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COMMENTARY

TOWARD AN UNDERSTANDING OF THE DIHYDROPYRIDINE-SENSITIVE CALCIUM CHANNEL

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The molecular nature of dihydropyridine (DHP†)-sensitive calcium channels is being actively studied using multidisciplinary techniques. Pharmacology, biochemistry, immunology, and molecular biology have contributed to an understanding of the channel. The 1,4-dihydropyridines have been used as high-affinity probes for the purification of the calcium channel/drug receptor complex from skeletal muscle and as many as five putative subunits have been identified, and designated α_1, α_2, β, γ and δ [1–6]. The full-length amino acid sequences of three of these putative skeletal muscle subunits (α_1, α_2 and β) have been deduced from cDNA clones [7–9]. Important functional roles for the α_1 subunit have been established, but the roles of the other putative subunits are unclear. Tissue-specific isoforms of the α_1 subunit have now been isolated with different primary structures and transcript sizes. The identification of tissue-specific isoforms of the α_1 subunit necessitates the establishment of a new standardization of nomenclature.

Subunit composition

Proving the quaternary structure of the DHP-sensitive calcium channel remains a challenge to investigators who are characterizing this important regulatory protein. Conflicting subunit patterns and stoichiometries of the DHP-sensitive calcium channel have led to disagreements and confusion. However, we are in the early stages of understanding the structure of calcium channels and the identification of the subunits necessary for proper channel function. Such was also the case for the nicotinic acetylcholine receptor [10] and the voltage-dependent sodium channel [11]. Early in the study of these channels, there was disagreement about the subunit structure because of extensive degradation by proteases, anomalous migratory behavior on polyacrylamide gels, and poor staining of subunits. Proteolysis, however, did not alter appreciably drug and toxin binding, antibody cross-reactivity, sedimentation behavior, or morphology, and channel function could be reconstituted in lipid bilayers. Similarly, partially degraded calcium channel preparations retained functional characteristics, e.g. channel activity and calcium antagonist binding.

A pentameric calcium channel complex has become generally accepted. An abundance of evidence favoring five subunits exists, and there is no compelling reason at this time to discard any of the putative subunits. The word "subunit" is intended to designate the elementary units of a large macromolecular complex without indication of a functional role. It is implicit, however, that all of the subunits are required to bestow the native properties to the protein. The putative subunits have been inferred from the purification of skeletal and cardiac muscle voltage-dependent calcium channels. Figure 1 shows a silver-stained polyacrylamide gel of the DHP-sensitive calcium channel purified from rabbit skeletal muscle, under non-reducing and disulfide-reducing conditions. Although the stoichiometry among the putative subunits has not always been shown to be uniform, proteins corresponding to the five putative subunits are invariably present. The lack of stoichiometry or variable stoichiometry between purified preparations could result from many factors including proteolysis, partial dissociation of subunits, differential staining of proteins, and the co-purification of non-subunit protein.

Biochemical and immunological data strongly suggest that the DHP-sensitive calcium channel is an oligomer of subunits. Immunoprecipitation of the channel prelabeled with a [^{3}H]dihydropyridine yielded multiple subunits [12–15]. Subunit-specific antibodies (against any of the five putative subunits) can immunoprecipitate [^{3}H]dihydropyridine-calcium channel/drug receptor complexes [12, 13, 15–18], and some of these antibodies have been shown to modulate DHP-sensitive channels [14, 19–21].

Tissue-specific isoforms

Studies of calcium channels from different tissue sources show tissue-specific variation. Tissue-specific isoforms of DHP-sensitive calcium channels ("isochannels") have been shown to possess distinct electrophysiological, pharmacological, and structural properties. Marked differences in their interactions with divalent cations, conductance properties, and gating kinetics have been found for cardiac and skeletal muscle DHP-sensitive calcium channels (Table

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† Abbreviations: DHP, dihydropyridine; kb, kilobases; pS, picosiemans; DTT, dithiothreitol; and NEM, *N*-ethylmaleimide.

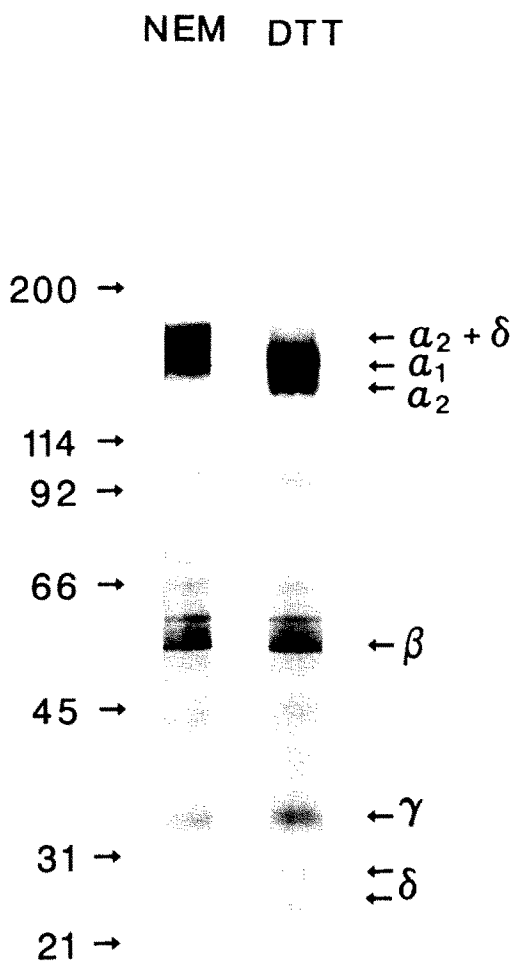

Fig. 1. Silver-stained sodium dodecyl sulfate–polyacrylamide gel showing the purified dihydropyridine-sensitive calcium channel under non-reducing (NEM) and disulfide-reducing (DTT) conditions. The positions of the molecular weight markers are indicated on the left side. The labels on the right side indicate the putative subunits.

Table 1. Properties of the cardiac versus skeletal muscle DHP-sensitive calcium channels

	Cardiac muscle	Skeletal muscle
α_1 calculated M_r (kD)	242	212
α_1 apparent M_r (kD)	195	165
Mg^{2+} permeability	No	Yes
Divalent blockers	$La^{2+} > Cd^{2+} > Co^{2+} > Ca^{2+}$	$Ca^{2+} > Cd^{2+} \sim Co^{2+}$
Conductance (110 mM Ba^{2+})	22.7 pS	10.6 pS
Activation kinetics	"Fast"	"Slow"
Mean open time	"Short"	"Long"
Activity at −100 mV	No	Yes

1 summarizes some of these differences) [22]. For example, the dissociation constants of dihydropyridines for the receptor in skeletal muscle are greater (i.e. lower affinity) than those in brain and cardiac tissue, with the cardiac receptor having the highest affinity [4]. This difference is preserved among various species of animals. Based upon biochemical evidence, it appears that cardiac and skeletal muscle calcium channels have similar quaternary structures. Although definitive subunit compositions have not been proven for any tissue, the cardiac channel subunits appear to correlate with skeletal

muscle subunits. Reports of purified cardiac preparations [23, 24] do not clearly define putative subunits, but two α subunits can be visualized. Like many skeletal muscle preparations, the presence of smaller subunits is difficult to determine.

Molecular biological studies have now revealed the primary sequences of both the skeletal muscle [7, 8] and cardiac muscle [25, 26] α_1 subunits. These studies show that cardiac and skeletal muscle α_1 subunits are similar and share structural features with other voltage-dependent cation channels. These α_1 subunits, like the α subunits of Na^+ channels, are arranged in four repeating hydrophobic motifs, each consisting of six conserved transmembrane domains. Within each motif is a segment (S4) which contains conserved positively charged amino acid residues (Arg or Lys) at every third or fourth position. The S4 segment is highly conserved between these two α_1 isoforms and also cloned Na^+ and K^+ channels [27, 28] and is probably the voltage-sensor of these voltage-dependent cation channels [7, 29]. Partial amino acid sequences from rat brain and rat aorta α_1 subunit isoforms reveal structures [30] similar to rabbit skeletal muscle and cardiac α_1. Differences among the species probably represent tissue specific variations of the α_1 subunit. Figure 2 shows partial amino acid comparisons of these four tissue-specific α_1 subunits.

Since the full-length sequences of the cardiac and skeletal muscle α_1 subunits have been deduced, the primary structures have now been compared. Interesting differences exist between cardiac and skeletal muscle α_1s. In particular, five of the potential protein kinase A phosphorylation sites identified in the skeletal muscle α_1 primary sequence are absent in the cardiac sequence, and four new sites appear [25, 26]. Among the absent sites is Ser687, which has been shown to be rapidly phosphorylated in skeletal muscle and proposed to be the *in vivo* site of regulation [31]. Since regulation of the cardiac calcium channel by phosphorylation is well known, the absence of this putative regulatory site is noteworthy. This suggests three possibilities: (1) the phosphorylation of the skeletal muscle α_1 at Ser687 may not have physiological relevance, (2) skeletal muscle and cardiac calcium channels may be regulated differently, and (3) β subunit phosphorylation may be involved in the regulation of both of the channels. Major structural differences also exist in regions of the proteins which are proposed to be extracellular to the membrane. The lack of similarity in these regions may help to explain the lack of cross-reactivity of antibodies which are raised against the skeletal muscle α_1 subunits of other tissues [24]. Also, differences exist in the length of both 5′ and 3′ ends of the cDNA clones encoding the skeletal muscle and cardiac isoforms. Both ends are considerably longer in the cardiac α_1 isoform and encode for a much larger open reading frame [25, 26], which is also consistent with the biochemical data [24].

Northern blot analysis with tissue specific probes reveal multiple transcript sizes in the tissues studied. Table 2 summarizes the transcript sizes detected under stringent washing conditions. Three different size range of transcripts, (6.5 kb), (8.5–8.9 kb), and (10.5–15.5 kb), were found. Of the tissues we have studied, only skeletal muscle has a single transcript size. It is unknown whether the different transcripts are derived from different genes with tissue-specific promoters or alternate splicing of a single gene. The sporatic nature of the similarity between the tissue-specific clones isolated is evidence in favor of the former (see Fig. 2).

Calcium channel activity

The importance of the α_1 subunit for calcium channel activity has been demonstrated using indirect approaches, which do not preclude the requirement of other subunits. For example, injection of skeletal muscle α_1 subunit cDNA into dysgenic mouse cells restored excitation–contraction coupling and dihydropyridine-sensitive calcium channel activity [32]. These dysgenic mouse cells have a low level or apparent lack of the α_1 subunit, but other subunits may be present. Further, inhibition of the calcium current by cadmium did not prevent excitation–contraction coupling which indicates that voltage-sensor activity was also restored. *Xenopus* oocytes injected with mRNA isolated from cardiac tissue show robust voltage-dependent calcium channel activity [33]. In hybrid depletion studies, anti-sense oligonucleotides prepared from segments of the skeletal muscle [33] or cardiac [25] α_1 subunit mRNA suppressed this calcium channel activity. For unexplained reasons skeletal muscle poly(A)$^+$ RNA when injected into oocytes results in only small and undefined calcium currents in oocytes. Not surprisingly, no one has successfully expressed dihydropyridine-sensitive activity in oocytes using the skeletal muscle α_1 subunit mRNA. Therefore, although the skeletal muscle and cardiac α_1 isoforms exhibit strong structural similarities, they are inherently different in inducing the expression of calcium channel activity in oocytes.

The α_1 subunit appears to contain structural features found in other cation channels. For the sodium [27] and the potassium [28] channels, a single subunit directed the expression of channel activity. In one recent study [34] calcium channel activity was expressed with the skeletal muscle α_1 subunit in mouse L cells; dihydropyridine-sensitive (Bay K 8644) calcium currents were detected with a unitary conductance of 6 pS and low levels (50–60 fmol/mg of protein) of dihydropyridine binding. A unitary conductance that is about half the value of the native channel conductance and the low binding capacity may be caused by the lack of other subunits or the inefficient processing of the protein by the expression system. The expressed α_1 subunit was larger (kD) than that which is isolated from fresh tissue (165 kD). Also, the activation half-times of the observed currents were as much as ten times slower than that of the native channel.

mRNA made from cardiac α_1 subunit cDNA directed the expression of a dihydropyridine-sensitive calcium channel in *Xenopus* oocytes [26]. The calcium current exhibited more typical activation kinetics and sensitivity to both dihydropyridine agonist and antagonist. However, single channel measurements were not reported. Coinjection of the skeletal muscle α_2 subunit cDNA with the cardiac α_1 subunit cDNA resulted in a larger calcium current, but did

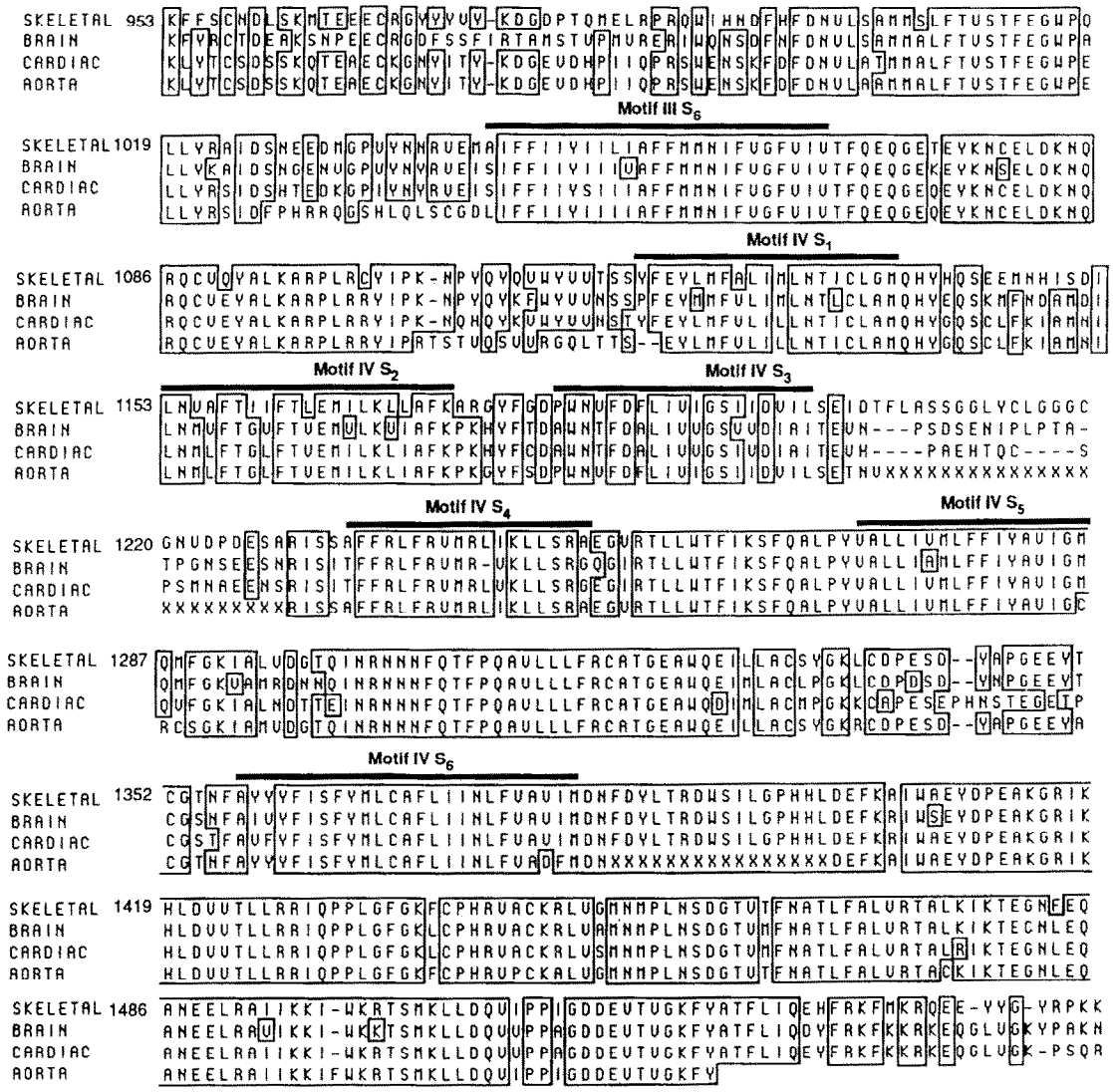

Fig. 2. Amino acid sequence comparison of the tissue-specific α_1 subunits of skeletal muscle, brain, heart and aorta. The area shown is comprised of parts of the third and fourth motifs of the skeletal muscle α_1 subunits [7]. Solid bars represent transmembrane regions. Blocked in areas represent regions of similarity in all four sequences. The Xs represent an area that has not been cloned.

not change the drug-sensitivity or voltage-dependency of the calcium channel. The mechanism underlying the increase in calcium current by coinjection of the α_2 subunit cDNA is unclear, but this result suggests that the α_2 subunit is intimately associated with the α_1 subunit and, therefore, part of the dihydropyridine-sensitive calcium channel. This association is probably shared by cardiac and skeletal muscle. We have suggested [8] that the α_2 subunit facilitates the insertion of the α_1 subunit into the membrane. Alternatively, the α_2 subunit may stabilize the α_1 subunit within the membrane or allosterically modulate the properties of the calcium channel, such as conductance or gating kinetics. These hypotheses could be tested using quantitative measurements such as radioligand binding or antibody techniques and single channel measurements respectively.

Table 2. Tissue-selective transcript sizes reported for the α_1 subunit of the DHP-sensitive calcium channel

Species	Organ	Size (kb)	Reference
Rabbit	Skeletal muscle	6.5	[7] [8]
Rabbit	Cardiac muscle	8.9 and 15.5 8.5 and 13.0	[26] [25]
Rabbit	Brain	8.9 and 15.5	[26]
Rat	Brain	8.6 and 10.5	[30]
Rat	Aorta	6.5 and 8.6	[30]

Nomenclature

The existing nomenclature (α_1, α_2, β, γ, δ) of the putative calcium channel subunits with its usage of

numerical subscripts to designate the two large molecular weight proteins needs to be modified to allow the naming of isoforms of the α_1 subunit. The utilization of numerical subscripts for the α subunits resulted from the preservation of the original nomenclature set by Curtis and Catterall [35]. The resolution of two distinct high molecular weight proteins necessitated the use of numerical subscripts when, ordinarily, numerical subscripts should be reserved for isoforms of a gene family of proteins. Structurally, the two α subunits are quite distinct and products of different genes [8]. Considering the divergent sequences of the skeletal muscle [7, 8] and cardiac [25, 26] α_1 subunits, the nomenclature should be modified to reflect the differences in α_1 structures. Partial sequencing of rat brain and rat aorta α_1 subunits [30] indicates some structural differences from the cardiac and skeletal muscle α_1 subunit sequences as well. For this reason, we propose that the new nomenclature should allow for expansion as different isoforms are discovered. Also, it should allow for the possibility that one "tissue specific" isoform could be expressed in another tissue (e.g. the cardiac isoform being expressed at some level in vascular smooth muscle). For these reasons, we suggest naming the skeletal muscle isoform α_{1A} and the cardiac isoform α_{1B}; subsequent isoforms should be named similarly, according to the order in which they are cloned and shown to be isoforms of the dihydropyridine-sensitive calcium channel. Also, if isoforms of the α_2 subunit are found, then letters should be employed for these isoforms as well.

Alternatively, the existing nomenclature could be abandoned and a new system adopted. Confusion arises from naming two structurally distinct proteins α_1 and α_2, when the use of subscripts generally denotes isoforms of one protein. An example of this is found in the three forms of the α subunit of the Na^+, K^+-ATPase, α_1, α_2 and α_3 [36]. All three isoforms perform the same function; however, they differ in their pharmacology. This is entirely analogous to the different α_1 subunits of the DHP-sensitive calcium channel. For this reason, it may be prudent at this point to rename the subunits so that α designates only the 165 kD protein, which previously had been named α_1. The subunit that until now was named α_2 would then become β, the β would be called γ, the γ would be called δ, and the δ would be called ε. Isoforms of the DHP receptor would then be called α_1 for skeletal muscle, α_2 for cardiac, and subsequently cloned isoforms would follow suit. If isoforms of the other subunits are found, subscripts would also be used. While initially creating confusion, this standardization might help to clarify the subject. Any adaptation of new nomenclature should be standardized and approved by the scientific community.

Summary

The dihydropyridine-sensitive calcium channel continues to fascinate scientific investigators. Each new discovery leads to more complexity. Further work elucidating the molecular structures and functions of the various isoforms of the channel will lead us to a better understanding of its nature. We are well on our way towards understanding the molecular structure and mechanisms involved in calcium permeability, and the coming decade promises to reveal numerous breakthroughs in our understanding of this channel.

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