variant of the processed α_2 protein. Immunoblots (Norman et al 1987) and Northern blots (Ellis et al 1988, Biel et al 1991) show that similar or identical α_2/δ subunits exist in skeletal muscle, heart, brain, vascular, and intestinal smooth muscle, suggesting that the α_2/δ subunit is expressed together with the various α_1 subunits. However, this conclusion may be not valid for all cell types. Differentiating BC3H1 cells express DHP binding sites (Biel et al 1991) and skeletal muscle L-type calcium channel (Caffrey et al 1987). As expected, only the differentiating BC3H1 cells contain the mRNA for skeletal muscle α_1, β and γ subunits. They do not contain transcripts specific for the α_2/δ subunit (Biel et al 1991), suggesting that, at least in this cell line, the expression of the α_2/δ gene is not coordinated with that of the other calcium channel subunits.

The β Subunit

The skeletal β subunit (CaB1) is a membrane protein, located intracellularly, consisting of 524 amino acids (Ruth et al 1989). Its deduced amino acid sequence contains stretches of heptad repeat structure characteristic of cytoskeletal proteins. Transcripts of two other genes (CaB2 and CaB3) encoding β proteins different from the skeletal muscle β subunit have been isolated from a cardiac cDNA library (Hullin et al 1992). A fourth β subunit, CaB4, has been cloned from rat brain (Castellano et al 1993). The deduced amino acid sequence of CaB2 and CaB3 show an overall homology to CaB1 of 71% (CaB2) and 66.6% (CaB3). Differential splicing of the primary transcript of CaB1 results in at least three isoforms: CaB1a through CaB1c (Ruth et al 1989, Pragnell et al 1991, Williams et al 1992b, Powers et al 1992). CaB1a is expressed in skeletal muscle whereas two other isoforms are expressed in brain, heart, and spleen (Powers et al 1992). Four different splice variants have been characterized for the CaB2 gene (CaB2a through CaB2d); CaB2a and CaB2b have been isolated from a rabbit cardiac cDNA library whereas CaB2c and CaB2d have been cloned from rabbit and rat brain libraries (Hullin et al 1992, Perez-Reyes et al 1992). Like the CaB1 gene, the CaB2 and CaB3 genes are expressed tissue specifically with transcripts of CaB2 existing abundantly in heart and to a lower degree in aorta, trachea, and lung, whereas transcripts of CaB3 genes are found in brain and smooth muscle containing tissues such as aorta, trachea, and lung (Hullin et al 1992). This suggests that the CaB3 gene product may be expressed predominantly in neuronal and smooth muscle cells. The CaB4 gene is expressed in the brain, predominantly in the cerebellum and kidney (Castellano et al 1993).

The γ Subunit

The γ subunit (CaG1) consists of 222 amino acids and is an integral membrane protein (Bosse et al 1990, Jay et al 1990). Its deduced amino acid sequence

contains four putative transmembrane domains and two glycosylation sites which are located at the extracellular side. Northern and PCR analysis have not identified the presence of γ subunit in other tissues; this fact suggests that the protein may be specific for skeletal muscle.

Other Subunits

The existence of additional subunits has been suggested from the copurification of unidentified peptides with the DHP-receptor protein (for example, see Kuniyasu et al 1992) without proving their functional necessity. In contrast, a novel subunit referred to as CCCS1 (candidate for calcium channel subunit) has been identified by suppression cloning in neuronal tissue of *Torpedo california* (Gundersen & Umbach 1992). The 21.7 kDa protein is required for the expression of the *Torpedo california* N-type calcium channel in *Xenopus* oocytes. The sequence of this protein is not related to any of the above described calcium channel subunits. It is similar to two cysteine rich proteins from *Drosophila* which were localized to nerve terminals. It is not known if mammalian N-type channels contain a related subunit, since small molecular weight proteins have not been detected in purified (McEnery et al 1991, Witcher et al 1993) nor in immunoprecipitated (Sakamoto & Campbell 1991, Ahlijanian et al 1991) rat brain ω-conotoxin binding sites.

FUNCTIONAL INTERACTION OF THE CALCIUM CHANNEL SUBUNITS

All cDNAs of Table 1 have been expressed singly or in combination with other subunits in *Xenopus laevis* oocytes or cell culture cells as functional ion channels. Transient expression in *Xenopus laevis* oocytes of CaCH2a cRNA (Mikami et al 1989) and CaCH2b cRNA (Biel et al 1990) induces DHP-sensitive currents with electrophysiological properties similar to those reported for cardiac and smooth muscle. Heterologous coexpression of the cardiac α_1 subunit together with the skeletal muscle β subunit and α_2/δ subunit enhanced consistently the inward current to amplitudes greater than 1 *mu*A/oocyte (Singer et al 1991). The α_2/δ or the β subunit alone or the combination of both decreased the activation time of the barium current twofold (Singer et al 1991, Wei et al 1991). Oocytes containing all four subunits (α_1, α_2/δ, β, γ subunit) had fast inactivating barium currents. The coexpression of the γ subunit shifted the steady state inactivation of I_{Ba} by 40 mV to negative membrane potentials (Singer et al 1991). Under each condition inward currents were increased severalfold by the calcium channel agonist BayK 8644. Homologous coexpression of the cardiac α_1 subunit with the cardiac β (CaB2) or the neuronal/smooth muscle β subunit (CaB3) with or without the α_2/δ

subunit results in an increase in the amplitude of I_{Ba} as well as in an acceleration of channel activation (Hullin et al 1992, Castellano et al 1993).

The four neuronal α_1 subunit cDNAs, i.e. the neuroendocrine L-type CaCh3 gene, the neuronal P-type CaCh4 gene, the neuronal CaCh5 gene, and the neuronal CaCh6 gene induce barium currents only when coexpressed with the α_2/δ and β subunit (Mori et al 1991, Williams et al 1992a,b; Fujita et al 1993, Soong et al 1993). The increase in current occurred always in the presence of the β subunit, most likely by an increased number of plasmalemmal calcium channel molecules (see also below). These results suggest that the skeletal muscle and other β subunits interact with different α_1 subunits by a common interaction site and mechanism.

Similar effects of the subunits were obtained by stable coexpression of the skeletal muscle α_1 and β subunit in mouse fibroblasts (L cells), which do not contain an endogenous calcium channel. The β subunit decreased the activation time of the expressed channel over 50-fold, and increased the number of DHP binding sites 2-fold (Lacerda et al 1991). In contrast to these results, coexpression of all four skeletal muscle subunits in L cells resulted in a decreased amplitude of the barium current and in a diminished response toward the calcium channel agonist BayK 8644 (Varadi et al 1991). In a later publication (Lory et al 1992) the same group reported that the decreased sensitivity toward the channel agonist BayK 8644 was caused by an overexpression of the β subunit in a 10:1 ratio over the α_1 subunit. The relative ratio of the α_1 and β subunit was estimated from Northern blots. Whether these Northern blots reflected the real mRNA and protein levels of the transfected α_1 and β subunits is unknown, making it difficult to evaluate the significance of this report. A similar phenomenon has not been observed by other groups using transient or permanent coexpression of other L-type α_1 subunits together with various β subunits in *Xenopus* oocytes, L or CHO cells.

The smooth muscle α_1 (CaCh2b) subunit expressed in CHO cells causes barium currents that are identical to those of native smooth muscle: the single channel conductance was 26 pSi in the presence of 80 mM Ba2+, the open probability increased with membrane depolarization, and the voltage-dependence of activation and inactivation was similar to that of the native smooth muscle channel (Bosse et al 1992). Stable expression of the CaCh2b with the skeletal muscle β gene (CaB1) increased in parallel the number of DHP binding sites and the amplitude of whole cell barium current; thus the amplitude of the inward current is probably directly related to the number of functional α_1 protein molecules (Welling et al 1993a). Coexpression of the α_1 subunit and the β subunit did not increase transcription or translation of the α_1 subunit gene, but apparently affected "maturation" of a functional channel (Nishimura et al 1993). The coexpression of the α_1 subunit with the CaB2 or CaB3 β subunit increased in parallel the inward current and the

density of the DHP binding sites. The BayK 8644 sensitivity of the barium currents was retained in all cell lines, although different concentrations of β subunit protein were expressed (Welling et al 1993a). The coexpression of the CaB1 β subunit decreased the channel activation time two-fold and shifted the voltage dependence of steady-state inactivation by 18 mV to −13 mV. Coexpression of the skeletal muscle α_2/δ subunit together with the smooth muscle α_1 and skeletal muscle β subunit produced channels that inactivated faster when calcium was used as a charge carrier.

The expression of the cardiac α_1 subunit (CaCh2a) in the same cells induces currents indistinguishable from those induced by the smooth muscle α_1 subunit (Welling et al 1992a). This electrophysiological similarity is not surprising, since the primary sequence of both channels is 95% identical (Biel et al 1990). The only difference noted was a faster activation of the cardiac channel and a higher sensitivity of the smooth (CaCh2b) than cardiac (CaCh2a) muscle channel toward the block by the DHP nisoldipine (Welling et al 1993b).

HORMONAL MODULATION OF EXPRESSED CALCIUM CHANNELS

High voltage activated L- and N-type calcium channels are modulated by neurotransmitters and hormones through G proteins and protein kinases. The precise regulation of cloned neuronal channels is not known. In vitro experiments show that the α_1 subunit of an immunoprecipitated N-type channel is phosphorylated stoichiometrically by cAMP kinase and protein kinase C (Ahlijanian et al 1991). No detailed analyses are available for the hormonal modulation of expressed neuronal calcium channel, in contrast to results obtained with the cardiac L-type calcium channel.

In heart, β adrenergic stimulation increases the calcium current 3- to 7-fold either by cAMP-dependent phosphorylation of the channel (Osterrieder et al 1982, Kameyama et al 1985, Hartzell et al 1991, Hartzell & Fischmeister 1992) or by the activated α subunits of the trimeric GTP binding protein G_s (Yatani & Brown 1989) or a combination of the activated α subunit of the trimeric GTP binding protein G_s and the active cAMP kinase (Cavalié et al 1991). The L-type calcium current of isolated tracheal smooth muscle cells is also stimulated by activation of the β-adrenergic receptor (Welling et al 1992b). This β-adrenergic receptor effect is mediated directly by a G-protein and not by cAMP-kinase activation. These results suggest that the CaCh2 gene α_1 subunit may be regulated in vivo by the α subunit of a G protein and by cAMP-dependent phosphorylation.

Two subunits of the purified skeletal muscle calcium channel, the α_1 and β subunit, are substrates for cAMP-kinase in vitro (Jahn et al 1988, Ruth et al 1989, DeJongh et al 1989, Röhrkasten et al 1988, Rotman et al 1992).

Both splice variants of the CaCh2 calcium channel α_1 subunit contain an identical number of predicted cAMP kinase phosphorylation sites. One or two of these sites are phosphorylated by cAMP kinase in the expressed full length α_1 subunit of the CaCh2a gene (Yoshida et al 1992). The purified cardiac α_1 subunit is not phosphorylated by cAMP kinase (Schneider & Hofmann 1988, Chang & Hosey 1988). The differing result could be explained if the purified cardiac α_1 subunit protein lacks part of the carboxyterminal phosphorylation sites due to a posttranslational processing of the protein as described for the skeletal muscle α_1 subunit. The potential importance of phosphorylation sites was supported by experiments that showed (i) that rat cardiac poly(A+) RNA induced the expression of an L-type calcium current in *Xenopus laevis* oocytes, which was stimulated 1.34-fold by isoproterenol (Dascal et al 1986) or 2- to 3-fold by cAMP (Lory & Nargeot 1992); (ii) that in *Xenopus laevis* oocytes expressing the cardiac α_1 subunit, cAMP increased barium currents only in the presence of the skeletal muscle β subunit (Klöckner et al 1992), and (iii) that dibutyryl-cAMP stimulated two-fold barium currents in CHO cells expressing the CaCh2a gene (Yoshida et al 1992). Perfusion of a CHO cell expressing the CaCh2a gene with the catalytic subunit of cAMP kinase increased the inward current 1.6-fold, but did not affect the barium current in cells expressing the CaCh2b gene (G Mehrke & F Hofmann, unpublished results). However, the inward barium current was not significantly affected when the pipette solution contained 3 mM ATPγS, 3mM ATPγS and 10 or 100 μM 8Br-cAMP or 3 mM ATPγS and 10 μM GTPγS (Hofmann et al 1993). CHO cells contain a functional adenylyl cyclase, G_S and cAMP-dependent protein kinase. Isoproterenol failed to increase the barium current in a CHO cell line which expressed the β_2 adrenergic receptor at a concentration of 1 pmol/mg protein and the CaCh2 calcium channel. Perfusion of these CHO cells with up to 0.1 mM inhibitor peptide of the cAMP kinase inhibitor protein did not decrease the inward current (G Mehrke & F Hofmann, unpublished results). These negative findings could suggest that the α_1 subunit alone is not sufficient to restore the hormonal regulation of the native calcium channel.

Similar conclusions were made by Klöckner et al (1992) who injected the cardiac α_1 subunit alone or together with the skeletal muscle β subunit into *Xenopus laevis* oocyctes. These authors reported that cAMP increased barium currents only in *Xenopus laevis* oocytes expressing the cardiac α_1 and the skeletal muscle β subunit. However, the reported inward currents were small, and their sensitivity toward Bay K 8644 or a 1,4 dihydropyridine blocker was not tested. Therefore, the results reported by Dascal et al (1986), Lory & Nargeot (1992), and Klöckner et al (1992) do not exclude the possibility that the β subunit associated with the endogenous *Xenopus laevis* oocyte calcium channel (Singer et al 1991, Singer-Lahat et al 1992) and increased the inward

barium current. The endogenous channel is insensitive to the 1,4 dihydropyridines but can be stimulated by cAMP in the presence of the skeletal muscle subunits (Dascal et al 1992). Furthermore, perfusion of CHO cells expressing the CaCh2 α_1 subunit and the skeletal muscle β subunit with cAMP or 8Br-cAMP had no effect on the size of the inward current; this observation suggests that, at least in CHO cells, the combination of these two subunits, which are not expressed in vivo in the same tissue, does not restore the hormonal control of the cardiac calcium channel.

These negative results are not due to a general inability of protein kinases to modulate the expressed channel, because different results were obtained with protein kinase C. The cardiac L-type calcium current is enhanced and subsequently inhibited by the activation of protein kinase C (Lacerda et al 1988). Similar results were obtained in *Xenopus laevis* oocytes after injection of rat heart mRNA (Bourinet et al 1992). The skeletal muscle α_1 subunit is rapidly phosphorylated by protein kinase C in vitro (Nastainczyk et al 1987). Currents through the CaCh2a α_1 subunit expressed in *Xenopus laevis* oocytes were modulated biphasic by the activation of protein kinase C (Singer-Lahat et al 1992). Initially the current increased, followed by a marked inhibition. The biphasic modulation was not modified significantly by the coexpression of the cardiac α_1 subunit with the α_2/δ, β and γ subunits from skeletal muscle suggesting that protein kinase C affected the current by phosphorylation of the α_1 subunit.

These results suggest that protein phosphorylation can modulate the properties of the expressed calcium channel. The failure to reconstitute the β adrenergic/cAMP kinase modulation of the cardiac calcium channel with the cloned CaCh2 gene probably indicates that an unphysiological combination of channel subunits has been used. The inability to see cAMP-dependent modulation of the channel expressed in *Xenopus laevis* oocytes could be caused by a constant phosphorylation of the α_1 subunit because oocytes have a high basal activity of the cAMP kinase. The primary sequences of cardiac and smooth muscle α_1 subunits are almost identical and contain identical potential phosphorylation sites. It is therefore conceivable that the cAMP-dependent stimulation of the cardiac calcium channel depends not solely on the phosphorylation of the α_1 subunit but also on the tissue-specific coexpression of other proteins, e.g. the β subunit. The deduced amino acid sequence of the skeletal muscle β subunit (CaB1) contains several phosphorylation sites. Two of these sites, Ser-182 and Thr-205, are phosphorylated in vitro by cAMP-dependent protein kinase (Ruth et al 1989, DeJongh et al 1990). The equivalent of Thr-205 is conserved in the "cardiac" β subunit (Thr-165 in CaB2a and Thr-191 in CaB2b) but is not present in the "smooth muscle" β subunit CaB3 (Hullin et al 1992). The sequence following this potential phosphorylation site is highly variable and determines several splice variants.

This variable region may be responsible for the tissue specific regulation calcium currents by hormones and neurotransmitters.

CONCLUSION

High voltage activated calcium channels are oligomeric complexes of four different subunits: α_1, α_2/δ, β, and γ. So far six different genes encoding α_1 subunits and four distinct genes encoding β subunits have been isolated from various tissues. The specific electrophysiological and pharmacological characteristics of calcium currents in different cells result from the expression of tissue-specific subunits of the calcium channel, leading to differences in functional interaction. This genetic polymorphism explains also the different regulatory mechanisms and possibly the different pharmacology of various calcium channels. An important observation appears to be that the expression not only of distinct α_1 subunits but also of small splice variations of one α_1 subunits changes significantly the sensitivity of the expressed channel against channel blockers. This nurtures the hope that the development of cell specific drugs is possible and may be of benefit in the treatment of disabling brain diseases.

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