Forward Genetic Analysis Reveals Multiple Gating Mechanisms of TRPV4*

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TRPV4 is a polymodal cation channel gain-of-function (GOF) allele which causes skeletal dysplasia in humans. To better understand its gating, we screened for additional GOF alleles based on their ability to block yeast proliferation. Repeatedly, only a limited number of such growth-blocking mutations were isolated. Expressed in oocytes, wild-type channels can be strongly activated by either hypotonicity or exposure to the potent agonist 4α-porphol 12,13-didecanoate (4αPDD), although the GOF channels behaved as if they were fully prestimulated as well as lacking a previously uncharacterized voltage-dependent inactivation. Five of six mutations occurred at or near the inner ends of the predicted core helices, giving further direct evidence that this region indeed forms the main intracellular gate in TRP channels. Surprisingly, both wild-type channels as well as these GOF channels maintain strong steady-state outward rectification that is not due to a Ca2+ block, as has been proposed elsewhere. We conclude that TRPV4 contains an additional voltage-dependent gating mechanism in series with the main intracellular gate.

Transient receptor potential (TRP)2 channels are a functionally and evolutionarily diverse group of cation-selective channels characterized by polymodal gating to chemical, thermal, mechanical as well as other stimuli (1). Predicted transmembrane topology and cryomicroscopic images (2) indicate that TRPs are structurally similar to other cationic channels such as the voltage-gated K+ channels (3). Each of the four subunits (4) is modeled to have six transmembrane helices (S1–S6), where S1–S4 forms the peripheral domain and the four S5–S6 domains converge to form the core, with the S6 lining the ion pathway. An ion filter is found in the pathway near the outer half (5). Structural homology to K+ channels (3) indicates (6, 7) and direct mutational analysis supports (8) that the cytoplasmic ends of the four S6 conditionally converge to occlude the pathway, creating an intracellular gate.

TRPV4, of the vanilloid class of TRP channels, originally drew attention and was isolated based on its ability to respond to hypotonic stress (9, 10). It was subsequently found to also be activated by heat (11), the arachidonic acid (AA) metabolite 5′,6′-epoxyeicosatrienoic acid (5′,6′-EET) (12) as well as other compounds (13, 14), but the most potent activator was found to be an exogenous agonist, 4α-phorbol 12,13-didecanoate (4αPDD) (15), 4αPDD and hypotonicity were found to activate TRPV4 through distinct pathways (16). In cultured kidney cells, hypotonicity is thought to activate TRPV4 through the production of 5′,6′-EET, possibly by activating phospholipase A2 (16). There must be other mechanisms by which TRPV4 is activated by hypotonicity, since it maintains robust hypotonic activation when expressed in yeast, which is incapable of synthesizing polyunsaturated fatty acids such as AA and its metabolites (17). TRPV4 is expressed in a broad range of tissue types that have varying physiological relevance (18). TRPV4−/− knockout mice are compromised in systemic-osmolality regulation (19). The functioning of TRPV4 has recently gained clinical attention, as GOF mutations were found to cause three kinds of skeletal dysplasias (20, 21) and mutations in its N-terminal ankyrin domains cause dominant hereditary motor and sensory neuropathies (22) in humans.

In an attempt to further understand the structure/function relationship of TRPV4 we used the budding yeast Saccharomyces cerevisiae to select for non-targeted mutations that alter gating. We have previously used such a strategy to successfully uncover gating mutants in bacterial mechanosensitive channels (23), the native yeast potassium channel Tok1 (24) as well as the native yeast vacuolar TRP channel, TRPV1 (Yvc1) (8, 25). More recently, Myers et al. (26) used a similar strategy to isolate several diverse GOF alleles of TRPV1. Focusing on one class of mutants found in that screen, it was determined that the pore helix of TRPV1 (as well as TRPV2) plays a critical role in the gating of that channel, possibly as a gating entity itself. In the present study, only a limited number of GOF mutations in TRPV4 caused severe growth-suppressing phenotype. Examination and consideration of these mutants suggest the existence of multiple gating mechanisms in TRPV4.

**EXPERIMENTAL PROCEDURES**

*Mutagenesis and Yeast Expression—*Error-prone PCR was used to amplify the open reading frame of rat TRPV4 (NM_023970) using standard low-fidelity Taq polymerase from a plasmid generously provided by Dr. W. Liedtke (10) and integrated into the inducible URA-selectable yeast expression plasmid p416GAL1 (27) using standard techniques. Several random clones were sequenced and found to have on average one to two nucleotide substitutions per open-reading frame; primarily transitions, but transversions were also encountered. Approximately 106 bacterial transformants were pooled, plasmids were isolated, and retransformed into the yeast strain
BY4742 derivative, BYYT (MATa, his3Δ1 leu2Δ0 lys2Δ0, ura3Δ0, tok1::KanMX4, yvc1::HIS3). Approximately 40,000 yeast transformants resulted, which were pooled and replated at ~200 CFU/plate all on a repressive dextrose-based CMD ura– medium (28). This entire procedure starting from the initial PCR was repeated in duplicate to generate two independent pools of screened plasmids. Colonies were replica-plated onto plates of expressive (galactose-, raffinose-based) CMGR ura– plated, and colonies were isolated that grew well on CMD but very poorly on CMGR. Strains that grew poorly due to genomic defects were weeded out by discarding those that failed to form polyps when uracil was added back to the CMGR plate. Plasmids were isolated from the remaining strains by standard techniques and both sequenced and re-transformed into fresh BYYT yeast to verify that the phenotype is plasmid-borne. TRPV4 open-reading frames were sequenced using Big Dye sequencing reagents (Applied Biosystems) and processed at the University of Wisconsin Biotechnology Center DNA sequence facility.

**Oocyte Expression**—Selected GOF alleles were PCR amplified using high-fidelity PfuUltra polymerase (Stratagene) and integrated into pGH19 for synthesis of cRNA (24). cRNA was synthesized from Xho1-linearized templates using a mMessage mMachine T7 kit (Ambion). Stage V and VI Xenopus oocytes were injected with 40 nl of diluted RNA solutions. Because expression of the GOF alleles was toxic to oocytes, 1 μM ruthenium red (Sigma) was added to the ND96 incubation buffer (29) in all cases. Conductances, particularly those of wild-type-expressing oocytes, increased over the course of several days, and currents were measured after 1–5 days depending on experimental requirements.

**Electrophysiological Techniques**—Two-electrode recording was performed using HS2A headstages and a VG-2A×100 virtual bath clamp connected to a Gene Clamp 500 amplifier interfaced through a Digidata 1440A digitizer acquired using pClamp10 software (all Axon Instruments). The base bath solution contained 66 mM KCl, 100 mM sorbitol, 1.8 mM BaCl2, and 5 mM K–HEPES pH 7.2 (all from Sigma). 3 μM 4αPDD (Sigma) or 10 μM 5′-EET (Biomol) were added directly to the bath chamber. Sorbitol was omitted from the hypotonic solutions. Data were analyzed using both pClamp10 and Sigma Plot 2000 (SPSS software).

**RESULTS**

**Isolation of Growth-blocking GOF Alleles of TRPV4**—We have successfully expressed rat TRPV4 in yeast (17). In an attempt to isolate rTRPV4 mutants that interfere with channel gating, we screened for GOF mutants that inhibit yeast growth under the assumption that hyperactive channels that could not gate properly and leak ions would be toxic. Plasmids containing mutagenized rTRPV4 open-reading frames under the control of the galactose-inducible GAL1 promoter (27) were transformed into yeast, and colonies were allowed to form under repressive conditions (dextrose plates). Colonies were isolated that failed to proliferate when replicated onto galactose plates due to the presence of the plasmid (see “Experimental Procedures”). Two independent mutageneses and screenings were conducted to assess mutagenic saturation. *Bona fide* toxic plasmids were rare, occurring at a frequency of less than 0.1% out of ~20,000 colonies screened. The TRPV4 blocker ruthenium red restored growth under inducing conditions (Fig. 1A), indicating that it is excessive channel conductance and not some other facet of expression that is toxic.

In the first screen, five growth-inhibiting alleles were identified. They included W733R (Fig. 1B, 6), L619P (2), L623P (3), M713I (4), and a double R151Q/D456G (1) substitutions. The R151Q/D456G mutant had a weaker growth inhibiting phenotype than the other GOF mutants (Fig. 1A), and in separate experiments it was found that R151Q alone did not inhibit growth (data not shown), making it likely that D456G is the effective mutation. In the second screen, three growth-blocking alleles were isolated, W733R, M713V (5), and M713I. The repeated isolation of W733R and M713I from the two independent screens indicates that the screen is at least near saturation. This conclusion is corroborated by the isolation of multiple aliphatic substitutions at Met-713 (Ile, Val) as well as the similar nature of L619P and L623P, indicating that not many alleles are capable of significantly blocking yeast proliferation.

It is notable that 5 of 6 of these mutations occur in or near the predicted S5/S6 channel core (Fig. 1B). M713I and Val occur right at the predicted cytoplasmic end of S6, and W733R occurs 20 amino acids downstream. L619P and L623P both are predicted to cause kinks in the cytoplasmic half of the S5 helix at points that are approximately one helical turn apart (Fig. 1B). The location of these mutations strongly suggests that they directly affect the channel main intracellular gate (see “Discussion”).
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Toxic Alleles Encode Pre-activated GOF Channels—TRPV4 alleles were expressed in Xenopus oocytes for electrophysiological analysis. We focused on selected GOF alleles in the channel core, which had the stronger growth-inhibiting phenotypes: the S5 kink mutant L619P, M713I at the end of S6, and W733R, 20 residues downstream. For a negative control, we also expressed a M680K, a mutation engineered into the channel presumptive filter that disrupts ion permeation (30, 31). Expression of the yeast-selected GOF alleles was toxic to oocytes as well, with the majority of the oocytes injected with ~10 ng of L619P or M713I cRNA dying within 24 h after injection. Wild-type cRNA was also toxic, but to a much lesser extent. It was indeed TRPV4 channel activity that was responsible for this toxicity, because the addition of 1 μM ruthenium red to the oocyte incubation medium alleviated this toxicity and was added to all subsequent oocyte incubations for the sake of consistency.

Oocytes injected with moderate amounts (~1 ng) of wild-type TRPV4 cRNA and incubated from 1 to 3 days produced currents that were largely dependent upon the application of the potent agonist 4αPDD (15) (Fig. 2A). 4αPDD activation was slow, requiring several minutes to become apparent after application of 3 μM and did not reach saturation even after 30 min. 4αPDD activation was irreversible, with removal from the bath only preventing further current increase. This stands in contrast to 4αPDD activation in TRPV4 expressed in cultured mammalian cells, where currents rapidly inactivate even when 4αPDD is maintained (32). Ruthenium red partially blocked the outward currents as expected (data not shown, but see Fig. 3). The non-conducting M680K channel (30) did not produce such currents, even when the cRNA was injected at a 5-fold excess (Fig. 2B). We were not able to activate oocyte-expressed wild-type TRPV4 with 5′,6′-epoxyeicosatrienoic acid (data not shown) in whole oocyte two-electrode recordings, which activates TRPV4 expressed in cultured cells (12).

In contrast, injection of ~10-fold lesser amounts (~100 pg) of M713I (Fig. 2C), L619P (2D), or W733R (data not shown) cRNA induced larger currents that were not further stimulated by 4αPDD application to a substantial degree. Both the GOF and wild-type channels displayed strong outward rectification (Fig. 2, bottom row). In other words, a gating mechanism that closes the channel in response to lack of agonist is defective but which closes in response to negative voltage remains intact in these mutants. This rectification was not due to a block of inward currents by residual ruthenium red carried over from the oocyte incubation buffer as evidenced by the fact that GOF channels expressed at low (subtoxic) levels incubated without...

FIGURE 2. GOF channels are in a pre-activated state but retain steady-state rectification. Oocytes injected with either 1 ng (wild type), 5 ng (M680K), or 0.1 ng (M713I, L619P) cRNA were subjected to 1-s voltage steps from a −60 mV hold to test potentials between −100 and +60 mV in 20 mV increments either not exposed (top row), or pre-exposed to 3 μM 4αPDD for 20 min (middle row). Peak responses from these traces are plotted against test potential in the presence (up triangle) or absence (down triangle) of 4αPDD. Current calibration bars for the traces are shown on top, and zero current levels are indicated at the left.

FIGURE 3. Hypotonicity does not further activate GOF channels. Hypotonic response from oocytes expressing high levels of wild-type (A, D), M713I (B), or L619P (C) cRNA was elicited by removal of 100 mM sorbitol from the 250 mOsm bath solutions. Responses were measured by repeated 250-ms pulses to +20 mV from −20 mV hold every 10 s. Insets in the upper right are the raw traces of the initial hypotonic response. The main figures plot the peak response occurring at the end of the +20-mV pulse during the initial hypotonic perfusion (hypo), restoration of 100 mM sorbitol (iso), and repeat of the hypotonic perfusion in the presence of 3 μM ruthenium red. In D, wild-type expressing oocytes were pre-incubated in 3 μM 4αPDD for 1 h. The calibration bar for all the main figures is at the top, and 0-current levels are indicated by the solid horizontal line in each figure (neither applies to the insets).
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GOF Alleles Also Fail to Respond to Hypotonic Stress Indicating a General Gating Defect—TRPV4 is also activated by hypotonic stress (9, 10), and the wild-type channel maintains this response in oocytes (33). Removal of inert osmoticum from the bath resulted in a large reversible increase in current (Fig. 3, left, center, and inset). This response could be elicited multiple times from the same oocyte (data not shown). That this activity is due to TRPV4 is verified by the fact that it did not occur in oocytes injected with M680K cRNA (data not shown) and did not occur when ruthenium red was included with the hypotonic stimulus (Fig. 3A, right).

As was the case with 4αPDD, GOF channels appear to be pre-activated with respect to hypotonic stimulus as well. Significant conductance through these channels occurs before hypotonic stimulus, does not increase during hypotonic stimulus, and is blockable by ruthenium red (Fig. 3, B and C). This provides further evidence that the defect in the GOF mutants is directly in the intracellular gate itself, as it has previously been shown that hypotonicity and 4αPDD activate channel gating through distinct pathways (16).

The currents from wild-type-expressing oocytes preincubated in 4αPDD for 1 h show an intermediate response to hypotonicity between that of untreated wild type and the GOF currents (Fig. 5D). It should be noted that the 4αPDD response did not saturate even after 1 h, and further incubations were increasingly toxic. That 4αPDD and mutation have the same general effect on hypotonic gating supports the conclusion that the GOF channel is in a state analogous to that induced by 4αPDD application.

GOF Channels Lack an Inactivation-like Behavior—When high levels of cRNA are injected and/or longer incubation times are employed, enough channel expression occurs such that wild-type currents can be analyzed in the absence of 4αPDD or ruthenium red also rectified (data not shown). Furthermore, these channels are quite capable of conducting inward currents as evidenced by transient tail currents observed upon repolarization (e.g. see Fig. 5, below).

As described in Fig. 5A, TRPV4 inactivation behavior is seen in wild-type channels from oocytes injected with high levels of cRNA, particularly when stepped from more negative holds (voltage protocol upper left inset). This inactivation is largely lacking in the GOF mutants and reduced when wild-type-expressing oocytes are preincubated in 4αPDD. B, degree of inactivation can be estimated by the reduced current levels observed upon stepping from increasingly positive holds (voltage protocol middle inset; note tests are held for 5 s as opposed to 1 s in A). C, plot of the peak responses elicited in protocols described in B as a function of the holding potential. Error bars are S.D., n is between 4 and 7. Calibration bars for each allele/condition are at the top, and zero current levels for all traces are indicated at the left.

FIGURE 4. Wild-type inactivation is lacking in the mutants. A, TRPV4 inactivation behavior is seen in wild-type channels from oocytes injected with high levels of cRNA, particularly when stepped from more negative holds (voltage protocol upper left inset). This inactivation is largely lacking in the GOF mutants and reduced when wild-type-expressing oocytes are preincubated in 4αPDD. B, degree of inactivation can be estimated by the reduced current levels observed upon stepping from increasingly positive holds (voltage protocol middle inset; note tests are held for 5 s as opposed to 1 s in A). C, plot of the peak responses elicited in protocols described in B as a function of the holding potential. Error bars are S.D., n is between 4 and 7. Calibration bars for each allele/condition are at the top, and zero current levels for all traces are indicated at the left.

The level of inactivation can be roughly quantified by measuring peak response upon depolarization to a common test from varying holds (Fig. 4B). Note that this will underestimate the true extent of inactivation in the wild-type channel because it starts to inactivate before the true peak conductance is reached. As can be seen, wild-type conductances are highly dependent on the holding potential, M713I and L619P channels have little dependence, and 4αPDD-treated wild-type channels have intermediate behavior (Fig. 4C, note a high level of variability in the 4αPDD-treated response, likely due to variability in the extent of 4αPDD saturation, see above). That the GOF channels lack inactivation suggests that it is also due to closure of the main intracellular gate as considered in the “Discussion” below.

Steady-state Rectification Remaining in the GOFs Is Not Due to Divalent Blockage—As mentioned above, GOF channels still clearly possess the ability to gate, permitting only significant outward yet little or no inward current at steady state. It has previously been reported that the rectification of TRPV4 is due to a voltage-dependent block of the predominant monovalent current by external Ca<sup>2+</sup> ions (5). Ca<sup>2+</sup> was replaced with Ba<sup>2+</sup> in all experiments described here to avoid activation of the native oocyte Ca<sup>2+</sup>-dependent Cl<sup>−</sup> current (34). Ba<sup>2+</sup> removal had little effect on steady-state outward rectification, slightly increasing outward, and, at most, nominally increasing inward currents in both wild-type and GOF channels (Fig. 5A). Ba<sup>2+</sup> does indeed partially block inward currents, as evidenced by the much larger tail currents upon repolarization in Ba<sup>2+</sup>-free conditions, e.g. compare inward tails in the presence of Ba<sup>2+</sup> (e.g. Fig. 4A) to those in its absence (Fig. 5B). Mg<sup>2+</sup> could substitute for Ba<sup>2+</sup> in reducing tail current magnitude upon repolarization (data not shown), indicating a general divalent ion blockage of monovalent conductance. It should be noted that in earlier works, Ca<sup>2+</sup>-dependent rectification was generally assessed by brief ramps from neutral holds (31, 32), which would not reflect steady-state behavior under such conditions. Divalent block is not responsible for the steady-state rectification, however.

To assess whether there was a significant change in the nature of the voltage-dependent closed state of wild type and those of the GOF mutants, activation, and deactivation kinetics were measured. Generally, two time constants were required to...
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A.

WT

-100

-60

-20

0

20

60

B.

-100mV

-40mV

60mV

6s

1s

-1000mV

-500mV

500mV

1000mV

60 ms

14 ms

21 ms

FIGURE 5. Mutant channels maintain steady-state rectification but have increased transient currents upon divalent ion removal. A, steady-state currents elicited from oocytes injected with high levels of cRNA and tested with protocols identical to those in Fig. 1 in the normal bath containing 1.8 mM Ba2+ (closed circles) or in its absence (open circles), B, tail current relaxations in Ba2+-free solutions seen upon 5 s returns to hyperpolarizing test potentials after 1-s depolarizations. Although Ba2+ removal did increase the magnitude of transient tail currents in both wild type and in GOF-expressing oocytes, it did not prevent their ultimate steady-state relaxations. The bottom plot and traces are from oocytes injected with wild-type cRNA preincubated in 3 μM 4αPDD for 1 h.

fit activations to peak from −80 hold to +60 mV test. The composite activation time constants were similar, being 20 ± 14 ms (weighted avg. ± std, n = 10 oocytes) for untreated wild-type, 21 ± 8 ms (n = 10) for wild-type channels pretreated with 4αPDD, 23 ± 14 ms (n = 10) for M713I and 23 ± 19 ms (n = 10) for L619P. Despite substantial oocyte-to-oocyte variance, wild-type channels activate at a similar rate to either PDD-stimulated of untreated mutant channels upon depolarization. Conglomerate deactivation rates from +60 to −80 mV were however distinct, being 230 ± 60 ms (n = 16) for wild-type, 730 ± 260 ms (n = 12) for 4αPDD-treated wild-type, 850 ± 210 ms (n = 8) for M713I, 802 ± 316 ms (n = 21) for L619P-expressing oocytes. The majority of the increase in the wild-type rate was due to the appearance of a fast decay (65 ± 17 ms) of about half the current (wild-type deactivations generally required an extra component to fit adequately). Thus wild type, both 4αPDD treated and untreated, and GOF channels activate at nearly the same rate upon depolarization but a significant fraction of the wild-type currents deactivate more rapidly upon repolarization.

DISCUSSION

In an unbiased screen for TRPV4 mutants that blocked yeast growth, we identified a limited number of alleles that encode hyperactive GOF channels. These GOF mutants are not further activated by hypotonic stimulation nor by 4αPDD application and lack voltage-dependent inactivation observed in wild-type channels. As discussed here, we conclude that hypotonic, 4αPDD, and possibly inactivation gating result from closure of the main intracellular gate. Curiously though, these GOF channels maintain robust steady-state outward rectification indicating the existence of an additional gating mechanism.

GOF Mutants Are Defective in Intracellular Gate Closure—Four of six isolated GOF alleles had mutations that would be predicted to directly affect the intracellular gate. Based on topological prediction and the known structure of K+ channels (3), M713I and Val are predicted to be at or near the bundle crossing of the closed intracellular gate. L619P and L623P would both be predicted to cause kinks in the lower half of the S5 helix that would have significant effects on this intracellular gate as well, because the S5 and S6 helices are presumably in intimate contact throughout (3). The location of these mutations alone is strong evidence that the specific defect is in the functioning of the intracellular gate itself. The GOFs no longer respond to either 4αPDD or hypotonicity, which have previously been shown to open the channel gate through distinct pathways (16). These observations further support the conclusion that the defect caused by these mutants is in intracellular gating itself as opposed to upstream events.

The genetic term gain-of-function refers to the measurable phenotype, in this case ensemble conductance. In a mechanistic sense, though, gating control is lost rather than gained. Although it is possible that the open state has been hyperstabilized in the mutants, it is far more likely that the closed conformation has been destabilized based on the simple fact that mutations usually destabilize protein structure (35). That 4αPDD could partially mimic these mutations in all respects support the conclusion that the intracellular gates are in essence in a chronically stimulated open state in the GOF mutants.

Robust Rectification in the GOF Mutants Indicates an Additional Gating Mechanism—Despite the conclusion that their intracellular gates are unable to stably close, the GOF channels maintain the ability to efficiently close in the face of negative membrane potential. At least three broad types of mechanisms could account for this. First is that negative voltage also closes the intracellular gate similar to lack of 4αPDD or hypotonicity, but that voltage has a much greater energetic contribution such that it can overcome any closed-state instability caused by GOF mutation. This seems unlikely. There is no detectable change in the opening rate between the mutant and wild-type channels upon depolarization and only a ~3-fold difference in the deactivation rate. If the closed conformation of the intracellular gate was so destabilized to the extent that the absence of potent activators such as 4αPDD could not encourage distribution into it, a substantial slowing of the deactivation and/or hastening of the activation rates upon voltage steps would be expected. Furthermore, TRPV4 has at best a remnant S4 voltage sensor (18), with only two charged residues at the less important cytoplasmic end of S4 (36). Such a sensor would not be predicted to have particularly strong energetic contributions toward gating.
A second possibility is that the voltage-dependent gating indeed results from a closure of the intracellular gate, but the contact points of the voltage-closed state are very different than those of the 4uPDD/hypotonicity closed states, and the GOFs isolated here only interfere with the latter. That depolarization activation rates are not, and repolarization deactivation rates are only mildly affected by GOF mutation similarly argue against this model, and it would furthermore be very difficult to envision how both the S5-kink mutants would have such surgical effects on individual closed state stabilities. Given the problematic nature of both these models that rely on voltage-dependent closure of the intracellular gate, we conclude that the robust steady-state outward rectification that remains intact in the GOF mutants is not due to the activity of the intracellular gate, but another distinct gating process.

Although this work does not provide direct evidence as to what the nature of the voltage-dependent gate might be, constraints exist. It has been proposed that rectification of TRPV4 is due to blockage by external Ca\(^{2+}\) ions. We indeed found that Ba\(^{2+}\) (and Mg\(^{2+}\)) partially blocks inward tail currents, but these currents eventually decay even in their absence demonstrating the existence of a steady-state gating mechanism not dependent on divalent block. It is notable that rectification in previous works was often assessed by fast voltage ramps from neutral holds (31, 32), and thus the lack of rectification in the absence of divalent ions may simply have reflected increased transient inward current, but not ultimate steady-state behavior.

Two other attractive candidates for the voltage dependent gating would be a filter-gating mechanism or an intrinsic or extrinsic anionic block of the inner pore. There is evidence that the filter region plays an important role in the gating of the closely related TRPV1 channel (26, 37), and filter region gating underlies C-type inactivation in other cation channels (38). If such a mechanism is at play here, then it is tempting to speculate that voltage is not directly controlling gating but instead the direction of the driving force (i.e. the filter collapses or by some other mechanism becomes non-conducting when the net ionic flow is inward). We have proposed such a mechanism for the outward rectification of the yeast K\(^{+}\) channel Tok1 (39).

Alternatively, voltage-dependent gating could result from blockage of the inner pore by residence of an anionic-blocking entity in response to a negative voltage field. This could be akin to an N-type ball-and-chain inactivation mechanism (although N-type inactivation is not directly voltage dependent) or reflect blockage by an extrinsic factor. An appealing aspect of this model is that it could neatly account for the lack of inactivation in the GOF channels, if it was presumed that inactivation was the result of a stochastic closure of the main intracellular gate (lacking in the GOF mutants) in response to inner pore evacuation of the blocking entity upon depolarization.

The present work focuses on mutations near the main intracellular gate (Fig. 1). The remainder of the mutations harvested from the yeast screen as well as recently discovered GOF mutations that cause heritable diseases (20, 21) in bone development will likely be informative in the further understanding of TRPV4 gating. Further mutageneses starting with the GOF mutants isolated here may in the future lead to unequivocal identification of the remaining voltage-dependent gating.

In the mean time, our findings that TRPV4 possesses multiple gating mechanisms, intrinsic strong steady-state rectification, and voltage-dependent inactivation further our understanding of the gating of this clinically significant ion channel.

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