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It Is Calmodulin After All! Mediator of the Calcium Modulation of Multiple Ion Channels

Minireview

Irwin B. Levitan*
Biochemistry Department and
Volen Center for Complex Systems
Brandeis University
Waltham, Massachusetts 02454

Just over 20 years ago, Paul Brehm and the late Roger Eckert reported a curious finding in a curious organism: voltage-dependent calcium channels in the ciliate *Paramecium* are not only opened by membrane depolarization, they also are inactivated during a sustained depolarization, by the very calcium that enters through the open calcium channels (Brehm and Eckert, 1978) (see Figure 1). This kind of calcium-dependent inactivation was very different from the time- and voltage-dependent inactivation of axonal sodium channels that had been described a generation earlier by Hodgkin and Huxley. Thus, their initial report was greeted with a touch of skepticism, but Eckert and his colleagues soon put all doubts to rest with a series of lovely papers confirming the essential role of calcium in the inactivation process, and extending the finding to neurons as well (e.g., Eckert and Tillotson, 1981).

With the demonstration that calcium channels come in multiple flavors with different properties, and the subsequent cloning of cDNAs encoding the pore-forming subunits for many of these channel types (see Catterall, 1995, for references), came the realization that only some calcium channels undergo calcium-dependent inactivation. Prominent among them is the L-type calcium channel, consisting of the pore-forming α_{1C} subunit together with accessory subunits. This channel is critical for such diverse phenomena as the prolonged cardiac action potential, smooth muscle contraction, and regulation of gene expression in neurons, and hence its feedback regulation by calcium-dependent inactivation is of fundamental biological significance. The molecular mechanism of calcium-dependent inactivation of L-type calcium channels has been elusive, but several recent papers suggest that the ubiquitous calcium transducer calmodulin plays a central role (Peterson et al., 1999 [March issue of *Neuron*]; Qin et al., 1999). Astonishingly, at least four papers implicating calmodulin in the regulation of different kinds of ion channels have appeared in the literature in the several weeks between the commissioning and the writing of this minireview. Rumor has it that more are still to come.

Calmodulin Mediates Activation of Some Calcium-Activated Potassium Channels

The idea that calmodulin might act as the mediator of calcium-dependent modulation of ion channels may seem rather obvious, given the ubiquitous role of calmodulin in so many cellular processes. The knowledgeable reader may even snort with derision and wonder why it took channelologists so long to come up with this concept. In fact, channelologists have had this idea for some time, but a rather general experimental finding has been that standard calmodulin inhibitors that block the calcium-dependent binding of calmodulin to its target proteins do not interfere with calcium regulation of ion channels. Ching Kung and his colleagues proposed more than a decade ago (reviewed by Saimi and Kung, 1994) that calmodulin might mediate the activation of calcium-activated potassium (and calcium-activated sodium) channels in *Paramecium*, but until recently there was no indication that this is the case other than in this curious organism.

Calcium-activated potassium channels, which use potassium as their charge carrier but require intracellular calcium to help them open, also come in several flavors. One of these is a relatively small conductance channel, the SK channel, that is responsible for the slow afterhyperpolarization that follows an action potential and thereby regulates the frequency of action potential firing in neurons. John Adelman and his colleagues (Xia et al., 1998) used a yeast two-hybrid assay and biochemical techniques to show that calmodulin binds constitutively to this channel, even in the absence of calcium. This is reminiscent of the enzyme phosphorylase kinase, of which calmodulin is an obligatory subunit that can be removed only under denaturing conditions (Picton et al., 1980). The coexpression of the SK channel with mutant calmodulins that exhibit altered affinity for calcium changes the calcium sensitivity of the channel in a way consistent with the hypothesis that calmodulin is indeed

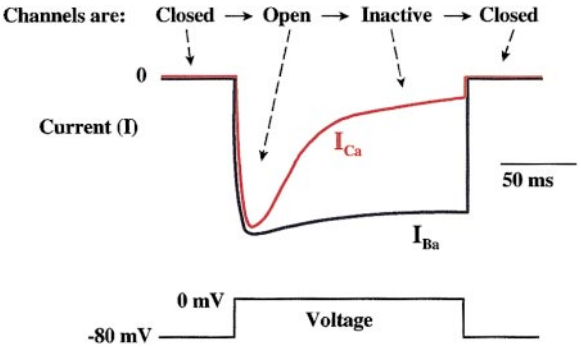

Figure 1. Calcium-Dependent Inactivation of Calcium Current

In response to membrane depolarization, for example from −80 mV to 0 mV (bottom voltage trace), calcium channels open and calcium ions flow into the cell down their electrochemical gradient. The inward Ca^{2+} current is shown, by convention, as a negative deflection. When calcium is used as the charge carrier, the calcium current (I_{Ca}, red trace) decreases with time during the depolarization, as a result of calcium-dependent inactivation of the calcium channels. Barium ions permeate calcium channels even better than calcium ions do but do not produce inactivation. Hence, when Ba^{2+} is used as the charge carrier, the barium current (I_{Ba}, black trace) is sustained throughout the membrane depolarization (the barium and calcium currents have been normalized to their peak amplitudes to facilitate comparison of their inactivation kinetics). This lack of inactivation with barium is one of many pieces of evidence that the inactivation is indeed calcium dependent (Brehm and Eckert, 1978).

*E-mail: levitan@brandeis.edu.

the endogenous calcium sensor (Xia et al., 1998). A very similar result has been reported recently for the intermediate conductance (IK) calcium-activated potassium channel family that is found predominantly in peripheral cell types including hematopoietic cells: calmodulin is bound constitutively to the channel, and mutant calmodulins can act as dominant negative suppressors of channel current (Fanger et al., 1999).

Where does this leave the large conductance (BK) calcium-activated potassium channels encoded by the Slowpoke gene family? A domain in the extended carboxy-terminal region of the α subunit, termed a "calcium bowl," has been implicated in the calcium sensitivity of these channels (Schreiber and Salkoff, 1997). My laboratory found long ago that calmodulin inhibitors do not influence the calcium sensitivity of BK channels—but nor do these same inhibitors affect SK or IK channels, apparently because the calmodulin is bound constitutively. Thus, although no evidence of a role for calmodulin in BK channel activation has yet emerged, one wonders whether this issue should not be revisited in the light of the recent findings with SK and IK channels summarized above.

Calmodulin Mediates Calcium-Dependent Inhibition of Cyclic Nucleotide–Gated Ion Channels

Phototransduction in retinal rod photoreceptors and olfactory transduction in olfactory sensory neurons are mediated by members of the family of cyclic nucleotide–gated (CNG) ion channels, whose opening requires the binding of cAMP or cGMP to the channel. CNG channels are permeant to both sodium and calcium and regulate the entry of these ions into the sensory cells, as a result of signal-induced changes in the intracellular concentrations of cAMP (olfactory neurons) or cGMP (rod photoreceptors). Calcium has long been known to play a key role in both visual and olfactory adaptation, and work from several laboratories, including those of Robert Molday, King-Wai Yau, and Anita Zimmerman, suggests the involvement of calmodulin in the regulation of CNG channel activity (see Zimmerman, 1995, and Molday, 1996, for references).

Calmodulin affects the rod and olfactory CNG channels in similar ways: it decreases channel activity by decreasing the sensitivity of channel gating to cyclic nucleotides. However, there are some interesting differences in the details. For example, calmodulin binds to the α subunit of the olfactory channel, whereas it is the β subunit of the rod channel that interacts with calmodulin (Molday, 1996; Grunwald et al., 1998). Amphibian rods may contain an endogenous constitutively bound modulatory calcium-binding protein that is distinct from calmodulin, but their CNG channels are also sensitive to calmodulin (Zimmerman, 1995). Such constitutive calcium-independent binding is reminiscent of the results for other channel types described in this minireview.

Calmodulin Mediates Calcium-Dependent Inhibition of NMDA Receptor Ion Channels

The N-methyl-D-aspartate (NMDA) class of glutamate receptor/channel plays critical roles in such fundamental phenomena as synaptic transmission, synaptic plasticity, synapse formation, and excitotoxic cell death in the mammalian central nervous system. A key property of the NMDA receptor ion channel that contributes to these multiple roles is that it exhibits a high permeability to calcium. Work by Mark Mayer and Gary Westbrook (Mayer and Westbrook, 1985), and subsequently by many other laboratories (see Zhang et al., 1998, and Krupp et al., 1999, for references), has demonstrated that receptor-mediated calcium influx leads to rapid calcium-dependent inactivation of NMDA receptors. More recent evidence implicates calmodulin in this calcium-dependent inactivation process.

NMDA receptor/channels are heterooligomeric complexes containing members of the homologous NR1 and NR2 subunit families. Richard Huganir and his colleagues found that calmodulin binds to a portion of the carboxy-terminal domain of the NR1 subunit, and that this interaction reduces the open probability of NMDA receptor/channels in a manner that can account for calcium-dependent inactivation (Ehlers et al., 1996). Inactivation in neurons may actually be more complicated and more interesting, involving yet another NR1 binding partner, the actin-binding protein α-actinin that competes with calmodulin for binding to NR1 (Wyszynski et al., 1997). Experiments from the Huganir and Westbrook laboratories suggest that inactivation may occur when calcium-bound calmodulin competitively displaces α-actinin from the NR1 subunit, thereby releasing the receptor/channel from its attachment to the actin cytoskeleton (Zhang et al., 1998; Krupp et al., 1999). Calcium may also bind directly to α-actinin and thereby reduce its affinity for NR1, independent of the displacement by calcium/calmodulin (Krupp et al., 1999).

Calmodulin Mediates Calcium-Dependent Inactivation of L-Type Calcium Channels

These indications that calmodulin may be used widely as a calcium sensor by other calcium-dependent ion channels provide the context for the recent flurry of work on calcium-dependent inactivation of L-type calcium channels. Many investigators have favored the hypothesis that calcium produces inactivation by binding directly to the α_{1C} pore-forming subunit (see Peterson et al., 1999, for references), in part because there is a putative EF hand calcium-binding motif in the carboxy-terminal domain of this subunit that is essential for calcium-dependent inactivation (Zühlke and Reuter, 1998). In addition, it is difficult to block calcium-dependent inactivation with calcium chelators, suggesting that the site of calcium binding for inactivation must be very close to the channel pore through which the calcium enters. Finally, calmodulin inhibitors do not interfere with inactivation—but then again, it is evident from many of the studies cited here that negative results with such inhibitors can be misleading.

Several intriguing pieces of evidence did indeed point to calmodulin as a player in L-type calcium channel phenomena. For example, Richard Tsien and his colleagues demonstrated that calcium entry into hippocampal neurons through L-type (but not other) calcium channels causes calmodulin to translocate to the nucleus, where it participates in the regulation of gene expression (Deisseroth et al., 1998). In addition, a mutational analysis from Harald Reuter's laboratory of the carboxy-terminal domain of α_{1C} identifies a so-called IQ calmodulin-binding motif as one of several sequences in this region important for calcium-dependent inactivation (Zühlke and Reuter, 1998).

Now, groups led by Lutz Birnbaumer (Qin et al., 1999)

and David Yue (Peterson et al., 1999) demonstrate that calmodulin binds to α_{1C} and mediates calcium-dependent inactivation. Both groups use biochemical approaches, including fusion protein pull-down experiments and gel shift assays, to demonstrate direct interaction of the IQ motif with calmodulin in a calcium-dependent manner. They also carry out mutational analysis (of both calmodulin and α_{1C}), and find that calcium-dependent inactivation can be eliminated either by mutations in calmodulin that diminish its affinity for calcium (Peterson et al., 1999) or by mutations in the α_{1C} IQ motif that interfere with its binding to calmodulin (Qin et al., 1999). The Yue group shows the interesting experiment of coexpressing with α_{1C} a mutant calmodulin that lacks high-affinity calcium binding; this abolishes calcium-dependent inactivation, leading to the suggestion that endogenous calmodulin normally mediates inactivation and that it can be displaced by the mutant acting in a dominant negative manner (Peterson et al., 1999; the Birnbaumer group reports a similar result as a note added in proof). The key conclusion from this dominant negative knockout of inactivation is that calmodulin must be tethered constitutively to the α_{1C} subunit, in a calcium-independent manner, at some site other than the IQ motif. Calcium that enters as a result of channel opening then binds to the tethered calmodulin and leads to its interaction with the IQ motif, thereby producing channel inactivation (see Figure 7 of Peterson et al., 1999). Local tethering of calmodulin seems, in retrospect, so logical a way to ensure a rapid response to a local calcium signal that one wonders why no one thought of it sooner. In any event, several of the ion channels discussed here clearly thought of it a long time ago!

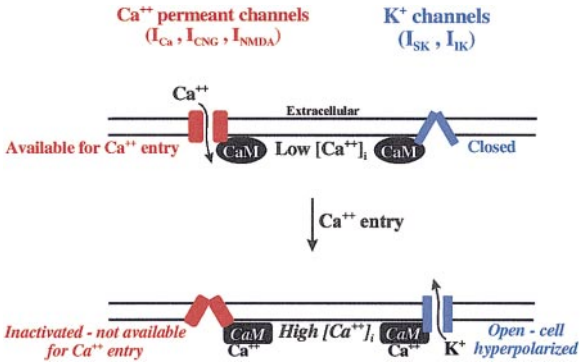

Figure 2. Calmodulin as a Negative Feedback Regulator of Calcium Entry Into Cells

As summarized in this minireview, calmodulin (CaM) can regulate the activity of several different kinds of ion channels. At rest (top), when intracellular calcium ($[Ca^{2+}]_i$) is low and calmodulin is not active, calcium-permeant channels (red) are available for calcium entry and calcium-activated potassium channels (blue) are closed. Hence, calcium entry is enabled. After calcium entry, calmodulin binds calcium and is activated (bottom). As a result, the red calcium-permeant channels are inactivated, and the blue calcium-activated potassium channels are open and can hyperpolarize the cell and thereby oppose voltage-dependent calcium entry. A common feature of these actions of calmodulin on different ion channels is that it acts as a calcium sensor and limits the amount of calcium that is allowed to enter the cell. I_{Ca}, I_{CNG}, and I_{NMDA} refer to calcium currents carried through L-type calcium channels, cyclic nucleotide–gated channels, and NMDA receptor/channels, respectively. I_{SK} and I_{IK} refer to potassium currents carried through the small and intermediate conductance calcium-activated potassium channels, respectively. In the case of the I_{Ca}, I_{SK}, and I_{IK} channels, calmodulin is tethered constitutively to the channel even at low intracellular calcium, as shown in the top part of the figure.

Concluding Remarks

What can we make of the apparently diverse group of channels whose activity is regulated by calcium, acting via calmodulin? An important feature common to all of these examples is that calcium feeds back to limit its own entry into the cell (Figure 2). In the case of the L-type voltage-dependent calcium channels, the NMDA receptor/channels, and the CNG channels that are permeant to calcium, this negative feedback is an obvious consequence of channel inactivation (Figure 2, left). It is also widely believed that at least some calcium-activated potassium channels are located in close proximity to voltage-dependent calcium channels, and so can act directly as sensors of the calcium that flows in through the latter channels. Because potassium channel activation will lead to membrane hyperpolarization and the closing of voltage-dependent calcium channels, calcium-activated potassium channels can act as feedback regulators of calcium entry (Figure 2, right). This occurs, for example, in presynaptic nerve terminals, where it may provide an important mechanism for regulating neurotransmitter release.

The curious finding of Brehm and Eckert finally has a molecular explanation, and a simple and satisfying one at that. The explanation is that L-type calcium channels use calmodulin as their calcium sensor, but in an unusual way: each channel has its own personal stash of calmodulin, which can be activated very quickly by the calcium that the channel itself conducts. Interesting problems of course remain; for example, the nature of the calcium-independent constitutive interaction of calmodulin with ion channels and the role of the essential EF hand motif in the L-type calcium channel (Zühlke and Reuter, 1998) should be elucidated. It is notable that several other flavors of calcium channels, the so-called R- and P/Q-type channels encoded by α_{1E} and α_{1A} subunits, respectively, also contain IQ-like motifs in their carboxy-terminal domains and bind calmodulin in a calcium-dependent manner (Peterson et al., 1999). These channels do not exhibit calcium-dependent inactivation, but certainly it is conceivable that some other key channel property is regulated by calcium/calmodulin. I very much doubt that we have seen the last of calmodulin as a modulator of membrane ion channels.

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