J. Membrane Biol. 161, 207–213 (1998)

The Journal of
Membrane
Biology

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Topical Review

The Molecular Basis of Voltage-gated Ca^{2+} Channel Diversity: Is It Time for T?

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Received: 14 July 1997/Revised: 4 November 1997

Abstract. The existence of diversity in the voltage activated Ca^{2+} channel populations of vertebrate cells has been long recognized. More recently, the molecular cloning of a considerable number of Ca^{2+} channel subunits from cDNA libraries has indicated that the range of possible Ca^{2+} channel phenotypes a cell can express may be even greater than was previously appreciated. A challenge of recent years has been to resolve how the properties of recombinant channels correspond with their counterparts experimentally characterized in native cells. In this short review I will outline the properties of both native and recombinant Ca^{2+} channels, and will then describe the current agreements and controversies concerning their relationships to each other.

Key words: Calcium channels — Pharmacology — Cloning — Review — Ion channels

Ca^{2+} Channel Diversity at the Cellular Level

It has long been understood that cytosolic Ca^{2+} ions play a fundamental role in the control of most biological processes [5, 20, 44]. At rest, the free Ca^{2+} concentration of cytoplasm is very low compared to that found both outside the cell and within many intracellular organelles. Rapid and large increases in cytosolic free Ca^{2+} can, therefore, be produced by increasing the Ca^{2+} permeability of the lipid bilayers that delineate the cytoplasm from these other compartments. Such changes in Ca^{2+} permeability primarily arise from the ligand- or voltage-dependent gating of a diverse range of Ca^{2+}-selective proteinaceous pores [44]. These ''Ca^{2+} channels'' are found in both the plasma membrane and the membranes of intracellular organelles. Although release of Ca^{2+} into the cytoplasm from intracellular organelles plays an important role in cell signaling, such Ca^{2+} pools can be rapidly depleted [5, 20]. In contrast, the extracellular pool of Ca^{2+} ions is regarded by the cell as essentially infinite. As such, the passage of Ca^{2+} ions across the plasma membrane is responsible for both providing and sustaining cellular Ca^{2+} signaling [5, 20].

One of the most rapid and Ca^{2+}-selective means of generating Ca^{2+} fluxes across the plasma membrane is via the activation of voltage-sensitive Ca^{2+} channels (VSCCs). Ca^{2+} entry generated in this way has been shown to play a pivotal role in the control of a wide spectrum of biological activities. These include synaptic transmission, gene expression, muscle contraction, hormone release, cell motility, cell division and cell death [44, 61]. To efficiently control such a broad range of processes, each with its own specific Ca^{2+} demands, it seems likely that cells require an equally diverse range of temporal and spatial patterns of Ca^{2+} entry and removal. Many mechanisms (e.g., Ca^{2+} buffering and Ca^{2+} channel localization) combine to produce a wide range of different voltage-gated Ca^{2+} signals. However a crucial source of heterogeneity of Ca^{2+} signal lies within the biophysical diversity present within the VSCC population itself [4, 43, 61, 103, 104].

The first indications of this diversity within cellular VSCC complements came from studies characterizing how Ca^{2+} currents turned on (activated), turned off (deactivated) and desensitized (inactivated) at different membrane potentials [4, 14, 31, 52, 58, 59, 75]. An important finding of these early biophysical studies, which has rigorously stood the test of time, is that there are some VSCCs which require only small depolarizations from rest to become active, whereas others require comparatively larger depolarizations before they permit the

Table. A summary of data from many laboratories on the biophysical and pharmacological properties of different Ca^{2+} channels in mammalian cells

	L-type	N-type	P-Type	Q-type	R-type	T-type
Biophysical properties						
Activation range	HVA	HVA	HVA	HVA	HVA	LVA
Inactivation rate (0 mV)	Slow	Intermediate (τ ~ 100 msec)	Very slow	Intermediate (τ ~ 100 msec)	Fast (τ ~ 30 msec)	Fast (τ ~ 20 msec)
Midpoint of inactivation	~−25 mV	−50 to −70 mV	~−25 mV	~−60	~−75	−55 to −80
Ca^{2+} dep. Inactivation	Yes	Yes	No	ND	ND	No
Deactivation rate (−80 mV)	Fast ($\tau \leqslant$ 200 μsec)	Fast ($\tau \leqslant$ 200 μsec)	Fast ($\tau \leqslant$ 200 μsec)	Fast ($\tau \leqslant$ 200 μsec)	Fast ($\tau \leqslant$ 200 μsec)	Slow τ ~ 2 msec
Pore properties						
pBa^{2+}/pCa^{2+}	~2	~2	~2	ND	~1.3	~1
Cd^{2+} block	Potent (IC_{50} ~ 1 μM)	Potent (IC_{50} ~ 1 μM)	Potent IC_{50} ~ 1 μM	Potent (IC_{50} ND)	Potent (IC_{50} ~ 1 μM)	Weak ($IC_{50} \geqslant$ 50 μM)
Ni^{2+} block	Weak IC_{50} ~ 230 μM	Weak IC_{50} ~ 270 μM	Intermediate IC_{50} ~ 90 μM	ND	Strong IC_{50} ~ 50 μM	Strong IC_{50} ~ 35 μM
Pharmacology						
DHP agonists/ FPL64176	Potentiate	NE	NE	NE	NE	NE
DHP antagonists	Voltage-dependent block	NE	NE	NE	NE	Weak block
ω-CTx-GVIA	NE	Blocks	NE	NE	NE	NE
ω-Aga-IVA	NE	NE	Blocks IC_{50} ~ 1 nM	Blocks IC_{50} ~ 100 nM	NE	NE
ω-Aga-IIIA	Blocks IC_{50} ~ 1 nM	Blocks IC_{50} ~ 1 nM	Partial Block IC_{50} ~ 0.5 nM	Blocks IC_{50} ND	Partial Block IC_{50} < 3 nM	NE

Due to the numerous recording conditions and preparations employed, the entry in each location can only be regarded as an approximate value or as representative of the majority of reports. Observations are best regarded as corresponding to experiments in which the extracellular divalent ion is either Ca^{2+} or Ba^{2+} at concentrations of 2 to 5 mM.

Abbreviations HVA, high voltage activated ($V_{1/2}$ ~ 0 mV); LVA, low-voltage activated ($V_{1/2}$ ~ −25 mV); ND, not determined; NE, no effect; pBa^{2+}/pCa^{2+} ratio of Ba^{2+} and Ca^{2+} current amplitudes for equimolar concentrations near peak of current-voltage relationship.

passage of Ca^{2+} ions [14, 31, 58]. In modern parlance, these have come to be known as low-voltage activated (LVA) and high-voltage activated (HVA) Ca^{2+} channels, respectively.

These initial indications of the existence of at least two types of Ca^{2+} channel were soon supplemented by a wealth of experimental evidence demonstrating additional Ca^{2+} channel diversity within the HVA channel population. Although the major experimental indications of these greater levels of VSCC diversity were pharmacological, they were also supported by biophysical data, particularly those pertaining to inactivation kinetics, permeation and block [33, 34, 45, 56, 64, 66, 72, 75, 79, 81, 105, 115]. At the time of writing, such experimental approaches have provided evidence for at least 6 clearly separable classes of Ca^{2+} channel in mammalian cells. The LVA Ca^{2+} channel group contains only one member, the T-type Ca^{2+} channel. Increasing evidence for diversity within the LVA channel population of mammalian cells, however, [45*a*, 100*a*]), indicates that a subclassification of the T-type channel family is likely to be necessary in the future. The remaining 5 VSCC types are all members of the HVA family. These are known as L-, N-, P-, Q- and R-type channels [56, 72, 79, 104, 115]. A summary of the differences and similarities that underpin this classification of VSCCs is shown in the Table.

Routes to Experimental Assessment of Channel Diversity

Using the information in the Table, one may formulate boundaries that permit the differentiation of individual channel types in electrophysiological experiments. For instance T-type currents are relatively easy to identify because they share three characteristics not present in any other channel type, namely (i) activation at quite negative potentials, (ii) slow deactivation and (iii) insensitivity to micromolar concentrations of Cd^{2+} [14, 33, 58]. Although one could conceivably adopt a variety of routes to distinguish between the 5 HVA VSCCs, most biophysical criteria express too much crossover between channel types to be very useful in channel definition.

As such, pharmacology remains the most rigorous approach for the delineation of HVA VSCC subtypes [59, 66, 72, 79–81].

L-type channels are best defined by their voltage-dependent block by dihydropyidine antagonists and their facilitation by BAY K 8466 and FPL 64176 [72]. N-type channels are also easy to identify because they are selectively and irreversibly blocked by the snail toxin ω-CTx-GVIA [2, 8, 50, 60, 98]. The P-type channel exhibits little or no inactivation and is very potently blocked ($IC_{50} \sim 1$ nM) by the spider toxin ω-Aga-IVA [64–66, 79, 105]. The Q-type channel inactivates at a similar rate to the N-type channel, and like the P-type channel is sensitive to ω-Aga-IVA. The dose of this toxin required to produce half block of the Q-type channel, however, is about 100 times greater than that required by the P-type channel [79]. R-type channels inactivate quite rapidly and are insensitive to all of the above-mentioned compounds [79, 115]. Indeed, no selective antagonists for the R-type channel exist, although it may be blocked by certain nonselective antagonists such as mibefradil and ω-Aga-IIIA [80].

Molecular Insights into the Basis of Ca^{2+} Channel Diversity

Today we know that the phenotypic diversity of VSCCs is predominantly produced by the selective expression of different Ca^{2+} channel subunit genes [17, 18, 61, 93, 103]. When VSCC diversity first became apparent, however, a number of explanations for its molecular basis were considered feasible. One possibility was that only a single Ca^{2+} channel molecule existed, and the various biophysical and pharmacological phenotypes observed in cells were produced by post-translational modifications such as phosphorylation or interactions with, for instance, the cytoskeleton or other membrane proteins.

The disciplines of molecular biology and protein chemistry have both been crucial in arriving at the molecular appreciation of Ca^{2+} channel diversity we have today. However, as we shall see later, certain quandaries remain unresolved. Early breakthroughs came from protein purification studies on the abundant Ca^{2+} channels of skeletal muscle. This work, which has subsequently been extended to the heart and brain, indicated that Ca^{2+} channels were heteromultimeric proteins [9, 17–19, 21, 23, 24, 27, 90, 101, 113]. In most organs, Ca^{2+} channels seem to consist of α_1, α_2, β and δ subunits, seemingly in a 1:1:1:1 ratio. An additional 222 amino acid glycoprotein, the γ subunit, is present only in skeletal muscle [10, 17, 18, 48, 99].

Classical protein chemistry demonstrated that α_1 was a large (~210 kD) glycosylated hydrophobic subunit [9, 21]. Interestingly, truncated forms of both muscle and brain α1 subunits have been described [24, 25, 40, 41, 54, 108], with the smaller form thought to be produced by Ca^{2+}-dependent proteolysis [39]. In addition to representing a further layer of VSCC diversity, the long and short forms of these α1 subunits are differentially phosphorylated [38, 42, 54] and, at least in skeletal muscle, seem to perform distinct cellular roles [3]. The α_2 (125 kD) and δ (16 kD) subunits are both glycosylated hydrophobic molecules which are transcribed from the same gene [13, 23, 30, 49, 99]. These two subunits are invariably linked by a disulfide bridge, and as such are now commonly regarded as a single molecular entity $\alpha_2\delta$. The β subunits are hydrophilic nonglycosylated proteins with molecular weights of about 58 kD [21, 85]. The β subunits are exclusively intracellular and bear numerous protein kinase consensus sequences permissive for a wide range of protein kinases [22, 47, 85].

Following on from channel purification, the next major advance was the cloning of the first Ca^{2+} channel subunits. Using library screening based on amino acid sequence data, the first subunit to be cloned was the dihydropyridine (DHP)-sensitive α_1 subunit from skeletal muscle [30, 100]. With time this was followed by the isolation of genes for 5 additional α_1 subunits [35, 62, 67, 70, 92, 95, 110–11], 4 β subunits [15, 16, 46, 73, 76, 78, 85], and single $\alpha_2\delta$ [30, 51, 111] and γ(48) subunits. In addition splice variants of the majority of subunits have also been described [12, 51, 74, 86, 89, 94, 112]. To cope with this considerable level of subunit diversity, a widely accepted all-encompassing system of nomenclature is now in place [7]. In this system α_1 subunits are known as α_{1A} to α_{1E} plus α_{1S} for the skeletal muscle DHP receptor. The β subunits are known as β_1 to β_4. Splice variants of each subunit are indicated by additional subscripted lower case characters (e.g., $\alpha_2\delta_a$,$\alpha_2\delta_b$ and $\alpha_2\delta_c$). There appears to be only a single form of the γ subunit which, commensurate with earlier protein purification work, is expressed only to any significant degree in skeletal muscle [10, 48].

Although it is generally believed that all Ca^{2+} channels are heteromultimers in vivo, it has been reported that, in vitro, some α_1 subunits are capable of producing functional Ca^{2+} channels in the absence of β or $\alpha_2\delta$ subunits [26, 53, 69, 95–97, 102]. However, this may not be the case for all α_1 subunits [71, 111]. These observations of autonomous function are consistent with the fact that the large α_1 subunit (much like that of the voltage gated Na^+ channel) encodes the pore, the voltage sensor and the inactivation machinery of the Ca^{2+} channel [69, 114, 115]. In addition, the binding sites for all Ca^{2+} channel antagonists thus far characterized in terms of their subunit interaction are also located on the α_1 subunit [29, 94, 96, 97, 102]. Recent work has also characterized the sites of interaction of a number of proteins with the α_1 subunit. These include the β and $\alpha_2\delta$ Ca^{2+}

channel subunits, G-proteins and the vesicle core complex protein syntaxin, [37, 77, 91].

Progress in Matching Cloned and Native Ca^{2+} Channel Phenotypes

As described above the α_1 subunit contains the major structural motifs that shape the permeation properties, the voltage-dependence of channel opening and closing, and the channel pharmacology. This subunit, therefore, clearly represents the major determinant of VSCC phenotype. To date, attempts to link the properties of recombinant channels containing defined α_1 subunits with the properties of channels observed in native tissue have met with mixed success. The least controversial parallels are those between recombinant α_{1B} containing channels and N-type channels [35, 110] and recombinant α_{1S} containing channels and the skeletal muscle DHP receptor [100]. The α_{1C} and α_{1D} genes both encode L-type channels, with the former being responsible for the classical cardiac L-type channel [62]. The α_{1D} subunit, in contrast, encodes a L-type VSCC more prevalent in neuronal and endocrine tissue [111]. As discussed below, the cellular correlates of the remaining two α_1 subunits, α_{1A} and α_{1E}, remain somewhat more controversial [11, 67, 79, 80, 87, 95, 115].

Early studies of the localisation of α_{1A} subunit mRNA indicated that, although widespread throughout the brain, it was present at particularly high levels in the cerebellum [67]. More recent immunological studies have demonstrated that equivalent levels of the α_{1A} subunit protein are found in corresponding locations. Interestingly the cell bodies of cerebellar Purkinje cells, which functionally contain predominantly P-type VSCCs [64], exhibit substantial labeling with an antibody directed against the α_{1A} subunit [109]. The currents produced when α_{1A} message was expressed in *Xenopus* oocytes [67] were sensitive to the raw venom of the funnel web spider, from which is derived the P/Q-type channel blocker ω-Aga-IVA (along with a number of other toxins). These observations led to the assertion that α_{1A} encoded the P-type channel. Subsequent investigations, however, demonstrated that when expressed in either oocytes or mammalian cell lines, the α_{1A} subunit produced currents that inactivated substantially and exhibited an IC_{50} for ω-Aga-IVA of 100–200 nM [87, 97, 115]. These properties are much more like those of the Q-type channel [79] than those of the classical P-type channel, which is noninactivating and requires only ~1 nM ω-Aga-IVA to produce 50% current inhibition [56, 64–66, 105]. At present it still remains an open question as to whether the α_{1A} subunit is responsible for the P-type or the Q-type channel, or as many suspect, both.

The first report examining the electrophysiological and pharmacological properties of the α_{1E} subunit expressed in oocytes concluded that it encodes the low-voltage activated T-type VSCC [95]. This conclusion was primarily based on the activation voltage range, rapid current inactivation and sensitivity to Ni^{2+}. More recent support for this hypothesis is provided by analysis of the permeation properties of α_{1E} with respect to Ca^{2+} and Ba^{2+} [11]. An opposing view is that the properties of α_{1E}-containing channels are more commensurate with the properties of the R-type channel described by Tsien and coworkers in cultured cerebellar granule cells [79, 80, 88, 107, 115]. This latter view is supported by a range of pharmacological and biophysical experiments, among the most persuasive of which are (i) α_{1E} and R-type currents are blocked by ω-Aga-IIIA, whereas T-type channels are not [63, 68, 80], (ii) both α_{1E} and R-type currents deactivate ~10 times faster than T-type currents [58, 68, 80, 112], and (iii) in contrast to α_{1E} and R-type channels, T-type channels are sensitive to dihydropyridines [1, 79, 80, 83]. Although both the ''α_{1E} is T-type'' and the ''α_{1E} is R-type'' lobbies have their supporters the overriding point of view from both camps remains that additional experimental work is required to finally resolve the cellular counterpart of the α_{1E} subunit.

β and α2δ Subunits and Channel Diversity

Although α_1 subunits are predominantly responsible for determining the phenotype of Ca^{2+} channels, the β and $\alpha_2\delta$ subunits are both able to modify certain channel properties that form part of the framework commonly used in the differentiation of VSCC subtypes. For instance, both the activation voltage range and rate of inactivation can be significantly altered by coexpression of different β subunits with any given α_1 subunit [26, 46, 96, 97, 106, 107]. This seems to be a physiologically relevant tier of additional VSCC diversity, since recent work clearly indicates that in vivo α_1 subunits are capable of forming interactions with the whole range of β subunits [90]. Is it therefore possible that the presence of different β subunits in different cells can reconcile the problems of assigning the molecular counterparts of P-, Q-, R- and T-type channels? Unfortunately the answer to this question seems to be no. Combination of α_{1E} with a range of different β-subunits in vitro never produces channels with the slow deactivation that is so characteristic of T-type channels [68]. Coexpression of the β_{2a} subunit with α_{1A} does produce currents with inactivation rates approaching those of the classical P-type currents of cerebellar Purkinje cells [26, 56, 64, 97, 105], however, neither β_{2a} nor any other β subunit combine with α_{1A} to produce VSCCs that exhibit the subnanomolar IC_{50} for ω-Aga-IVA that is the main pharmacological hallmark of the P-type channel [65, 66, 87, 97].

The only well described biophysical change produced by co-expression of the $\alpha_2\delta$ subunit is a glyco-

sylation-dependent ~10-fold increase in current amplitude [12, 37, 46, 62, 67, 111]; an effect that relies on the presence of a β subunit for its full expression [26]. Pharmacologically the co-expression of this subunit has been reported to increase ω-CTx-GVIA affinity of α_{1B} channels [12] and to slightly increase the ω-CTx-MVIIC affinity of α_{1A} channels. It is thought, however, that the major role of $\alpha_2\delta$ is to stabilize the plasma membrane incorporation of the Ca^{2+} channel complex and as such it seems this subunit contributes little to the functional diversity of Ca^{2+} channels.

Some Remaining Challenges

Although much work remains to be done concerning the biological utility of possessing so many possible VSCC subunit combinations (let alone splice variants and post-translational modifications), the question at the front of many researchers minds remains ''what is the molecular basis of the T-type channel?'' The importance of this question to both academic and industrial scientists recently prompted an entire meeting and book specifically devoted to the better understanding of the T-type Ca^{2+} channel [68]. Naturally, like so many other seemingly elusive channels and neurotransmitter receptors, the T-type channel will not escape the clutches of the molecular biologists forever. The LVA channel diversity described to date at the cellular level [45a, 100a] indicates that the T-type channels are likely to be encoded by a range of related genes, much like their HVA counterparts. The rather distinctive biophysical properties of LVA channels compared with other Ca^{2+} channels suggests that T-type channels may well escape traditional homology cloning approaches. Indeed it already seems likely that expression cloning or even purification and sequencing may be required to crack this particularly tough experimental nut. Support for use of the former method comes from the observation that seemingly *bona fide* T-type channels can be generated in *Xenopus* oocytes following injection of mRNA isolated from mammalian neurones [28].

The other important question in the pigeonholing of Ca^{2+} channels is ''do both P- and Q-type Ca^{2+} channels contain the α_{1A} subunit, and if so, how are the distinctive biophysical and pharmacological properties of the P-type channel produced?'' Of those workers who accept that there is a difference between P- and Q-type channels, the majority would predict that the α_{1A} subunit will be shown to encode both channel types; a prediction which is supported by recent experimental results [36]. At present, however, it would be foolish to rule out the possibility that a separate, as yet undiscovered, P-type channel specific α_1 subunit exists.

Finally we must not turn our backs on the fact that, although the actual subunit compositions of Ca^{2+} channels are clearly central to their phenotype, there are many other possible sources of VSCC diversity. In this regard much can be learned from previous studies of other receptors and channels. These have demonstrated that some of the most basic properties of these membrane proteins can be altered by a wide range of activities including (i) interactions with other proteins such as those of the cytoskeleton [84] or vesicular release machinery [6], (ii) phosphorylation [55], (iii) glycosylation [82], and (iv) the effects of small endogenously synthesised molecules such as polyamines [32, 57]. The roles, if any, similar activities play in the generation of Ca^{2+} channel diversity is largely unclear at present.

In conclusion, the efforts of protein chemists and molecular biologists have done much to clarify the basis of the Ca^{2+} channel diversity first brought to light by electrophysiological and pharmacological studies. When any remaining undiscovered genes are cloned, and the full molecular basis of Ca^{2+} channel diversity is realized, the main questions will again swing to the implications and functional necessity for such a wide-ranging diversity.

Andy Randall's laboratory is supported by the Medical Research Council (UK). I would like to thank Ms. Nicolle McNaughton (MRC, Cambridge) and Dr. Anna Williamson (UCHSC, Denver) for their comments on this review.

Note added in proof

Since submitting this review Perez-Reyes and Colleagues (Perez-Reyes, E., Cribbs, L.L., Daud, A., Lacerda, A.E., Barclay, J., Williamson, M.P., Fox, M., Rees, M. and Lee, J.H.) have made the major advance of cloning an α1 subunit that encodes a bona fide T-type Ca^{2+} channel. Unlike the α1E subunit, when expressed in oocytes this novel subunit (α1G) produces a conductance that bears all of the classical signature properties of T-type channels described in this review. These include true low-voltage activation, rapid inactivation, slow deactivation and a small single-channel conductance (7.5 pS). These important new results support the hypothesis of Randall and Tsien that α1E does not encode the T-type channel, but rather produces a channel akin to the R-type Ca^{2+} channel seen in cerebellar granule cells.

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