Commentary

Ion Trafficking through T-type Ca^{2+} Channels: A Way to Look at Channel Gating Position

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Voltage-gated Ca^{2+} channels play a key role in controlling Ca^{2+} entry during cell depolarization. At least 10 genes encode the main (α_1) subunits of voltage-gated Ca^{2+} channels, which have been grouped into two main classes: the high voltage–activated (HVA) and the low voltage–activated (LVA) channels. HVA channels are primarily involved in muscle contraction, synaptic transmission, and hormone secretion, while LVA channels are associated with action potential generation and repetitive electrical activity. Structurally speaking, the Ca^{2+} channel α_1 subunits forming the pore share strong similarities with other voltage-gated ion channels, in particular with Na^+-conducting pores (Hille, 2001). Each α_1 subunit has four domains (I–IV) linked together in a single polypeptide chain and each domain contains six putative transmembrane segments (S1–S6), plus a loop (P) that dips partially into the pore to, presumably, form the pore lining. Functionally speaking, the most striking similarity between Ca^{2+} and Na^+ channels is represented by the LVA (T-type) channels, which have comparably low threshold for activation (−50 to −40 mV in 5 mM Ca^{2+}) and inactivate fully and rapidly, if at a 20- to 40-fold lower rate than Na^+ channels. Like Na^+ channels, fast inactivation of T-type channels is strictly voltage rather than Ca^{2+} dependent, as in the case of channel types of the HVA family (L, N, P/Q, and R). T-type channels, however, possess other properties that are unique in comparison to other Ca^{2+} channels: (a) they deactivate more slowly (τ_{deact} = 2.5 ms at −110 mV in 5 mM Ca^{2+}; Carbone and Lux, 1984a); (b) they inactivate at relatively negative holding potentials; (c) they are equally permeable to Ca^{2+} and Ba^{2+}; (d) they have small single channel conductance; and (e) they outlast membrane-patch excision since they do not require specific metabolic factors to preserve their activity (Carbone and Lux, 1984b, 1987).

T-type channels were identified 20 years ago by several groups. Since then their biophysical, pharmacological, and functional properties have been widely investigated (for reviews see Huguenard, 1996; Perez-Reyes, 2003). A main drawback that significantly limited the analysis of ion permeability and gating properties of T-type channels was the lack of selective toxins or drugs that allowed for their pharmacological isolation. New impetus for approaching these issues was provided by the molecular cloning of three different pore-forming α_1 subunits (α_{1G}, α_{1H}, α_{1I} also denoted as $Ca_V3.1$, $Ca_V3.2$, $Ca_V3.3$) with biophysical properties that clearly identify them as T-type channels (Perez-Reyes et al., 1998). This opened for a new era of biophysical studies of LVA channels, which led important new insights into the features of ion selectivity and gating that distinguish LVA from HVA channels.

Among the new findings on ion permeation through cloned T-type channels, those concerning the blocking action of divalent and trivalent cations deserve particular attention. They show clear evidence for the following: (a) a voltage-dependent blocking action of Ni^{2+} (Lee et al., 1999), which is more effective on inward Ca^{2+} currents through α_{1H} channels compared with other T-type channel subunits; (b) a more effective blocking capability of Mg^{2+} on inward Ba^{2+} as compared with Ca^{2+} currents in α_{1G} channels (Serrano et al., 2000), uncovering a Ca^{2+}/Ba^{2+} selectivity that is absent in Mg^{2+}-free media; and (c) the existence of potent T-type channel blockers among trivalent cations, with yttrium (Y^{3+}) being the most potent blocker of α_{1G} currents (Beedle et al., 2002). The common aspect of these studies is that T-type channels can be blocked by multivalent ions larger or more hydrated than Ca^{2+} and that blocking ions can be effectively removed from their blocking position in a voltage- and current-dependent manner by strong depolarization. This is particularly evident in the case of Ni^{2+}, in which effective unblocking occurs while outward ion currents clear the channel. The unblock persists in the absence of permeating ions, proving that "ion–ion repulsion" (or single file diffusion) in a multi-ion pore favors but does not fully

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Abbreviations used in this paper: HVA, high voltage-activated; LVA, low voltage-activated.

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account for clearing the blocking ions from open channels. Inward and outward currents facilitate the clearing of Ca^{2+} channels (Kuo and Hess, 1993) but, in the case of Ni^{2+}, this effect accounts for only part of the relief of block. It is worth noticing that voltage-dependent unblocking is not a property limited to T-type channels. Blocking of HVA channels by Mg^{2+}, Cd^{2+}, and La^{3+} possesses the same features. Strong positive voltages can effectively remove the Mg^{2+}, Cd^{2+}, and La^{3+} block of inward Ca^{2+} currents also in the case that permeant ions are absent (Thévenod and Jones, 1992; Carbone et al., 1997; Block et al., 1998).

The most reasonable explanation of the voltage-dependent blocking and unblocking by divalent or trivalent cations is that ion permeation through T-type channels is controlled, as in HVA channels, by a single intrapore-binding site sufficiently deep into the pore to experience a fraction of membrane voltage (Fukushima and Hagiwara, 1985; Lux et al., 1990; Armstrong and Neyton, 1991; Yang et al., 1993; Dang and McCleskey, 1998). Application of strongly positive or negative voltages would significantly lower the outer or inner entry energy barriers, thus facilitating the escape of the blocking ion outside or inside the pore (Woodhull, 1973). The present view of the intrapore binding site controlling both ion selectivity and channel block (the selectivity filter) consists of a ring of four negative charged groups inside the pore. In HVA channels, each of the four P loops contains a glutamate forming the EEEE locus (Yang et al., 1993), in LVA channels, two glutamates are substituted by two aspartates in the corresponding position (EEDD locus; Talavera et al., 2001). The spatial arrangement of the four negative charges in the P loops is postulated to closely coordinate two Ca^{2+} ions whose sequential entrance and subsequent interaction should induce high Ca^{2+} fluxes while preserving high affinity for the pore site. This would explain the dual nature of Ca^{2+} ions as blockers of Na^+ currents at micromolar concentrations and as permeant ions at millimolar concentrations (Almers and McCleskey, 1984; Hess and Tsien, 1984). Removal of Ca^{2+} block at very positive or very negative voltages may be favored by a still not well resolved combination of current-dependent ion–ion interaction (Kuo and Hess, 1993) and voltage-dependent lowering of the energy barriers at both sites of pore entry (Fukushima and Hagiwara, 1985; Lux et al., 1990). The recent availability of T-type channel clones has allowed for closer comparisons between LVA and HVA channel permeability properties, highlighting the role that the EEEE or EEDD locus plays in the regulation of ion selectivity in the two channel groups. T-type channels have apparently the narrower pore size (5.1 Å diameter) compared with the α_{1C} L-type with a larger pore (6.2 Å diameter; Cataldi et al., 2002). Replacement of the two aspartates with two glutamates confers to the α_{1G} channel the same Cd^{2+} sensitivity of cardiac α_{1C} L-type channels but not the same Ca^{2+}/Ba^{2+} selectivity, suggesting that other structural elements besides the EEEE or EEDD locus contribute to the differences in selectivity and permeation properties between Ca^{2+} channels (Talavera et al., 2001).

Studies on ion permeation through open channels are relevant not only for determining the pore structure of the selectivity filter but also for determining the location and movements of groups responsible for channel gating. Interactions between ion permeation and activation–inactivation gating of Na^+, K^+, and Ca^{2+} channels are well documented (Hille, 2001), suggesting strong coupling between pore structure, ion passage, voltage-sensor movements, and channel gating. However, it is also possible to derive indirect information concerning the location of channel gating by simply looking at the properties of channel blocking and unblocking under suitable conditions (Swandulla and Armstrong, 1989; Thévenod and Jones, 1992). This is the main focus of the work by Obejero-Paz et al. (2004), which exploit the voltage-dependent blocking properties of Y^{3+} on α_{1G} T-type channels to infer about the intracellular position of the activation gate.

Looking at the literature on voltage-gated Ca^{2+} channels over the last two decades, it is evident that very little is known about the location of the Ca^{2+} channel activation gate. The little information available comes from the kinetics of HVA channels block by Cd^{2+}. Extracellular Cd^{2+} is thought to block the pore by binding with high affinity to the Ca^{2+}-selectivity filter, thus preventing Ca^{2+} flux. For HVA channels, Cd^{2+} block of open channels is voltage dependent, so that strong hyperpolarization (−80 mV) drives Cd^{2+} through the open channel into the cytoplasm (Swandulla and Armstrong, 1989), while strong depolarization (+80 mV) drives Cd^{2+} out into the extracellular space (Thévenod and Jones, 1992). However, Cd^{2+} also potently blocks the resting closed channel, even at high-hyperpolarized voltages (−80 mV) where open channels unblock rapidly. This simple observation implies that Cd^{2+} cannot easily escape a closed channel to the intracellular space, as expected if the closed portion of the gate is on the intracellular side of the pore. This is not surprising since it is likely that the main structural arrangement of the activation gate is well conserved among voltage-dependent ion channels (K^+, Na^+, and Ca^{2+}) and that the gate (or one of the gates) indeed is located at the intracellular vestibule of the channel, as inferred for voltage-gated K^+ channels by electrophysiological, spectroscopical, and crystallographic data (see Hille, 2001).

The present paper by Obejero-Paz et al. (2004) brings new evidence about the cytoplasmic location of

the activation gate of α_{1G} T-type channels by looking at the kinetics of ion entry while the channel is either open or closed. To do this, the authors exploited two unique properties of the elements under question: first, the voltage-dependent blocking properties of Y^{3+}, which unselectively block LVA and HVA channels with extremely high affinity (IC_{50} = 28 nM for the α_{1G} subunit; Beedle et al., 2002); and second, the peculiar gating properties of T-type channels that deactivate more slowly than HVA channels, allowing a comparison of the rates of Y^{3+} entry into open versus closed channel at the same potential. As for Cd^{2+} and La^{3+}, block of α_{1G} channels by Y^{3+} can be nearly abolished by strong depolarization (>100 mV). Thus, after brief depolarization (1 ms) to +200 mV, in which unblocking is almost complete, it is possible to estimate the degree and rate of reblocking of open channels at different voltages by returning to either positive or negative potentials.

Given these conditions, the authors could answer the question: can an extracellular Y^{3+} enter a closed T-type channel? Obviously, if the activation gate is on the cytoplasmic side of the pore, Y^{3+} should be able to enter the pore with no particular constraint independently of whether the gate is open or closed. An intracellular gate predicts equal access to open and closed channels (see Fig. 1 in Obejero-Paz et al.). Thus, a direct comparison of entry rates of Y^{3+} when the channel is mainly open or closed should answer the question. Since comparison of reblocking rates must be done at the same potential, the critical issue is to find a channel that is either open or closed for a sufficiently long time in the two states at the same potential. This is the case for α_{1G} T-type channels that in 2 mM Ca^{2+} are fully closed at −100 mV when this voltage level is maintained for seconds but can stay open for few milliseconds (τ_{deact} = 2.5 ms at −100 mV for α_{1G}; Serrano et al., 1999) when the same potential is reached after a brief depolarization sufficiently positive to quickly open and clear the channel from blocking ions.

Obejero-Paz et al. (2004), show that the entry rate of Y^{3+} during reblock of open channels is fast and concentration dependent and that also closed channels are blocked by Y^{3+} at a concentration-dependent rate only eightfold slower (at −100 mV) than open channel block. A slower entry rate when the channel is closed may imply an extracellular activation gate, but a more reasonable explanation would be that Y^{3+} entry is conditioned by ion–ion interactions or ion competition for the occupancy of the selectivity filter inside the pore. In an open channel, permeant ions flow rapidly and Y^{3+} can enter at a rate approaching the diffusion limit while in a channel closed by an intracellular gate, ions will occupy more steadily the pore site. Entry of Y^{3+} therefore would be conditioned and delayed by the exit of permeant ions toward the extracellular side, which is less favorable at very negative voltages when the channel is closed. That ions compete for the selectivity filter was demonstrated by the fact that replacing Ca^{2+} with Ba^{2+} causes the entry of Y^{3+} to be speeded up by a factor 2.3, making the closed-blocked kinetics only a factor 3.2 slower than in the case of the open-blocked channel. Ba^{2+} binds less than Ca^{2+} to the selectivity filter and moves faster in and out of the pore. Thus, Y^{3+} can enter more easily when Ba^{2+} is the only permeant ion. If an extracellular blocker can enter rapidly and equilibrate with the closed pore, the main activation gate must be on the intracellular side of the selectivity filter and would exclude the existence of an extracellular gate. Consequently, Ca^{2+} channels do not close at both ends of the pore as previously suggested by studies of Cd^{2+} block on N-type channels (Thévenod and Jones, 1992). This also implies that closed channels can contain a blocking ion or, in more physiological conditions, a permeant Ca^{2+} ion, raising the question of whether a closed channel is indeed fully closed or weakly conductive. At present, there are no indications of detectable conductances associated with closed Ca^{2+} channels, but even a very low undetectable conduction through the closed channel would have significant consequences for the maintenance of intracellular Ca^{2+} levels.

A final remark concerns the possibility that Y^{3+} or other trivalent cations may directly affect channel gating. As illustrated in Fig. S2 and S3, Obejero-Paz et al. show that Y^{3+} apparently delays the α_{1G} inactivation gate at 0.3–1 μM concentrations. These low concentrations do not support unspecific screening effects of Y^{3+} on membrane negative surface charges but rather suggest interactions between the Y^{3+}-occupied selectivity filter and the inactivation gate. Modification of channel gating by the block of trivalent cations is not a property limited to voltage-gated Ca^{2+} channels. Gating modifications associated with changes of ion permeation have been recently reported also for the Na^+ TTX-resistant channels (Kuo et al., 2004). In this case, block of TTX-r Na^+ channels by La^{3+} and Cd^{2+} is effectively removed by strong positive potentials, but while La^{3+} markedly slows the inactivation kinetics and to a lesser degree the activation kinetics, Cd^{2+} produces only blocking effects. This indicates mutual and selective interactions between ion occupancy of the selectivity filter and channel gating. Consistent with this is the observation that point mutations of the EEDD locus of α_{1G} channels induce changes to channel gating. Aspartate-to-glutamate substitutions in domain III (EEED) and domain IV (EEDE) speed up the activation, inactivation, and deactivation kinetics (Talavera et al., 2003), indicating that changes of the structural arrangement of the selective filter can affect the movement of voltage sensors and the rate of gating transitions.

The bottom-line message is that despite the impressive work done on ion permeability and channel gating over the last 30 years, a large number of basic questions remain unanswered. This is particularly true for the Ca^{2+} channels where studies on the molecular mechanisms of gating are less advanced than for K^+ and Na^+ channels. Works on ion permeation through cloned T-type channels like the one discussed here favor the understanding of the overall channel gating organization. Learning more about the molecular arrangements controlling ion flows through the selectivity filter and how changes of ion permeation influence the proteic groups controlling voltage sensors and gating movements will help focus on the critical links between the pore structures responsible for channel function. This is crucial for understanding the physiological roles of Ca^{2+} channels, which are involved in the transient and resting control of intracellular Ca^{2+} that regulates vital activities of body function.

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