Splitting the Two Pore Domains from TOK1 Results in Two Cationic Channels with Novel Functional Properties*

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Carlos Saldaña‡, David Naranjo‡, Roberto Coria‡, Antonio Peña‡, and Luis Vaca§

From the ‡Departamento de Genética Molecular, ¶Biofísica y §Biología Celular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Ciudad Universitaria, México D.F. 04510, México

Potassium channels are membrane-spanning proteins with several transmembrane segments and a single pore region where ion conduction takes place (1). Biggin et al. (2000) Curr. Opin. Struct. Biol. 4, 456–461; Doyle, D. A., Morais Cabral, J., Pfuetzner, R. A., Kuo, A., Gulbis, J. M., Cohen, S. L., Chait, B. T., and MacKinnon, R. (1998) Science 280, 69–77). TOK1, a potassium channel identified in the yeast Saccharomyces cerevisiae, was the first described member from a growing new family of potassium channels with two pore domains in tandem (2P) (Ketchum, K. A., Joiner, W. J., Sellers, A. J., Kaczmarek, L. K., and Goldstein, S. A. (1995) Nature 376, 690–695). In an attempt to understand the relative contribution of each one of the 2P from TOK1 to the functional properties of this channel, we split and expressed the pore domains separately or in combination. Expression of the two domains separately rescued a potassium transport-deficient yeast mutant, suggesting that each domain forms functional potassium-permeable channels in yeast. In Xenopus laevis oocytes expression of each pore domain resulted in the appearance of unique inwardly rectifying cationic channels with novel gating and pharmacological properties. Both pore domains were poorly selective to potassium; however, upon co-expression they partially restored TOK1 channel selectivity. The single channel conductance was different in both pore domains with 7 ± 1 (n = 12) and 15 ± 2 (n = 12) picosiemens for the first and second domain, respectively. In light of the known structure of the Streptomyces lividans KcsA potassium channel pore (see Doyle et al. above), these results suggest a novel non-four-fold-symmetric architecture for 2P potassium-selective channels.

Potassium channels play key roles in the physiology of prokaryotic and eukaryotic organisms. In many cells, potassium channels are responsible for maintaining the resting membrane potential and for the modulation of firing properties in excitable cells (4). The exceptionally diverse functional properties of potassium channels are matched by a large number of genes identified over the last few years (5).

Genome sequencing and molecular cloning has allowed the identification of a significant number of potassium channels possessing two and six transmembrane domains (TM)

Despite the structural diversity, all these channels have a common feature consisting of a single pore-forming domain, which is essential for ion conduction and selectivity (1, 2). It is generally accepted that all these potassium channels aggregate as tetramers to form a functional channel, leaving the pore at the axis of a four-fold symmetry (1, 6).

Recently a new family of potassium channels characterized by the presence of two pore-forming domains in tandem (2P) has been identified (3, 7–9). These new potassium channels have either four (2P/4TM) or eight (2P/8TM) transmembrane segments and are highly conserved throughout evolution (7, 11, 12). Recent experimental evidence suggests that this family of potassium channels probably dimerizes to form functional channels (8). In general, the sequences of both selectivity filters of 2P channels are different, thus they are expected to make potassium-selective pores without the regular four-fold symmetry, but the functional significance of having two different pore-forming domains remains largely unknown.

TOK1 was the first member identified from the 2P family of potassium channels (3). This is the only member from this family possessing eight TM (2P/8TM). The arrangement of the putative TM and the 2P in TOK1 results in a structure that resembles a six TM Shaker-like channel attached to an inward rectifier-like channel. This potassium channel may be important for the maintenance of membrane voltage in yeast, which is essential for nutrient uptake and turgor regulation (10).

Despite over 60 genes identified so far encoding 2P potassium channels, very little is known about the functional significance of having two pore-forming domains in tandem. In an attempt to determine the role of each one of the two pore-forming domains in this new family of potassium channels, we divided TOK1 at the intracellular linker between the sixth and seventh TM to produce two structures that resemble single pore domain channels, one with 6TM and the other with 2TM, each one with its respective pore-forming domain.

The results presented here indicate that each construct can form functional potassium channels in yeast and Xenopus laevis oocytes showing poor ionic selectivity and novel gating and pharmacological properties. Co-expression of both constructs partially restored wild type TOK1 channel properties. Given the proposed pore structure for 1P potassium channels based on the crystal structure of the KcsA potassium channel (2), these results suggest a novel non-four-fold-symmetric architecture to attain potassium-selective pores.

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†To whom correspondence should be addressed: Departamento de Biología Celular, Instituto de Fisiología Celular, UNAM, Ciudad Universitaria, México D.F. 04510, México. Tel.: 525-622-5654; Fax: 525-622-5611; E-mail: lvaca@ifisiol.unam.mx.

The abbreviations used are: TM, transmembrane domain(s); 2P, two pore domains in tandem; 1P, one pore domain; P1, first pore; P2, second pore; HOMOPIPES, homopiperazine-N,N′-bis-2-ethanesulfonic acid; MES, 4-morpholineethanesulfonic acid; CHO, Chinese hamster ovary; HER, human embryonic kidney; pF, picofarads.

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Proposed membrane topology of wild type TOK1, TOK1A, and TOK1B channels. A, hypothetical TOK1 structure with eight transmembrane helices and two pore domains in tandem. The first pore (P1) domain is located between the fifth and sixth transmembrane segments, and the second pore (P2) domain is located between the seventh and eighth transmembrane segments. S, segment. B, we introduced a stop codon after methionine 351 and the endonuclease restriction site (BglII) by mutagenic PCR to separate the two pore domains from TOK1 and divide the channel into two structures that resemble single pore potassium channels. The first structure contained six transmembrane segments (TOK1A), and the second contained two transmembrane segments (TOK1B). C, complementation of the potassium uptake-deficient S. cerevisiae yeast mutant (∆H9004 trk1, ∆H9004 trk2) by pYES2-TOK1, pYES2-TOK1A, and pYES2-TOK1B. The double mutant yeast cells were transformed with constructs indicated in the figure and grown in SD-URA medium supplemented with 100 mM KCl (data not shown). Transformed cells were replicated in the potassium-deficient medium (0.5 mM KCl containing 4% galactose to induce gene expression) LS-GAL and grown in this medium for 10 h. All constructs (TOK1, TOK1A, and TOK1B) rescued the double mutant, whereas the mutant transformed with the empty vector (pYES2) did not grow in potassium-deficient medium. The wild type S. cerevisiae yeast containing TRK1 and TRK2 (Wt) grew normally in potassium-deficient medium.
Materials and Methods

Reagents and Solutions—All salts used were analytical grade purchased from Sigma. HEPES and MES were purchased from Calbiochem, and HOMOPIPES was from Research Organics Inc.

Production of TOK1 and TOK2—The primers used for mutagenic PCR were 5′-AGGGCGACATCTACATTTATATGATC-3′ and 5′-AACGTTCATGTTCTGCTGGATGAGCT-3′ forward and reverse, respectively. In the forward primer, bases in bold show the nucleotides replaced, the stop codon is shown in italics, and the restriction site (BglI) is underlined. Universal primers were used to amplify the 5′- and 3′-ends of the respective constructs. The amplified fragments were cloned into pCR2.1 (Invitrogen), and constructions were sequenced in its entirety. TOK1 and TOK2 were cloned into the BamHI site of pYES2 (Invitrogen) and introduced into the Saccharomyces cerevisiae double mutant potassium uptake-deficient yeast CY162 (Δtrk1Δtrk2) using the lithium acetate method. For electrophysiology experiments all constructs were cloned into the BamHI and BglII sites of the pGEM vector for cRNA synthesis using the mMachine kit (Ambion).

Complementation Assays—The double mutant potassium uptake-deficient CY162 (Δtrk1Δtrk2) yeast cells were transformed with TOK1, TOK1A, and TOK1B constructs and grown in SD-Ura (yeast nitrogen base with 0.6% amino acids, 2% glucose, 20 mg/ml lysine, and 30 mg/ml histidine) medium supplemented with 100 mM KCl. Transformed cells were replicated in the potassium-deficient medium LS-GAL (0.5 mM KCl containing 4% galactose to induce gene expression) and grown in this medium for 2–3 days. The data shown were obtained at this time point.

Measurements of Potassium Transport in Yeast—The double mutant potassium uptake-deficient CY162 (Δtrk1Δtrk2) yeast cells were transformed with TOK1, TOK1A, and TOK1B constructs. Yeast cells were harvested by centrifugation and starved for 12 h before initiating potassium measurements. Starved cells were washed twice with distilled water, suspended at a density of 6 × 10^8 cells/ml, and kept on ice until use. Aliquots of 0.5 ml were applied to the recording chamber containing a potassium-selective electrode (Corning) adapted with a valinomycin membrane. The electrode was connected to a potentiometer (Selectron 2000, Beckman Instruments). The output signal from the potentiometer was acquired with a PC computer via an analog-digital interface and in-house-designed software (programmer Wilfrido Martinez). The data reported represent the concentration of potassium in the medium in mM after calibration with known concentrations of potassium dilutions.

Transient Expression of TOK1B in Mammalian Cells—The cDNA from TOK1 was introduced in the BamHI-NotI sites from the pcDNA 3.1 expression vector (Invitrogen). Chinese hamster ovary (CHO) and human embryonic kidney (HEK293) cells were purchased from the American Type Culture Collection (ATCC) as frozen passages. Cells were cultured using Dulbecco’s modified Eagle’s medium supplemented with antibiotics and 10% bovine fetal serum. CHO and HEK293 cells were transfected with 5 μg of the TOK1B construct mixed with LipofectAMINE plus (Invitrogen). Cells were studied 24–48 h post-transfection.

Electrophysiology—cRNA was prepared after linearization of pGEM containing TOK1, TOK1A, and TOK1B with NotI and transcribed with T7 as described previously (3). Transcript concentration was estimated spectrophotometrically. Oocytes were isolated as described elsewhere (11) and injected with 46 nl of distilled water containing 5–10 ng of cRNA of the construct to be studied. Whole oocyte currents were recorded 1–4 days after injection using a two-electrode voltage-clamp system (Oocyte Clamp, Warner Instruments). Electrodes filled with 3 mM KCl, 1 mM EGTA, 5 mM HEPES-KOH (pH 7.0) showed resistances of 0.3–1.0 megaohms. Recordings were performed under constant perfusion at room temperature. The low potassium bath solution contained 2 mM KCl, 98 mM NaCl, 1 mM MgCl₂, and 0.3 mM CaCl₂. In the high potassium bath solution the KCl concentration was 100 mM. The bath solutions were adjusted with 5 mM Hepes (pH 7), 5 mM MES (pH 6), or 5 mM HOMOPIPES (pH 5). Patch clamp experiments in the outside-out configuration were performed with an EPC-9 amplifier (HEKA Electronics). Ensemble averages were prepared with Igor Pro 4 (WaveMetrics). In these experiments the pipette contained the 100 mM KCl solution, and the bath contained the 2 mM KCl solution described above. The relative permeability calculations were performed according to the Goldman-Hodgkin-Katz equation as described elsewhere (4). In all cases, combined data represent the mean ± S.D. from n independent observations.

Results

Dividing the Two Pore Domains from TOK1 Produces Novel Cationic Channels—TOK1 possesses eight transmembrane segments with two pores in tandem. This structure resembles a six transmembrane domain Shaker-like channel attached to an inward rectifier-like channel (Fig. 1A), therefore, dividing the two pore-forming domains from this channel would result in two structures that resemble single pore potassium channels. The first structure consists of six TM segments with the first pore (P1) domain located between the fifth and sixth TM segments (TOK1A, Fig. 1B), and the second structure has two TM segments containing the second pore (P2) domain between the two segments (TOK1B, Fig. 1B).

Separate expression of each construct in the potassium transport-deficient S. cerevisiae double mutant (Δtrk1Δtrk2) (12) overcame the potassium auxotrophy as illustrated in Fig. 1C. This result indicates that both constructs may function as individual potassium channels in yeast. This yeast potassium transport-deficient mutant has been previously used to identify a novel 2P potassium channel from Drosophila melanogaster (ORK1) (13) and the voltage-dependent potassium channel from Arabidopsis thaliana (KAT1) (14) utilizing a complementation strategy similar to the one reported here. This strategy relies on the fact that this mutant cannot grow in potassium-deficient medium. Fig. 1C shows that the mutants transformed with wild type TOK1, TOK1A, or TOK1B are rescued only when exposed to a galactose medium to induce plasmid expression. Furthermore, transformed mutants grown in glucose, where plasmid induction does not occur, did not grow in potassium-deficient medium (data not shown).

Experiments performed with a potassium-selective electrode show a clear potassium uptake-deficient phenotype in Δtrk1Δtrk2 (Fig. 1D). This phenotype has been studied previously using similar measurements and reflects the role in potassium uptake played by the only two potassium transport systems functionally identified in this yeast (TRK1 and TRK2 (15)). The addition of glucose to the medium results in the activation of the H⁺-ATPase from yeast (PMA1) with the concomitant establishment of the cell potential via this proton transport system. Unlike animal cells, in yeast the membrane potential is largely determined by the activity of this electrogenic ATPase (16). Under these conditions, glucose addition establishes an electrochemical gradient initiating the uptake of potassium into wild type yeast cells via the high affinity potassium transport systems (TRK1 and TRK2).

As expected, the double mutant Δtrk1Δtrk2 yeast cells do not show potassium uptake due to the absence of both potassium transporters (Fig. 1D). This potassium uptake-deficient phenotype is reversed in the double mutant Δtrk1Δtrk2 yeast cells transformed with wild type TOK1 (Fig. 1D). The rescue of the potassium-deficient phenotype from the Δtrk1Δtrk2 double mutant by overexpression of TOK1 has been previously documented (17).

Interestingly, the separate expression of TOK1A or TOK1B also rescued the Δtrk1Δtrk2 phenotype (Fig. 1D). In fact, po-
tassium uptake from Δtrk1Δtrk2 yeast cells transformed with the three constructs (TOK1, TOK1A, or TOK1B) was indistinguishable by this technique (Fig. 1D). These results indicate that TOK1A and TOK1B contribute to potassium uptake in the double mutant either directly by producing potassium-permeable channels or indirectly via the activation of otherwise silent endogenous potassium transport systems from yeast. The later possibility is less likely since TRK1, TRK2, and TOK1 are the only known potassium pathways functionally identified and recognized by genome analysis in this yeast.

To further explore the possibility that TOK1A and TOK1B may produce functional channels, both constructs were expressed separately in X. laevis oocytes. Water-injected oocytes showed inward currents smaller than 0.3 μA at −160 mV (Fig. 2). The amplitude of these currents is in agreement with what other groups have observed in X. laevis obtained from different suppliers (18, 19).

Injection of mRNA from each construct (TOK1A or TOK1B) separately into oocytes resulted in the appearance of inwardly rectifying channels with novel functional properties (Fig. 2). Unlike TOK1, which is a depolarization-activated outwardly rectifying potassium channel, both constructs (TOK1A and TOK1B) produced hyperpolarization-activated channels showing a strong inward rectification (Fig. 2A). The rectification was so strong that no outward currents were detected even at potentials as positive as +100 mV (data not shown).

One cannot discard at this point, however, the possibility that expression of TOK1A and TOK1B may result in the activation of endogenous and otherwise silent oocyte channels. Weakly potassium-selective and chloride conductances activated by hyperpolarization have been previously described in oocytes injected with IsK (20, 21), Mat-8 (22), and the γ subunit from the Na−K−ATPase (23).

In an attempt to clarify this point, TOK1B was introduced in a mammalian expression vector (see “Material and Methods”) and transiently expressed in CHO and HEK293 cells. Expression of TOK1B in these two mammalian cell lines resulted in the appearance of inwardly rectifying, hyperpolarization-activated currents similar to those illustrated in Fig. 2 (data not shown).

Nontransfected CHO cells showed small inward currents of 5 ± 1.5 pA/pF at −160 mV measured at pH 6 (12 ± 2 pF/cell, n = 25). Approximately 50% of the CHO cells transfected with TOK1B displayed large inward currents (150 ± 12 pA/pF at −160 mV, n = 15). Similar results were obtained in HEK293 cells where inward currents under control conditions at −160 mV were 7 ± 3 pA/pF (17 ± 4 pF/cell, n = 22). After transfection with TOK1B ~60% of the cells showed large inward currents of 210 ± 52 pA/pF at −160 mV measured at pH 6 (n = 14).

Unfortunately these two cell lines did not tolerate extracellular acidification below pH 6, making any further studies of the effect of pH on gating and single channel conductance with these constructs extremely difficult. Nevertheless, these preliminary results provide additional information strongly suggesting that expression of TOK1A and TOK1B may produce functional channels rather than activate endogenous and otherwise silent channels in yeast, oocytes, rodent, and human cells.

Modulation of TOK1, TOK1A, and TOK1B by Extracellular Acidification—One common feature found in many 2P potassium channels is their modulation by protons. TOK1 (24) and the TWIK (25) subfamily of channels are inhibited by intracellular acidification, whereas increasing the extracellular pH above 7 activates the members of the TASK subfamily of channels (26).

We have found that TOK1 is also modulated by extracellular pH. Extracellular acidification increased the amount of inward current through wild type TOK1 with little effect on the outward current (Fig. 2A). Above pH 6 TOK1 showed a strong outward rectification, whereas at pH 5 a significant amount of inward current was detected (Fig. 2A). The inward current carried by wild type TOK1 at pH 5 resembled the current produced by the expression of TOK1A or TOK1B separately (Fig. 2A).

Extracellular acidification also increased the amount of inward current through TOK1A and TOK1B and reduced the time to reach the peak inward current at all voltages explored. Fitting the inward current obtained between −130 and −160 mV to a single exponential function produced the activation time constants (τ) shown in Table I. In general membrane hyperpolarization reduced the time to reach the peak inward current at the three pH values explored for TOK1A and TOK1B; however, the time constants obtained at pH 7 were 10–20 times slower compared with pH 5 (Table I).

Interestingly, despite the clear structural differences between TOK1A and TOK1B (TOK1A possesses six TM, and TOK1B has only two TM), both constructs produced channels with similar activation kinetics, pH effects, and inward rectification properties. If indeed these constructs produce functional channels, such results would suggest that the pore domain (the only conserved sequence in both structures) might determine these properties.

Co-injection of TOK1A and TOK1B Partially Restores TOK1 Channel Properties—Since the separate expression of TOK1A and TOK1B appear to produce functional channels, the next obvious question to explore is whether co-expression of both constructs may reconstitute functional TOK1 channels. To investigate this, oocytes were co-injected with equimolar concentrations of both constructs. Co-injection of mRNA from TOK1A and TOK1B (TOK1AB) partially restored wild type TOK1 channel properties as indicated by the appearance of a significant amount of outward current at voltages more positive than 0 mV in the coinjected oocytes (Fig. 3A). Partial co-assembly of TOK1A and TOK1B channels to form wild type TOK1 was further suggested by selectivity experiments where the current produced by TOK1AB showed a more negative reversal potential (−74 ± 3 mV, n = 10; Fig. 3C) than the individual injection of TOK1A (−22 ± 4.4 mV, n = 9; Fig. 3D) or TOK1B (−42 ± 2.6 mV, n = 8; Fig. 3E). Under these conditions the reversal potential observed for wild type TOK1 was −76.5 ± 3 mV (n = 10; Fig. 3E). Calculating the relative permeability ratios using the Goldman-Hodgkin-Katz equation gave a PK+/PNa+ of >200 for TOK1 ≥ TOK1AB, 5 for TOK1B, and 2.4 for TOK1. Due to the strong inward rectification observed with TOK1A and TOK1B, the reversal potentials were measured using voltage protocols to elicit tail currents (Fig. 3A). Thus, in addition to the fact that TOKA and TOKB are inward rectifiers, they are poorly potassium-selective.

Pharmacological experiments further strengthened the possibility of co-assembly between TOK1A and TOK1B to form wild type TOK1 channels. Wild type TOK1 is inhibited by triethanolamine with K50 = 5 mM (3). TOK1A and TOK1B were not blocked by triethanolamine concentrations up to 20 mM, whereas 10 mM triethanolamine inhibited 75% of the current produced by TOK1AB (data not shown). All these results strongly suggest that in the co-injection of TOK1A and TOK1B part of the protein may associate to reconstitute TOK1 channels.

Characterization of TOK1A and TOK1B Single Channels—Single outside-out patches obtained from TOK1A- and TOK1B-expressing oocytes demonstrated further novel properties from
both constructs. The single channel conductance for TOK1 is 35 picosiemens (3). Oocytes obtained from six different animals were injected with mRNA from TOK1A or TOK1B. Outside-out patches obtained from these cells showed multiple channels of equal amplitude for each construct. The single channel conductance calculated between -100 and -160 mV from patches with a single channel showed clear differences between TOK1A and TOK1B. The conductance obtained from
Novel Cationic Channels from Splitting Two Pores from TOK1

TABLE I

|                      | Time constants for the activation of TOK1A and TOK1B whole oocyte currents and single channels |
|----------------------|--------------------------------------------------------------------------------------------------|
|                      | The values shown are in milliseconds. All currents were fitted to a single exponential rise equation. Because of the current inactivation observed at the end of the voltage pulse at pH 5, the fit limit was set between the beginning of the voltage pulse and 250 ms. This applies for whole oocyte currents and the ensemble averages from single channels. Single channel ensemble averages were obtained from 20–30 sweeps like those shown in Fig. 4. |
|                      | −130 mV | −140 mV | −150 mV | −160 mV |
| TOK1A                |         |         |         |          |
| Whole oocyte         |         |         |         |          |
| pH 5                 | 100     | 63      | 35      | 18       |
| pH 6                 | 167     | 129     | 76      | 30       |
| pH 7                 | 1174    | 590     | 330     | 218      |
| Single channel       |         |         |         |          |
| pH 5                 | 99      | 70      | 42      | 21       |
| pH 6                 | 165     | 130     | 73      | 32       |
| pH 7                 | 1182    | 601     | 335     | 220      |
| TOK1B                |         |         |         |          |
| Whole oocyte         |         |         |         |          |
| pH 5                 | 60      | 39      | 20      | 6        |
| pH 6                 | 170     | 114     | 72      | 40       |
| pH 7                 | 373     | 318     | 229     | 169      |
| Single channel       |         |         |         |          |
| pH 5                 | 58      | 41      | 17      | 6        |
| pH 6                 | 169     | 120     | 71      | 44       |
| pH 7                 | 342     | 311     | 226     | 171      |

TOK1A was 7 ± 1 (n = 12) and was 15 ± 2 (n = 12) pico siemens for TOK1B (Fig. 4C).

The identification of single channels from TOK1A and TOK1B was based on the following observations. (a) These channels were observed only in outside-out patches from oocytes expressing the respective constructs and never in water-injected oocytes (n = 9 from three different animals) or in oocytes injected with the cRNA for the human bradykinin type II receptor (n = 12 from four different animals) used as control of injection, (b) both channels responded to changes in extracellular acidification similarly to whole oocyte currents, and (c) single channel ensemble averages from both constructs resembled whole oocyte currents (compare Fig. 2A with Fig. 4, B and E). Fitting the ensemble averages to exponential function produced time constants that were indistinguishable from those obtained with whole oocyte currents (Table I).

Although direct measurements of the reversal potentials for TOK1A and TOK1B were not possible due to the reduction in single channel amplitude and channel open probability near the reversal potential, fitting the data to the Goldman-Hodgkin-Katz equation produced estimated reversal potentials similar to those obtained with whole oocyte currents. The extrapolated reversal potential for TOK1A was −15 ± 5 mV (n = 12) and −50 ± 6 mV for TOK1B (n = 16; Fig. 4C). In both constructs, no measurable channel activity was detected in the outward direction, confirming the strong inward rectification observed with whole oocyte currents.

Modulation by extracellular acidification was evident also at the single channel level. Fitting the current produced by the ensemble average of many single channel recordings to a single exponential function gave τ values indistinguishable from those obtained at the three pH values explored with whole oocyte currents (Table I).

These experiments indicate that the increment in inward current amplitude though TOK1A and TOK1B observed with extracellular acidification is not the result of changes in the single channel conductance but rather due to an increment in open probability (P o). The effect of membrane hyperpolarization and extracellular acidification on TOK1A and TOK1B single channels is illustrated in Fig. 5. Single channel P o de-

cayed exponentially with membrane depolarization (Fig. 5B). No distinguishable single channel activity was observed at potentials more positive than −100 mV. The strong voltage dependence observed with TOK1A and TOK1B single channels may explain the inward rectification observed with whole oocyte currents.

Extracellular acidification increased channel P o at all voltages explored in TOK1A and TOK1B (Fig. 5, B and C), thus the modulation of single channel gating by acidification was consistent with the effects obtained with whole oocyte currents for both constructs (Fig. 2). The results presented here may explain why extracellular acidification unmasks inward currents through wild type TOK1 channels, suggesting that this may be the result of changes in channel gating and voltage dependence in the wild type channel.

The fact that water-injected oocytes and oocytes injected with the mRNA encoding the human type II bradykinin receptor (control of injection) did not produce hyperpolarization-activated currents or single channels similar to TOK1A or TOK1B taken together with the appearance of inwardly rectifying currents in CHO and HEK cells transfected with TOK1B suggest that these constructs may produce functional channels rather than activate endogenous channels. Furthermore, the reconstitution experiments using combined injections of TOK1A and TOK1B (TOK1AB) can only be satisfactorily explained if a fraction of both constructs interact to form TOK1 wild type channels.

DISCUSSION

We have divided a 2P potassium channel with eight TM to produce two constructs that resemble single pore potassium channels, one with two TM resembling a Kir-like inward rectifier potassium channel and the other with six TM resembling an outward rectifier of the Kv family of potassium channels.

Expression of the two domains separately overcame the potassium auxotrophy in the potassium transport-deficient yeast double mutant Δtrk1,Δtrk2. Furthermore, experiments measuring potassium uptake with a potassium-selective electrode showed a clear phenotype in the Δtrk1,Δtrk2 double mutant, consisting in a reduced potassium uptake compared with that in wild type yeast. This phenotype was rescued by transforming the double mutant with plasmids containing wild type TOK1, TOK1A, or TOK1B separately.

Electrophysiology experiments performed with X. laevis oocytes injected with TOK1A and TOK1B mRNA showed the appearance of inwardly rectifying, hyperpolarization-activated cationic currents not present in oocytes injected with mRNA from the human type II bradykinin receptor or in water-injected oocytes. Similar cationic currents were also observed in CHO and HEK293 cells transiently transfected with a plasmid containing the cDNA from TOK1B.

Although it is possible that TOK1A and TOK1B (which share limited amino acid homology and are structurally very different) may be activators of cationic channels in yeast, oocytes, rodent, and human cells, we believe this hypothesis is less likely than that in which both constructs (which have the general features found in 1P potassium channels) may indeed produce functional cationic channels.

Intersubunit interactions of the 2P from TOK1 may account for the differences in selectivity and single channel conductance observed between the wild type channel and both constructs (TOK1A and TOK1B). In this regard, it is worth mentioning that even when the amount of mRNA injected in the TOK1AB experiments was the same as that injected for TOK1A or TOK1B separately the amplitude of current produced by the co-injection was one-third of that obtained with the individual injection of the constructs.
This observation is particularly important for two reasons. 1) If TOK1A and TOK1B are activators of endogenous channels, one would expect equivalent current amplitudes in the co-injection experiments and not less current as we have observed. This observation further supports the hypothesis that TOK1A and TOK1B may produce functional cationic channels; and 2) these observations suggest that some tetrameric combinations might result in nonfunctional or nonconducting channels. Homotetramers of TOK1A or TOK1B and the heterotetramer TOK1AB result in functional channels; however, it is possible that heterotetramerization of three TOK1A plus one TOK1B or vice versa may result in nonfunctional channels. Ongoing experiments with TOK1A and TOK1B constructs cloned in tandem may help to elucidate this point.

If TOK1A and TOK1B are indeed producing cationic channels with similar properties, these results are particularly

**Fig. 3.** TOK1A, TOK1B, and TOK1AB produce channels with different potassium selectivity. A, representative whole oocyte currents from oocytes injected with 5–10 ng of cRNA of the wild type TOK1, TOK1A, TOK1B, and TOK1AB obtained with 2 and 100 mM KCl extracellular solutions. TOK1 and TOK1AB currents were elicited with the voltage protocol illustrated at the top left margin of the figure (the same protocol as used in Fig. 2). Because of the strong inward rectification, TOK1A and TOK1B current measurements were obtained from the tail currents elicited with the voltage protocol shown at the right. The arrows indicate the point at which the current measurements were obtained to construct the current-voltage curves. Current-voltage relationships for the wild type TOK1 (B), TOK1AB (C), TOK1A (D), and TOK1B (E) are shown. The insets show a magnification to demonstrate the reversal potential shift obtained with 2 mM KCl solution adjusted to pH 6.
suggestion that mutations that alter the symmetry of the pore tandem constructs (dimers) of the 1P potassium channel Drk1 suggests that the individual expression of TOK1A and TOK1B results in channels with poor selectivity for extracellular potassium ions whereas TOK1B and wild type TOK1 produced potassium-selective channels, one might speculate that the spatial rearrangement of the two pores from both constructs to form wild type TOK1 may provide a non-four-fold-symmetric potassium-selective architecture. This architecture contrasts with the proposed four-fold-symmetric architecture for 1P potassium-selective channels based on the crystal structure of the Streptomyces lividans KcsA potassium channel (2).

In this regard, recent experimental evidence obtained with tandem constructs (dimers) of the 1P potassium channel Drk1 suggests that mutations that alter the symmetry of the pore domain result in changes in selectivity, gating, and single channel conductance in the mutant dimers (27).

TOK1 is an outwardly rectifying potassium channel. Single channel openings are rarely observed in the hyperpolarized direction (3). These properties are the opposite of what we have observed with the expression of TOK1A and TOK1B. The strong inward rectification observed in TOK1A and TOK1B may result from the voltage dependence properties of both channels since single channels open scarcely at voltages approaching the reversal potential and no discernible opening events were observed in the outward direction.

In the family of inward rectifier potassium (Kir) channels, the unifying hypothesis to explain inward rectification proposes that the blockade by magnesium and/or polyamines produced by the depolarization is sufficient to account for the inward rectification in many members from this family (28). One cannot discard at the present time a similar mechanism involved in the strong rectification observed with TOK1A and TOK1B.

Alternatively, the voltage dependence and gating differences observed between wild type TOK1 and the constructs (TOK1A and TOK1B) might be the result of a different spatial arrangement of the pore architecture. We do not have a definitive explanation at the present time to account for these differences.

The structure of TOK1 resembles a six transmembrane domain Shaker-like channel attached to an inward rectifier-like channel, therefore the finding that separating the two pore-forming domains from TOK1 results in the appearance of potassium-permeable channels with novel selectivity, single channel conductance, and gating properties is very provocative.

If indeed TOK1A and TOK1B produce functional channels (as suggested by the evidence in yeast, oocytes, and mammalian cells presented here) then these new channels may provide an excellent experimental tool to explore structural determi-
nants of ionic selectivity, gating, and voltage dependence in 2P potassium channels and may help to understand the functional role of the sequence variations in the 2P found in this growing family of channels. Future experiments splitting the two pores from other 2P potassium channels may provide additional information about the non-four-fold-symmetric architecture of 2P potassium-selective channels.

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