Voltage sensor of ion channels and enzymes

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Abstract Placed in the cell membrane (a two-dimensional environment), ion channels and enzymes are able to sense voltage. How these proteins are able to detect the difference in the voltage across membranes has attracted much attention, and at times, heated debate during the last few years. Sodium, Ca^{2+} and K^{+} voltage-dependent channels have a conserved positively charged transmembrane (S4) segment that moves in response to changes in membrane voltage. In voltage-dependent channels, S4 forms part of a domain that crystallizes as a well-defined structure consisting of the first four transmembrane (S1–S4) segments of the channel-forming protein, which is defined as the voltage sensor domain (VSD). The VSD is tied to a pore domain and VSD movements are allosterically coupled to the pore opening to various degrees, depending on the type of channel. How many charges are moved during channel activation, how much they move, and which are the molecular determinants that mediate the electromechanical coupling between the VSD and the pore domains are some of the questions that we discuss here. The VSD can function, however, as a bona fide proton channel itself, and, furthermore, the VSD can also be a functional part of a voltage-dependent phosphatase.

Keywords Voltage sensor · Kv channels · Cav Channels · BK channels · Proton channels and VSP

Introduction

The fascinating story of the voltage sensor of voltage-dependent channels started in 1952, and is summarized in two visionary sentences appearing in the last paper of the series by Hodgkin and Huxley (1952) predicting both the existence of a voltage sensor and the gating currents. The voltage sensor predicted: “...it seems difficult to escape to the conclusion that the changes in ionic permeability depend on the movement of some component of the membrane which behaves as though it has a large charge or dipole moment”. The gating charge predicted: “For the movement of any charged particle in the membrane should contribute to the total current...”.

It took 21 years to demonstrate the existence of Na^{+} channel gating currents (Armstrong and Bezanilla 1973; Keynes and Rojas 1973; Bezanilla 2000), small (~1% of the ionic currents) transient currents appearing before the onset of the Na^{+} current. Ten years later, with the introduction of the molecular biology techniques and the cloning and characterization of the primary structure and functional expression of the voltage-dependent Na^{+} channel, we had the first hint about the structural determinants of the voltage sensor (Noda et al. 1984, 1986). The sodium channel protein was found to consist of four domains (I–IV), each containing six transmembrane (TM) segments (S1–S6). The fourth TM segment (S4) contains positively charged residues periodically separated by two hydrophobic residues. Numa and coworkers (Noda et al. 1984) proposed that this structure, S4, hosts the determinants for voltage-sensitivity and, together with Stühmer’s group, gave some of the first electrophysiological evidence that the positive charges in S4 were involved in voltage-sensing (Stühmer et al. 1989).

The actual “molecular biophysics” of voltage-dependent ion channels started, however, with the cloning of the

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Shaker K^+ channel that as a smaller protein is more amenable to genetic manipulations than the Na^+ channel. The Shaker K^+ channel consists of subunits, each containing six TM (S1–S6) segments, leaving the N- and C-terminal oriented towards the internal side of the membrane (Papazian et al. 1987). As in sodium channels, the S4 segment is rich in arginine residues (the S4 of Shaker contains seven positively charged amino acids residues) which later proved to be the channel gating charges (e.g., Aggarwal and MacKinnon 1996; Seoh et al. 1996). Voltage-dependent K^+ (Kv) channels consist of subunits that look like a single domain of voltage-dependent Na^+ (Nav) channels—unsurprisingly, Kv channels are tetramers (MacKinnon 1991).

Almost simultaneously with the identification of the gating charges in Shaker channels, the movements of the voltage sensor in Na^+ and K^+ channels, the piece lacking in the puzzle, were detected (Yang and Horn 1995; Larsson et al. 1996; Mannuzzu et al. 1996; Yusaf et al. 1996; Cha and Bezanilla 1997; Gonzalez et al. 2000, 2001; Latorre et al. 2003; Gonzalez et al. 2005). Fluorescence probes inserted in the neighborhood of S4 produce voltage-dependent fluorescent changes (ΔF) that, when plotted against voltage originated ΔF -V curves, precede the G(V) curves [like the Q(V) curves]. That observation demonstrates that either the S4 undergoes a displacement, or its accessibility is altered during channel gating. This saga was followed by the finding that in Shaker the first four charges in S4 contribute to the total gating charges as if they move across the entire electric field (Aggarwal and MacKinnon 1996; Seoh et al. 1996; Starace et al. 1997; Starace and Bezanilla 2004; reviewed in Bezanilla 2000).

In 2003, the MacKinnon group (Jiang et al. 2003) crystallized a bacterial voltage-dependent K^+ channel, KvAP, and determined its structure. They also found that the isolated voltage sensing domains (S1–S4) crystallize as a single well-defined structure. That finding, as well as the existence of a whole family of K^+ channels consisting of only two transmembrane segments (Doyle et al. 1998; Kubo et al. 2005), makes it clear that Kv channels are modular, consisting of a voltage sensor domain (VSD) and a separate pore domain. The determination of the structure of KvAP was followed by the crystallization and elucidation of the structure of a mammalian Kv channel (Kv1.2) as well as a chimera between the mammalian Shaker Kv1.2 channel and the Kv2.1 channel (Long et al. 2005, 2007). The chimeric channel was crystallized in the open conformation, giving important hints about how the electrical energy contained in the electric field is transformed into pore opening.

Surprisingly, Murata and coworkers (Murata et al. 2005) found a protein consisting of VSD followed by an intracellular domain coding for a phosphoinositide phosphatase (*Ciona intestinalis* voltage sensor-containing phosphatase,

Ci-VSP) based upon genomic information from the urochordate *Ciona intestinalis*. Nature had more surprises for us: in 2006, two groups cloned a voltage-gated proton (Hv) channel consisting of only the VSD (Ramsey et al. 2006; Sasaki et al. 2006). These channels have an S4 containing 3 arginines separated by two hydrophobic amino acids, as in Nav and Kv channels. As well as being extremely selective to protons, they are activated by depolarizing voltages (like Nav and Kv). Thus, it appears that the VSD on its own can give rise to voltage-dependent enzymes, as well as voltage-dependent channels.

Based upon what has been learned from the crystal structure of Kv channels and fluorescence studies, we discuss here our current knowledge of the conformational changes in the VSD in voltage-dependent channels, as well as how these conformational changes may be coupled to channel opening. We also review what is known about the voltage sensors in Ca^{2+} and Na^+ channels. We describe in some detail the voltage sensor of the BK channel since BK gating charge is decentralized. In contrast to Kv channels, only half of the total gating charge is contained in the S4 segment in BK channels. Furthermore, we look at the structure and function of proton channels, which has only recently been explored on a molecular level. Although it is most likely that the S4 segment is responsible for the voltage dependence in proton channels, how S4 movement is coupled to proton flux is still a mystery in these channels lacking a “pore domain”. Finally, we thought the case of Ci-VSP of interest because it provides a remarkable example of the coupling of the cell electrical activity with its metabolism.

Kv channels are modular

The Kv family consisting of 40 different genes is the largest and most diverse family in the S4 superfamily of ion channels, which also includes the Nav, Cav, and Slo family of voltage-dependent channels (Gutman et al. 2005). The first K^+ channel gene cloned was the Shaker K^+ channel from *Drosophila melanogaster*. The sequencing of Shaker (Kamb et al. 1987; Papazian et al. 1987; Tempel et al. 1987), and the similarity of the putative membrane topology (Fig. 1) of the Shaker protein to the domains of Nav channels (Noda et al. 1986), suggests a tetramer containing subunits consisting of 6 transmembrane segments (S1–S6), where the S4 contains 7 positively charged residues (5 arginines and 2 lysines, Fig. 2). Kv channels were soon confirmed to be tetrameric arrangements of subunits containing six transmembrane (MacKinnon 1991; Liman et al. 1992).

The cloning of inward rectifier K^+ channels and the elucidation of the structure of the bacterial K^+ (KcsA; Doyle et al. 1998) channel revealed that functional K^+ channels

Fig. 1 Membrane topology of voltage-dependent channels and enzymes. Membrane topology of voltage-dependent channels and enzymes discussed in this review. The extracellular side is up. The voltage sensor domain is colored in *blue* and the pore domain *light blue*. Regulatory domain of BK are shown in *gray*, and the phosphatase of C_i -VSD in *purple*

can be made of subunits consisting of only two transmembrane segments and a pore region similar to the pore domain (S5-pore-S6) domain of Kv channels. On the other hand, the ability to produce functional voltage-dependent chimeric K^+ channels by substituting the pore of KcsA into the Shaker K^+ channel (Lu et al. 2001) suggests that Kv channels are modular, composed of two distinct functional domains, the voltage sensor domain (VSD; S1–S4) and the pore domain (S5-pore-S6; Fig. 1). The strict structural requirements to generate functional chimeras between Shaker and KcsA reveal the existence of an electromechanical coupling: the voltage sensor transforms the electrical energy contained in the voltage gradient across the membrane into mechanical energy to open the pore. The bona fide proof that the voltage sensor of Kv channels is a separate structural domain appeared, however, with the crystallization of the voltage-dependent K^+ (KvAP) channel from the bacteria *Aeropyrum pernix* (Jiang et al. 2003). The S1–S4 transmembrane segments crystallizes as a unit, and its structure is similar to the voltage sensor contained in the chimeric Kv1.2–Kv2.1 channel resolved at 2.4 Å and crystallized in the presence of lipids (Long et al. 2007).

Kv gating charges revealed

Voltage-dependent K^+ channel gating currents were recorded almost 10 years after the measurements of the Na^+ channel gating current by the Bezanilla group (Bezanilla et al. 1982; White and Bezanilla 1985). K^+ gating currents develop before the ionic currents with a fast rising phase and an exponential decay. The gating charge-voltage curve is shifted to the left along the voltage axis with respect

to the conductance-voltage curve (Fig. 3), a clear demonstration that the gating charge moves between closed states. These two observations are most economically explained using linear kinetic schemes like the one shown below:

$$\begin{array}{ccc} K_0 & & K_n \\ C_0 \rightleftharpoons C_1 \cdots C_n \rightleftharpoons O & & \\ q_0 & & q_n \end{array} \quad (R1)$$

where the transition from closed state C_{i-1} to C_i is associated with charge movement q_{i-1} and an equilibrium constant $K_{i-1}(V)$ at the potential V and O is the open state.

Current fluctuation analysis provides a method to measure the number of channels (N) in a given membrane area (Sigworth 1980). By applying fluctuation analysis, if the total charge displaced in the same area is known, then the charge displacement per channel (Q/N) can be obtained. Measurements of total charge displacement applying this method have converged on the currently accepted value of 3–4 e_0 per K^+ channel voltage sensor. (Schoppa et al. 1992). The MacKinnon group (Aggarwal and MacKinnon 1996) improved the precision of these methods by engineering a specific radioactive toxin. The toxin binds in a one-to-one fashion to the channel enabling the determination of Shaker K^+ channel density (N) directly, yielding a Q/N value of 12–13 e_0 charges for the Shaker K^+ channel. Similar numbers have been obtained by the Bezanilla group (Seoh et al. 1996) using the Q/N and limiting slope method (Almers 1978; Schoppa et al. 1992; Sigg and Bezanilla 1997; Gonzalez et al. 2000). It is important to note here that the Q/N method detects all the charge displaced during channel activation whether or not this charge is associated to channel opening.

Fig. 2 VSD sequence alignments. Alignment of sequences recognized as the VSD of representative voltage-dependent channels and enzymes. The alignments include Shaker (uniprot.org access number: P08510), Kv1.2 (P63142), Kv2.1 (P15387), Ci-VSOP (Q1JV40), Ci-VSP (Q4W8A1), BK (Q12791), Cav1.1 (O57483) and Nav1.1 (Q25377)

On the other hand, the limiting slope method, introduced by Almers (1978), is able to give a good approximation of the number of effective gating charges directly associated with channel opening, under the assumption that, if there is more than one open state, transition between open states must be voltage-independent. This method yields a lower limit to the range of single channel gating charge displacement. The theory underlying the limiting slope method was refined by Sigg and Bezannila (1997) to include non-linear sequential models under the assumption described above. One way to derive the “limiting slope” is to start with the

Fig. 3 Gating currents of some channels and enzymes. Gating currents obtained from different kinds of voltage-dependent channels and enzymes. Examples of superimposed gating current traces at different voltages are shown on the left. *Q(V)* plots are shown in blue on the left. For voltage dependent channels, the *G(V)* curves are also shown, in green. **a** Ig from Shaker W434F recorded on-cell by a patch-clamp in symmetrical KMES. Voltage pulses are from −80 to 50 mV. The GV corresponds to a simulated Boltzmann. **b** Human BK channel (hslow) recorded with inside-out patches in symmetrical NMDG-MES. The GV was obtained in symmetrical KMES. Voltage pulses are from −90 to 350 mV. **c** Ig from CaV channels (CaV1.2) recorded with cut-open voltage clamp technique in 2 mM Co. The GV was obtained in 10 mM Ba2+. Voltage pulses are from −80 to 60 mV and from −80 to 110 mV, for gating and ionic currents respectively. **d** Ci-VSP modified from Murata et al. (2005)

linear sequential model R1 given above where the open probability when the voltage takes a large negative value is given by the relationship:

$$P_0 = Ke^{-QV/RT} \quad (1)$$

where K is the product of all the equilibrium constants at zero membrane voltage and Q is the sum of all charge movements, $Q = \sum q_i$. From Eq. (1), it is clear that: $d \ln(P_0)/dV \approx Q/kT$

The slope of the curve of P_o versus potential may thus be used as an approximation to the total charge which must be moved to open the conducting pore. The agreement between the values for the total charge per channel (12–13 e_0 ; Fig. 4a) using these two methods (limiting slope and gating charge/subunit) indicates that in Shaker channels all the gating charge is coupled to channel opening.

Charge-neutralizing mutations of residues in the VSD of Shaker K^+ channels show that only E293 (an acidic residue in S2, Fig. 2 and E2 in Fig. 5a), and R362, R365, R368, and R371 (in S4, Fig. 2, and R1, R2, R3 and R4 in Fig. 5b) contribute significantly to the gating charge. Neutralization of each of these residues lead to large decreases in the gating charge and in the effective valence of the voltage dependence as calculated using the limiting slope method (Aggarwal and MacKinnon 1996; Seoh et al. 1996). From these studies, it can be concluded that the first four charges of S4 contribute to the gating charge, but there is no unique explanation for the effects of neutralization. Neutralization can remove the charge and alter the extent of movement of the remaining charges, or it can modify the electrical profile seen by the remaining charges (Nonner et al. 2004; reviewed in Bezanilla 2008). Actually, it is most probable that neutralization of the negative residue contained in S2 induces a change in the electric field rather than contributing directly to the number of gating charges.

The first indications that gating charges move during activation came from accessibility studies in Na^+ channels showing that the rates of modification of cysteines replacing S4 charged residues are voltage-dependent (Yang and Horn 1995; Yang et al. 1996). Similar studies were also carried out in Shaker K^+ channels (Larsson et al. 1996), revealing that cysteines replacing arginines at positions 362 and 365 are only accessible from the outside at depolarizing voltages, while a cysteine replacing R368 can be modified from the inside only while the channel is closed. These results not

Fig. 4 Limiting Slope Analysis. Limiting slope analysis of ShakerIR and Hv channel. **a** Corresponding to the fit slope factor $z\delta$ at different voltages. the red line is the asymptotic value of z at very hyperpolarizing voltages obtained by the derivative with respect to the voltage of the monoexponential fit at low probability, in this case, $z=12.8 e$. **b** Fit for the slope factor $z\delta$ of an Hv Channel: the value is $z=6.1 e$

Fig. 5 VSD models for Kv Channels. Structural model of the resting/activated VSD of the Kv1.2. S1–S4 are colored individually in side (**a**) and front (**b**) views. Side chains of charged residues are also shown: carbons in yellow, oxygen in red and nitrogen in blue. The Kv1.2/Shaker corresponding positions are as follows: E226/E283 for E1, E236/E293 for E2 in S2; D259/D316 for D in S3; and R1 to R4 in S4. The structural model was generated for Isacoff's group (Pathak et al. 2007)

only support that the S4 moves but also point to the existence of a short hydrophobic septum in the closed state. In this conformation, position 362 is only accessible from the outside while 368 is accessible from the internal side. Thus, only 6 amino acid residues separate the internal from the external milieu, corresponding in an α -helix to a septum of $\sim 9 \text{ \AA}$. Histidine scanning mutagenesis yielded further insight by telling us how much of the electric field was traversed by the gating charges in Kv channels (Starace and Bezanilla 2001, 2004; Chanda and Bezanilla 2008). Accessibility of histidines which replaced arginines R365, R368 in S4 reveal that R365H and R368H move across the full length of the electric field, as shown by the proton transport induced when S4 moves between resting and active states. In other words, in the presence of a pH gradient, histidines in positions 365 and 368 are able to transport protons in a merry-go-around fashion characteristic of proton transporters. Remarkably, mutation R362H behaves as a voltage-dependent proton channel when the voltage sensors are in the resting configuration. Although the mechanistic explanation of this result is controversial, it indicates that the external and internal water crevices are only separated by a short septum. Finally, R371H also forms a proton pore, but in this case, the pore conducts only when the sensor is in the active

configuration, indicating that the short region where the field is concentrated is present in both the closed and the open states (Starace and Bezanilla 2001) (Figs. 2 and 5). These findings also support the notion that the S4 is lined by water accessible crevices separated by a thin hydrophobic septum where the electric field is concentrated, as well as the motion of the first four charges of S4 moving across the entire length of the electric field.

On the other hand, a histidine scanning mutagenesis of the S1 and S2 transmembrane helices identified two residues that, when mutated, form proton pores (Campos et al. 2007). One such residue was found in S1 (I241) and the other in S2 segment (I287); cysteines introduced at either of these two positions are able to form disulfide bonds with a substituted cysteine in R362 in the closed state of the Shaker channel. The close proximity of these two hydrophobic residues with the first arginine in S4 has led to the conclusion that I241 and I287 form part of the hydrophobic septum through which charges are transported.

Also in favor of a thin septum and accessible crevices is the finding that replacing S4 arginines in Shaker and in Nav channels by smaller uncharged amino acids gives rise to ionic currents (Sokolov et al. 2005; Tombola et al. 2005, 2007). These currents that go through the VSD were dubbed “omega” currents to distinguish them from the alpha current that go through the conducting pore. The conducting pores are large enough to allow the flux of guanidinium ions that, like arginine, contain a guanidinium moiety. This type of result indicates that the guanidinium moiety of arginine can translocate charge by moving through an aqueous pore during gating.

A decrease in the size of the gating charge at low ionic strength suggests that part of the electric field falls across these water crevices (Islas and Sigworth 2001). From this reduction, an intracellular conical cavity of 20- to 24-Å depth and 12-Å aperture, and a smaller extracellular cavity of 3-Å depth and the same aperture were estimated, leaving a septum with an expected thickness of 3–7 Å. Consistent with this figure, using a series of permanently charged MTS reagents with alkyl tethers ranging from methyl to hexyl, Ahern and Horn (2005) found that short adducts (<3CH₂) added to R362C can be dragged across the electric field during activation to carry additional charge across it, but charged adducts with 6 CH₂ or longer linkers cannot be dragged across the septum, suggesting that these linkers are long enough to stretch with the voltage sensor’s movement, but without shuttling the additional charge across the field. Therefore, the low dielectric septum can only be 4 Å across.

Coupling between VSD and the K-selective pore

The classical experiments of Armstrong (1969, 1971) revealed for the first time the existence of an internal gate

able to hinder the passage of ions through K⁺ channels. The voltage sensor movement that makes the opening of that gate possible appears to require physical interaction between residues 475–480 in S6 with residues in the S4–S5 linker (Lu et al. 2001, 2002). In fact, the structure of the Shaker mammalian orthologue Kv1.2 shows a surface of intimate contact between these two helical segments in the same subunit, suggesting an allosteric communication between the VSD and the pore domain (Long et al. 2005). The VSD of one subunit is located near the pore domain of the adjacent subunit. A model has been proposed in which the VSDs perform mechanical work on the pore through the S4–S5 linker helices’ contact with the S6 transmembrane domain, constituting the coupling machinery that constricts or dilates the inner entrance of the pore (Long et al. 2005) (Fig. 5). This structure and that of the Kv1.2/Kv2.1 chimera show all voltage sensors in the active position and the pore open. In the closed state, this interaction surface probably persists because the open probability in Shaker K⁺ channels maintains a tight coupling to the voltage sensor, not showing any hints of becoming less voltage-dependent, even at open probabilities as low as 10^{−8} (Gonzalez-Perez et al. 2010; Schoppa et al. 1992; Islas and Sigworth 1999). Thus, the allosteric binding energy between the S4–S5 linker and the C-terminal-end of S6 remains high in the closed–resting state, being at least 11 kcal/mol.

VSD conformation during slow inactivation

The gating charge-voltage (Q-V) curves from slow inactivated channels are shifted to more negative voltages (~−60 mV) as compared with the Q-V curves obtained from non-slow-inactivated channels (Olcese et al. 1997; Gonzalez-Perez et al. 2008). Thus, slow inactivation correlates with the appearance of a highly stable conformational state of the voltage sensor. Such a conformational change induced by voltage appears to be an intrinsic property of the VSD, since the shift in the Q-V curve that is seen in slow-inactivated channels can be also observed when gating currents are measured using the isolated VSD (Villalba-Galea et al. 2008). Periodicity analysis suggests a S4 transition from a mostly 310 helical packing at resting to mostly α-helical at positive voltages. This latter packing being the observed in the structure of Kv channels, crystallized in the absence of electric field.

Voltage sensor in Nav and Cav channels

Nav and Cav channels consist of four non-identical Kv-like repeats daisy-chained in a single polypeptide (Catterall 1996) and, as a consequence, the amino acids composition

of each VSD diverged during evolution. Comparing the sequences of a wide range of invertebrate and vertebrate species (Strong et al. 1993) revealed that individual repeats of Nav and Cav channels share more similarity with each other's corresponding regions than with other repeats within the same channels, suggesting a common ancestral channel with four repeats. Since plant and protozoa express Cav channels, but not Nav channels, Strong and coworkers (Strong et al. 1993) suggested that the first Cav channels emerged from two rounds of duplication of a gene encoding for a channel consisting of multiple subunits containing six transmembrane segments, which later diverged to give rise to Nav channels. Perhaps as a remnant of this process are the splice variants of Cav (Wielowieyski et al. 2001; Arikath et al. 2002) and Nav (Plummer et al. 1997) that generate transcripts encoding for only two repeats. These truncated isoforms that are not targeted to the membrane appear to suppress the expression of the full length homologue but are not targeted to the membrane. However, an engineered two repeat Cav channels truncated at the intracellular loop joining the first and second repeat can be targeted to the membrane. When expressed in cultured myocytes, this two subunit channel gives rise to bona fide gating currents about half the size of the full length protein (Ahern et al. 2001), suggesting a symmetric contribution of all VSD to channel gating as hinted by the full conservation of the four voltage-sensing arginines of the S4 segments of all these channels (Fig 1).

Contribution of individual VSD to activation and inactivation in Nav channels

Evidence for a voltage-dependent movement of the S4 in Nav channels come from experiments showing that cysteines replacing arginines of the fourth S4 have a voltage-dependent rate of modification by sulfhydryl reagents (Yang et al. 1994, 1996). These studies and others (Smith and Goldin 1997; Cestele et al. 1998) have also revealed that the voltage sensor in the fourth repeat is linked to channel inactivation rather than activation. Many Nav- and Cav-related channelopathies that arise from missense mutations neutralizing S4 arginines have biophysical consequences, thus linking the fourth VSD domain to inactivation (Chahine et al. 1994; Yang et al. 1994; Cannon 2010). To obtain direct evidence that different VSDs of Nav channels are coupled to activation and inactivation to different degrees, Bezanilla's group adapted voltage-clamp fluorometry to Nav channel research by attaching fluorescent probes to individual VSDs (Cha et al. 1999; Chanda and Bezanilla 2002). These experiments showed that fluorescence changes followed the time course of channel activation when the fluorophore is attached to any of the first three VSDs. In

contrast, fluorescence signals from Nav channels labeled in the fourth VSD are much slower and mirror the time course of current inactivation and charge immobilization. The picture that emerges is that the first three VSDs activate in parallel and precede channel opening, while the movement of the fourth VSD is rate limiting for inactivation. VSDs from neighboring subunits also appear to interact, as perturbation in the gating charges of the first S4 alters the behavior of the fourth VSD domain (Chanda et al. 2004). Undoubtedly, individual S4s are coupled to different degrees to Nav channel activation and inactivation.

Charges involved in voltage sensing in Na and Cav channels

Using noise analysis of gating currents recorded in macro-patches of *Xenopus* oocytes expressing Nav1.2, Conti and Stuhmer (1989) measured the number of gating charges per gate at approximately $2.3 e_0$. If the movement of all four VSD contributes to the gating noise, then the total number of charges per channel would be $9.2 e_0$. Using the limiting slope method on Nav channels carrying the QQQ mutation which removes inactivation, Hirschberg and colleagues (Hirschberg et al. 1995) showed that 12 elementary charges were necessary to open the channel. This number is slightly higher than predicted from Conti and Stuhmer but is consistent with measurements in Kv channels, suggesting that S4 segments of each repeat carries about 3 charges and all contribute equally to channel opening in an inactivation removed Nav.

In the case of Cav channels, the results of two methods were compared to determine the number of charges per channel (Noceti et al. 1996): the limiting slope analysis and Q/N method. For the limiting slope, membrane patches were subjected to slow voltage-ramps to obtain a stationary conductance versus voltage that was then adjusted to a monoexponential approximation of the Boltzmann function at very negative potential. For the Q/N, charge movement (Q) was measured at the onset of a pulse to the current reversal potential and the number of channels (N) was estimated via noise analysis of tail currents (Q/N). The limiting slope method yielded 8.6 elementary charges for two types of Cav channels (Cav1.2 and Cav2.3) while the Q/N method produced about 15 elementary charges for Cav2.3. Interestingly, when co-expressed with $\beta 2a$, one of the auxiliary subunits known to facilitate channel opening (Neely et al. 1993; Olcese et al. 1994), the number of charges per channel measured by the two methods converges to 8. Two factors may contribute to overestimating z when using the Q/N method: (1) not all charges are coupled to channel opening, and (2) since the maximal P_O is small, the number of channels may have been underestimated. It is

noteworthy that the subunits co-expressed to increase the Po also inhibit inactivation. Perhaps some VSDs are coupled only to the inactivation gate and thus excluded from limiting-slope measurement, yet they contribute to the total charge displaced per channel (Q/N). In this scenario, Q/N may be reduced in channels in which the inactivation process is hindered, such as those formed by the pore-forming subunit ($\alpha 1$) and the auxiliary subunit ($\beta 2a$).

Voltage-gated proton Hv channels

A family of voltage-sensing proton channels called Hv or VSOP channels were cloned from mouse, human and the sea squirt *Ciona intestinalis* (Ci-Hv) (Ramsey et al. 2006; Sasaki et al. 2006). Hv channel proteins contain 4 transmembrane segments (TMs) which are homologous to the 4 TMs of the VSD of voltage-gated channels. How is it possible that proteins encompassing a single VSD and lacking a pore domain produce a functional channel (Ramsey et al. 2006; Sasaki et al. 2006)? Lee et al. (2009) showed that purified Hv1 protein reconstituted into liposomes generates voltage-activated proton currents, confirming that the proton currents are indeed conducted by the Hv channel itself. An added complexity is that Hv channels are dimers, with dimerization inhibited by truncation of the C- and the N-terminus (Koch et al. 2008; Tombola et al. 2008). The sequence of the C-terminus suggests that the two C-termini from the two subunits in a Hv dimer form a coiled-coil structure known to mediate subunit interactions in other ion channels (Margeta-Mitrovic et al. 2000). However, the truncated mutant with reduced dimerization capabilities still conducts protons favoring the view that the permeating pathway is within the Hv subunit rather than at the interface between two neighboring subunits (Koch et al. 2008; Tombola et al. 2008). Hv channels are dimers with two pores, similar to voltage-gated CIC chloride channels (Koch et al. 2008; Tombola et al. 2008). MD simulations of Hv homology models, based on Kv channel crystal structures, suggest that water molecules penetrate deep into the protein and create a H^+ -conducting water wire across the channel (Ramsey et al. 2010; Wood et al. 2011). Recent mutagenesis experiments suggest that an aspartic residue in the middle of the channel is protonated/deprotonated during H^+ conduction and functions as the selectivity filter in Hv channels (Musset et al. 2011).

Structure and function of voltage sensor in Hv channels

The fourth TM (S4) segment in Hv channels has a motif where every third amino acid is a positively charged residue (arginine or lysine). The cysteine accessibility method,

developed in earlier work to study the S4 movement in Shaker K^+ channels (Larsson et al. 1996), has been used likewise to test the parallel hypothesis that in Hv channels, S4 moves across the cell membrane during activation. As predicted, depolarization promotes modifications of proton currents by externally applied MTSET to an Hv mutant carrying an external cysteine (I248), whereas no modification occurs under hyperpolarizing conditions that keep the Hv channels closed (Gonzalez et al. 2010). Similar results were obtained for a Cys substitution for Ala 246 and Val252 of the Ci-Hv channel (Gonzalez et al. 2010). The state-dependent modification of these three positions suggests an outward movement of the S4 segment or the rearrangement of the structural environment around these amino acid positions. Experiments on positions closer to the intracellular end of S4 segment, Ile 262 and Asn264 show MTSET modification only at hyperpolarized voltages (i.e., in the closed state of channel). These results suggest that the S4 movement transfers the equivalent of 2–3 gating charges across the membrane for each Hv subunit. Using the limiting slope method, Gonzalez et al. (2010) estimated the effective gating charges that couples to channel opening in the monomeric and dimeric Hv channel. They found that 2–3 charges were associated with the activation of monomeric channels, whereas 4–6 charges appear necessary to open dimeric channels. These findings on the cloned Hv channels are consistent with earlier studies where DeCoursey's group found that the movement of 6 equivalent gating charges appears necessary to open Hv channels in airway epithelia cells. The larger number of gating charges in dimeric channels suggests a high degree of cooperativity among Hv subunits (Decoursey and Cherny 1997).

Additional evidence for cooperativity came from fluorescence measurements. The Hv-S242C channel expressed in oocytes and labeled with the fluorophore Alexa488-maleimide reports on the movement of S4. Consistent with the hypothesis that Hv uses its positively-charged S4 as its voltage sensor, the fluorescence changes preceded the proton current during depolarization (Gonzalez et al. 2010). Furthermore, the square of the values of fluorescence traces reproduce the kinetics of the proton currents reasonably well, suggesting that there are at least two kinetic steps in each subunit before the channels open, or that the S4 of both subunits needs to activate before the channels can open. These results can be explained using an H–H type of kinetic model in which one S4 in the resting position inhibits the proton currents through both subunits (Fig. 6), and both S4s need to be activated before the proton current can flow through either subunit. Since there is no inhibition from a second subunit, in the monomeric Hv channel the activation of one S4 suffices to allow proton flow. In experiments using linked Hv subunits with different voltage dependences, Tombola and coworkers also showed that the two

Fig. 6 Hv channels kinetic models. Channel opening in a dimeric Hv channel. The model shows that each subunit has its own permeation pathway. The—assumed— independent activation of each S4 enables the proton current only when both S4s have activated

subunits in a Hv dimer activate with a high degree of cooperativity (Tombola et al. 2010).

Voltage sensor in BK channels

Ca^{2+} and voltage-dependent K^+ channels of large conductance (BK) are homo tetramers formed of α subunits possessing an extracellular N terminus and seven transmembrane segments (Fig. 1). The large C terminus region is organized as a gating ring composed of two ‘Regulators of conductance for K^+ ’ (RCK1 and RCK2) which bind Ca^{2+} and other divalent cations (reviewed in Latorre and Brauchi 2006; Cui et al. 2009; Latorre et al. 2010; Lee and Cui 2010). Despite having an extra transmembrane segment (S0), BK channels share considerable homology with Kv channels including a voltage sensor domain (S1–S4) and a pore region (S5–S6) (Atkinson et al. 1991; Adelman et al. 1992; Butler et al. 1993).

An intrinsic voltage sensor in BK channels requires the existence in the channel protein of charges or dipoles able to sense the changes in membrane voltage. The displacement of these charges, as in Kv channels, is the origin of gating currents. Fast gating currents preceding the ionic currents have been detected in the absence of Ca^{2+} , confirming the hypothesis that BK channel voltage sensitivity reflects the action of an intrinsic voltage sensor whose displacement inside the electric field promotes channel opening (Stefani et al. 1997; Horrigan et al. 1999) (Fig. 3). In contrast to other voltage-dependent cation channels, BK channels have a putative S4 segment containing only three positively charged residues (Fig. 2). This smaller number of charges is still large compared to the measured number of gating charge-per-channel, with estimates falling between 2.6 and 4 e_0 (Stefani et al. 1997; Horrigan et al. 1999). These measurements also stand in contrast to the fact that Kv2.1 has an identical set of positively charged residues in the proximal part of S4 as BK (Fig. 2), yet share the same number of gating charges as Shaker ($\sim 13 e_0$; Islas and Sigworth 1999). A systematic neutralization of the three charges contained in S4 revealed that only neutralization of residues R210 or R213 change the gating valence of the

channel when measured by the limiting slope method (Diaz et al. 1998). As we discuss below, and as was pointed out by Cui and Aldrich (2000), this approach should be used with caution for the BK channel.

The kinetic and equilibrium behavior of BK channels is best explained with allosteric models of the type set forth by the Aldrich group and Magleby group; for an excellent review, see Magleby (2003) and references therein. In contrast with Kv channels, where the voltage sensor is tightly coupled to channel opening (i.e. all gating charges are associated to the activation process; Fig. 7, Scheme I), BK channels can open when all the voltage sensors are at rest while charge can be displaced when moving between open states (Horrigan and Aldrich 2002; Fig. 7, Scheme II).

The experimental evidence supporting that type of model is large, but here we will present only the evidence related to the voltage-dependent aspects of BK channel activation. In contrast to Kv channels, the slope of the P_o versus voltage curves for BK decreases at very negative voltages, suggesting that the channel can open without voltage sensor

Scheme I

Scheme II

Fig. 7 BK Allosteric Model. Allosteric model of voltage-gating in BK channels. *Scheme I* General gating scheme in the absence of Ca^{2+} shows that pore opening $\text{C} \leftrightarrow \text{O}$ and voltage sensor activation $\text{R} \leftrightarrow \text{A}$ are connected through an allosteric constant D . L is the equilibrium constant between closed and open states when all voltage sensors are in the resting state (R) and J describes the equilibrium $\text{R} \leftrightarrow \text{A}$ for individual VSDs when the channels are closed. *Scheme II* is the expanded 10 states model accounting for the tetrameric structure of the channel

activation (*Co* to *Oo* in Fig. 7, Scheme II; Horrigan et al. 1999; Horrigan and Aldrich 2002). Ma and coworkers (Ma et al. 2006) found that the neutralization of only R213 of the BK S4 reduces the voltage sensor charges (z_j) from 0.58 e_0 to 0.28 e_0 . Recalling that the total gating charge, $z_T = 4z_j + z_L = 2.32 + 0.3 = 2.62$ e, the total reduction in gating charges induced by the mutant R213C amounts to 1.2 e. The rest of the gating charge appears to be contributed by three other residues distributed in different transmembrane segments: D153 and R167 in S2, and D186 in S3. Thus, as in Shaker, negatively charged residues also contribute to the voltage sensitivity of BK. However, the addition of a negative charge in the S2 of BK (Y263, equivalent to position 293 in Shaker) has no effect on BK voltage sensitivity. This (and other evidence we discuss below) indicates that the position and/or the displacement of gating charges are different between BK and Kv channels. It is important to note here that accessibility experiments indicate that R213 is altered by cysteine-modifying reagents from the internal side, whereas R210 and R207 residues are accessible only from the external solution at -80 mV (a voltage at which BK channels are closed; Hu et al. 2003). These results imply that, of these three charges, the only one able to move through the electric field is R213. Additionally, arginines 207 and 210 are exposed to the external solution independently of the BK channel activation state.

BK voltage sensor conformational changes revealed using voltage clamp fluorometry

Using voltage clamp fluorometry, movement of the voltage sensor of BK channels has been measured by the Olcese group (Savalli et al. 2006; Pantazis et al. 2010). The voltage-dependent fluorescent $[F(V)]$ changes detected by fluorophores bound to cysteines located in the short S3-S4 BK loop (L199C; N200C; R201C) precede the conductance-voltage curve, strongly suggesting that the fluorescent probes are following conformational changes in the voltage-sensing region of the channel. This hypothesis has been further confirmed using the mutant R201C, in which the measured $Q(V)$ curve almost superimposes the $F(V)$ curve.

The work of Ma and coworkers (Ma et al. 2006) showed that, in contrast to Kv channels, at least half the total gating charge is located in transmembrane segments other than the S4 segments. Moreover, the Olcese group (Pantazis et al. 2010) has shown that there is also cooperativity between the different transmembrane segments containing the voltage sensing residues. Using voltage clamp fluorometry and charge neutralization, they have shown that S2 and S4 have different voltage dependences, producing cross-dependent conformational changes: charge neutralization in the S2 segment reduces the voltage dependence of S4 and vice versa. These results are consistent with models that assume coupling in

which: (1) the activation of one segment displaces its neighbor; (2) the neutralization of S2 charges abridges the movement of S4 charges, and/or (3) dynamic field focusing is involved, where water crevices are created as a consequence of segment activation (for a review, see Bezanilla 2008).

The active configuration of the BK voltage sensor is stabilized by $\beta 1$ subunits and Mg^{2+}

In many different tissues, BK channels are accompanied by an auxiliary subunit $\beta 1$ (Knaus et al. 1994; McManus et al. 1995; Tanaka et al. 1997). The $\beta 1$ subunit produces a large increase in the BK channel's apparent sensitivity to Ca^{2+} as well as slowing down both activation and deactivation (reviewed in Orío et al. 2002). Surprisingly, $\beta 1$ modifies Ca^{2+} affinity only modestly (Bao and Cox 2005) and, moreover, Nimegean and Magleby (2000) have found that $\beta 1$ increases P_o even in the absence of the divalent cation. This Ca^{2+} -independent, $\beta 1$ -induced increase in P_o accounts for most of the apparent increased in Ca^{2+} sensitivity of the channel, visualized as a leftward shift of the $P_o(V)$ curves of the $\alpha + \beta 1$ channels as compared to channels formed by the α subunit alone. Gating current measurements indicate that $\beta 1$ coexpression also shifts the $Q(V)$ curve to the left without changing the size of the effective gating charge per voltage sensor (Bao and Cox 2005). These results strongly suggest that $\beta 1$ stabilizes the active configuration of the voltage sensor, and that such stabilization is more pronounced when the channel is open. These effects of the $\beta 1$ subunit on the voltage sensor increase the apparent Ca^{2+} sensitivity of the channel by diminishing the energy available to open the channel supplied by Ca^{2+} binding.

Yang and coworkers (Yang et al. 2008) found that Mg^{2+} is coordinated at the interface between the VSD and the RCK1 domain in activate BK channels. The side chain of the amino acids D99 (intracellular S0-S1 linker) and D172 (intracellular S2-S3 linker) in the VSD and E374 and E399 in the RCK1 domain of a different subunit together form a Mg^{2+} binding site, suggesting a close proximity between these two domains. This proximity enables the electrostatic interaction between the bound Mg^{2+} and R213 contained in the S4 segment, which in turn affects the displacement of the voltage sensor, explaining in a very economical manner the Mg^{2+} activation of BK channels.

A phosphatase activity controlled by membrane potential: the case of the *Ciona intestinalis* voltage-sensor-containing phosphatase

A genomic survey of the ascidian *C. intestinalis* led to the discovery of a gene whose product (dubbed *C. intestinalis*

voltage-sensor-containing phosphatase, Ci-VSP) has similarities to both an ion channel and an enzyme. Ci-VSP is a phosphatase having a great number of structural similarities with the tumor suppressor phosphatase (PTEN) (Steck et al. 1997; Lee et al. 1999; Murata et al. 2005). The protein consisted of a VSD similar to that of Kv channels with four transmembrane segments (S1–S4) followed by a cytoplasmic domain displaying phosphoinositide phosphatase activity. As in voltage-gated channels, S4 contains positively charged residues (four arginines), each separated by two hydrophobic amino acids, while S2 and S3 contain negative charges (Fig. 2). Once expressed in *Xenopus laevis* oocytes, this protein induces gating currents very similar to those generated by Kv channels and which are well described by a $Q(V)$ curve with $V_0=63$ mV and $z=1.0$ (Murata and Okamura 2007; Hossain et al. 2008; see Fig. 3). These gating currents are abolished if the two distal charges of the S4 segment are neutralized. Gating current can be recorded even when a Ci-VSP lacking the whole C-terminal is expressed in oocytes, showing that the VSD is a self-contained functional unit. As in PTEN, the phosphatase-like domain of Ci-VSP dephosphorylates phosphatidylinositol-3,4,5-trisphosphate [PtIns(3,4,5)P₃], producing PtIns(4,5)P₂. In contrast to PTEN, which is a soluble cytoplasmic protein, the phosphatase activity of Ci-VSP is voltage-dependent.

Murata and coworkers (Murata et al. 2005) inferred the voltage-dependence of the phosphatase activity by measuring the activity of inward rectifiers (IRK1) and the activity of voltage-dependent channels (KCNQ2/3) channels which rundown in the absence of PtIns(4,5)P₂. Murata and Okamura (2007) demonstrated unequivocally that Ci-VSP in contrast to PTEN can dephosphorylate both PtIns(4,5)P₂ and PtIns(3,4,5)P₃ and that the amount of PtIns(4,5)P₂ is reduced by depolarization. IRK1 and KCNQ2/3 channel activity is reduced in a graded manner as the membrane is depolarized, indicating that the phosphatase activity is regulated by the displacement of the voltage sensor of Ci-VSP.

The phosphatase in Ci-VSP removes the phosphate in position 5 of the PtIns(4,5)P₂. The enzymatic active site formed by the amino acid sequence CKGGKGR (Cys-X5-Arg) is different in only one residue as compared to that of PTEN, with the first glycine (G365) replaced by an alanine. Replacement of G365 by an alanine in the Cys-X5-Arg of Ci-CVP gives rise to a protein that is unable to dephosphorylate PtIns(4,5)P₂ or induce rundown of the inward rectifier, GIRK2. Thus, Ci-VSP is able to dephosphorylate both PtIns(4,5)P₂ and PtIns(3,4,5)P₂, where PtIns(4,5)P₂ dephosphorylation is the mechanism by which Ci-VSP modulates the activity of some K⁺ channels (Iwasaki et al. 2008). These results were confirmed by Halaszovich and coworkers (Halaszovich et al. 2009) in experiments that

combined patch clamp, fluorescent phosphoinositide-binding proteins and total reflection microscopy.

The remaining problem was to unveil the structural determinants of the allosteric coupling between the Ci-VSP voltage sensor and the phosphatase, and whether the conformational changes of the phosphatase during enzymatic activity can be transmitted to the voltage sensor's movements (bidirectional coupling). A hint of a possible bidirectional interaction between the VSD and the phosphatase was given by the functional characteristics of a truncated Ci-VSP missing the C-terminal. Gating kinetics of this VSD alone construct are much faster than those of the full-length protein (Murata et al. 2005). However, it was unclear whether this is a consequence of a new conformation adopted by the VSD in the truncated protein, perhaps affected by long-range electrostatic interactions due the dephosphorylation of phosphoinositides, or the result of the absence of phosphatase activity. To discriminate between these possibilities, Hossain and coworkers (Hossain et al. 2008) used a teleost VSP (Dr-VSP) that has an electrophysiological behavior qualitatively similar to that of Ci-VSP and made the phosphatase inactive by substituting the cysteine in the active center by serine (C302S). As in the truncated mutant, gating kinetics of C302S mutant is much faster than the wild-type Dr-VSP, albeit with no modifications of the $Q(V)$ curve, a result which supports the hypothesis that enzyme activity impacts gating kinetics.

Besides the high degree of homology between Ci-VSP and PTEN in their catalytic region (see above), the 16 amino acid region connecting the voltage sensor to the phosphatase domain (residues 240–255) of Ci-VSP shares a 50% identity with the first 16 amino acids of the N-terminal of PTEN. That interfacial region is known as PtIns(4,5)P₂ binding motif (PBM), since it is the structural determinant of the membrane anchoring of PTEN. Neutralization of arginines 11, 14 and 15 in PBM eliminates phosphatase activity and membrane binding concurrently (Campbell et al. 2003; Das et al. 2003; Iijima et al. 2004; Walker et al. 2004). Villalba-Galea and coworkers (Villalba-Galea et al. 2009) on the basis of this evidence mutated to alanine R253 and R254 of Ci-VSP (corresponding to R14 and R125 in PTEN) which disrupted phosphatase activity. It is important to note here that phosphatase activity is not rescued by introducing lysines in position 253 and 254, indicating that the interaction of the phospholipid binding motif (PBM) and the membrane is arginine-specific, presumably through the phosphate groups of the phospholipids. Interestingly, R253A and R254A mutations alter the kinetics and steady-state voltage dependence of the gating currents, another demonstration of the bidirectionality of the coupling between the voltage sensor and the phosphatase. Villalba-Galea and coworkers (Villalba-Galea et al. 2009) concluded that the activation of the phosphatase is induced by the

voltage sensor via the PBM and that this coupling requires at least arginines 254 and 255. These arginines interact with the phospholipids, allowing the phosphatase domain to acquire the most favorable orientation for catalysis (the active conformation) and, by locking the enzyme in the proximity to its substrate, this interaction limits the movement of the voltage sensor. However, it was found that PBM mutants reduce and PMB deletion eliminates the phosphatase activity of the isolated enzyme *in vitro* (Kohout et al. 2010). This direct action of the mutants on enzymatic function implies that phosphatase activity cannot be used to determine the role of the linker in coupling. The solution to this problem is provided by the use of voltage clamp fluorometry to measure the voltage-dependent movements of the voltage sensor. This approach shows unequivocally that mutations of basic residues in the PBM compromise coupling between the voltage sensor and the enzyme (Villalba-Galea et al. 2009; Kohout et al. 2010). Interestingly, PtIns(4,5)P2 appears to modulate a late step of voltage sensor motion that takes place at positive voltage. This step is also selectively affected by mutation of linker basic residues, suggesting that phosphoinositide binding stabilizes the linker in its activated configuration. Thus, as PtIns(4,5)P2 decreases, the linker loses membrane anchoring and reaches an uncoupled state (Kohout et al. 2010). Similar to Kv and BK channels, Ci-VSP is modular: the coupling between the voltage sensor and the phosphatase modules is allosteric. The results above have firmly established that the PBM is involved in that electrochemical coupling. The modularity of this protein has recently received further support from chimeras experiments, consisting of the voltage sensor of Ci-VSP and the phosphatase of PTEN behave as voltage-dependent phosphatases (Lacroix et al. 2011).

Concluding remarks

We have come a long way since 1952 when the existence of voltage-sensing structures was predicted by Hodgkin and Huxley. The combination of several techniques emerging in the last three decades, such as cloning, site-directed mutagenesis, voltage-clamp fluorometry, and structural analysis, has allowed us to build a fairly detailed picture of these voltage-sensing structures. In all cases, we can recognize an α helical (or 3_{10}) segment carrying charged residues, preferable arginines, that are repeated every third residue; the charges are thus oriented along a stripe on the α helix or toward one face of the 3_{10} helix. In all cases, membrane depolarization increases the number of S4 residues exposed to the external aqueous phase and decreases the number of S4 residues exposed to the internal aqueous phase; sometimes, residues (or neighboring residues) located in the middle can be made accessible to hydrophilic reagents from

both sides of the membrane by switching the voltage from positive to negative potentials. That S4 movement results in the net translocation of a maximum of 4 charges, giving rise to gating currents. The data suggest, however, that charge translocation involves a rather small movement across a narrowly focused electrical field along a thin hydrophobic septum within a protein core flanked by aqueous crevasses. However, not all VSDs are as charged as those found in voltage-dependent K^+ or Na^+ channels; for example, BK channels appear to have a decentralized VSD in which only half of the charge displaced during activation is localized in S4, and whose movement translocates at most one charge during voltage-activation.

From this review, we can also see that the coupling between the VSD and the effector domain share a common design whether the channel is a bona fide ion conducting pore-domain or is an enzyme. In most cases, the cytoplasmic end of S4, as in the S4–S5 linker of 6 TM channels or PBM in Ci-VSP, is a key structural determinant of electromechanical coupling.

What appears to be variable among voltage-sensing proteins is the energy involved in the coupling, spanning from the strict or tight coupling of Kv channels to the allosterical coupling of BK channels in which channels can open even in the absence of activated VSDs. Interactions between VSDs which contact each other also vary widely: from fully independent as in Kv channel to strongly cooperative as in Nav and Hv channels. What now appears on our horizon is a generation of detailed landscapes of voltage-dependent channels activation, as crystallization, spectroscopic techniques and computational methods capture images of these dynamic proteins.

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Conflict of interest None

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