Determination of Key Structural Requirements of a K⁺ Channel Pore*

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Among the highly conserved sites in K⁺ channel pores, the tyrosine-glycine sequence is believed to play an important role in selectivity. Here we describe a novel approach in which comprehensive mutagenesis of the YG sites of the voltage-gated K⁺ channel, Kat1, is combined with phenotypic screening in Saccharomyces cerevisiae and electrophysiological analysis in Xenopus oocytes to determine the roles of these sites in K⁺ selectivity. We show that structural constraints necessitate a tyrosine or phenylalanine at the first position to confer full K⁺ selectivity. Substitution to arginine creates a channel titratable by external pH, suggesting that the side group at this position may line the channel pore. Permeation is abolished by any increase in bulk at the adjacent glycine position unless accompanied by a compensatory mutation at the tyrosine site. These results suggest a model in which the selectivity filter of the K⁺ channel requires an aromatic residue paired with glycine within the pore loop in order to maintain maximal K⁺ selectivity.

Potassium uptake in Saccharomyces cerevisiae is mediated by two putative potassium transporters encoded by the TRK1 and TRK2 genes (1, 2). Cells in which both genes are deleted exhibit a 1000-fold increase in the requirement of potassium in the medium to sustain normal growth compared with wild-type cells (2). The phenotype of trk mutants facilitated cloning of Arabidopsis K⁺ channel cDNAs, KAT1 and AKT1, by their ability to mediate K⁺ uptake and thus restore yeast cell growth on potassium-limiting media (3, 4).

Kat1 and Akt1 are founding members of a class of voltage-gated inward rectifying K⁺ channels that harbor the six-transmembrane architecture of the Shaker superfamily of channels (5). Particularly noteworthy are the amphipathic helix (S4), thought to constitute the voltage sensor (6), and, between the S5 and S6, the conserved pore region containing the tyrosine-glycine signature sequence found in most K⁺-selective channels (Fig. 1).

Although structurally closer to Shaker than to Irk1 or RomK1, Kat1 was shown to be an inward rectifying K⁺ channel by its ability to elicit slowly activating, hyperpolarization-dependent, K⁺-selective currents when expressed in Xenopus oocytes (14). Similar results were obtained from recordings of KAT1 expressed in S. cerevisiae (15). The electrophysiological properties of Kat1 are characteristic of the inward rectifying K⁺ channels described in the guard cells of higher plants (16, 17), and KAT1 promoter-driven expression of a glucoronidase reporter gene in transgenic Arabidopsis was shown to occur only within guard cells of young plants (18). Thus, KAT1 likely encodes a guard cell-specific inward rectifying K⁺ channel associated with stomatal opening and closing.

The ion selectivity of Kat1 is typical of a K⁺-selective channel with monovalent cation permeability following the order K⁺ > NH₄⁺ > Rb⁺ > Na⁺ > Li⁺ > Cs⁺ (14, 19). Like most K⁺ channels, Kat1 contains within its pore loop the highly conserved glycine-tyrosine-glycine (Gly262-Tyr263-Gly264) triplet, which has been shown to be intimately involved with ion selectivity (20–22). Recent investigations of K⁺ channel structure using cysteine substitution and modification, toxin binding, and mutant cycle analysis (23–27) support a model in which an hour glass-shaped pore is formed from wide external and internal vestibules, which converge rapidly to a short, narrow pore. Pascual et al. (24) have proposed that the Gly375-Tyr376-Gly377 triplet of the Kv2.1 channel forms the narrowest region, because cysteine mutants at the adjacent residues Val374 and Asp378 are accessible to probes from the inside and outside of the membrane, respectively. Ranganathan et al. (26) further specified that the highly conserved Tyr445 of the Shaker channel (analogous to Tyr263 of Kat1) is centrally located within the pore and likely interacts directly with potassium ions.

We previously described how the expression of an inward rectifying K⁺ channel in S. cerevisiae can be used as a model system to identify and analyze mutations that alter the ability of the channel to discriminate between physiological ions (28). This system offers the ability to screen large numbers of mutant channels for changes in ion selectivity observed as an increase in sensitivity of the yeast cells to potentially toxic ions such as sodium. Uozumi et al. (29) have recently used this strategy to identify several mutations within the pore region of Kat1 that alter ion selectivity of the channel. Here, we show that each of the 400 possible amino acid sequences at the highly conserved tyrosine-glycine sequence of the K⁺ channel pore can be generated and tested in a combinatorial fashion to assess the role of these sites in ion selectivity.

The results of this comprehensive mutagenesis screen revealed extremely stringent structural requirements for the YG region of the K⁺ channel pore. Single mutations at Gly264 never produced functional channels in S. cerevisiae. Furthermore, although many amino acid substitutions at Tyr263 retain channel function, only a single mutation, Tyr → Phe, retained wild-type levels of selectivity for K⁺ over other physiological ions.

EXPERIMENTAL PROCEDURES

Construction of the YG Library—The KAT1 coding sequence was cloned into the multicopy yeast expression vector pYES2 (Invitrogen)
under the control of the galactose-inducible promoter GAL1. The resulting plasmid was modified to include a poly(A) tail at the 3′-end of the gene. To facilitate subcloning of mutant pore region fragments, unique HindIII and BglII sites that did not alter the amino acid coding sequence of Kat1 were introduced at nucleotide positions 726 and 1239, respectively. A degenerate primer was designed such that the codons corresponding to amino acids Tyr263 and Gly264 were changed to NN(G/T). This combination of 32 codons is sufficient to encode all 20 amino acids. Polymerase chain reaction was used to create the degenerate fragments, which were cloned into the HindIII-BglII sites. 14,500 individual Escherichia coli transformants were obtained in the YG library. This was sufficient to ensure a 14-fold redundancy for representing each of the possible 1024 combinations of the YG codons in the library.

Plasmid DNAs from 46 bacterial transformants were isolated at random and sequenced to verify the quality of the library. Mutations to all amino acid codons were found at both positions 263 and 264. Each of the codons corresponded to the amino acids Tyr263 and Gly264, consistent with the degenerate primer used to generate the library. The frequencies of the expected and observed ratios of each amino acid codon are shown in Table I.

Additional site-directed mutations were generated using a recombinant polymerase chain reaction approach (30). Mutant polymerase chain reaction fragments were cloned into the unique HindIII-BglII sites.

Media and Strains—The transport characteristics of the wild-type (strain R757) and K′-uptake-defective (strain CY162, trk1Δ trk2Δ) strains of S. cerevisiae used in this study have been previously described (1, 2). Yeast transformations were performed by electroporation (31), and the Ura′ transformants were plated on yeast nitrogen-based medium supplemented with the required amino acids, 100 mM KCl, and 1 M sorbitol. After several days of growth at 30 °C, colonies that developed were replica-plated to various test media. These media all contained 2% galactose and 2% sucrose for induction of the GAL1 promoter. Yeast nitrogen-based medium supplemented with the required amino acids but lacking uracil, pH 5.9, contained 7 mM KC1. Low salt medium was made as described previously to 0.2 mM KC1 (1, 32). Some media were supplemented with NaCl to 400 or 700 mM. Low salt media were made essentially free of ammonium. Plasmids were isolated from yeast (33) and transformed into E. coli. Plasmid DNA was prepared and sequenced by dyeodeoxy methods (34) (U.S. Biochemical Corp.). Plasmids were retransformed into S. cerevisiae to confirm phenotypes.

Media used to analyze the pH sensitivity of the RG mutant (see Fig. 5) were low salt, containing 1 mM potassium. These media were buffered with 20 mM Tris base and 20 mM MES, which maintained the pH of the media over several days of growth with tested with pH paper.

Electrophysiology—RNA from Kat1 and mutant KAT1 cDNAs was synthesized using mMessage mMachine capped RNA transcription kit (Ambion Inc.). Oocytes were isolated from Xenopus laevis (Nasco), defolliculated, injected with RNA, and incubated at 18 °C in modified Barth's solution containing 96 mM KC1 (35). Between 2.5 and 25 ng of RNA was injected, depending on the expression or activity level of the channel. Recordings were made 1–4 days after RNA injection using an oocyte clamp OC-725B amplifier (Warner Instruments), filtered at 1 kHz (Frequency Devices 902). Data were recorded and analyzed using Axon Instruments software AxoData and AxoGraph on a Macintosh PowerPC 7100/80. Leakage currents were subtracted using a P/6 subtraction method (36).

Bath solutions contained 100 mM KC1 ([X] = K′), Rb+, NH4+, or Na+, 1.8 mM CaCl2, 1.0 mM KHCO3, 1.0 mM MgCl2, 10 mM HEPES (pH 7.4 with Tris base; solutions at low pH were prepared by the addition of HCl). At least 10 ml of bath solution was perfused during solution changes.

Conductance Ratio and Permeability Ratio Calculations—The amplitude of current was determined at the end of a 1-s pulse to ~150 mV in the bath solutions containing potassium, rubidium, ammonium, and sodium. Conductance ratios were determined by dividing the current measurement of the test ions by the potassium current. All values are means ± S.E. Reversal potentials were measured by first activating channels with a 1-s hyperpolarizing pulse to ~150 mV and then stepping to different test potentials. Conductance measurements for the low pH experiments were taken at the end of a 3-s pulse to ~130 mV. The permeability ratio P/K was determined using the equation P/K = [X]/[K]/[X]/[K] based on the Nernst-Plank electrodeffusion equation (37).

RESULTS

A yeast genetic screen was utilized to study the effects of amino acid substitutions at the highly conserved tyrosine-glycine amino acid pair in the pore of the Arabidopsis potassium channel Kat1. A potassium uptake-deficient strain of S. cerevisiae (CY162, trk1Δ trk2Δ) can grow normally on media containing 100 mM K′ but exhibits slow growth on media with 7 mM K′ and virtually no growth on media containing 0.2 mM K′ (Fig. 2A) (2). Expression of the wild-type KAT1 allele from the galactose-inducible promoter GAL1 confers strong growth of trk1Δ trk2Δ cells on media containing as little as 50 μM K′ (3). In contrast, on glucose-containing medium, the GAL1 promoter is repressed and the KAT1 cDNA cannot suppress the trk1Δ trk2Δ phenotype.

A yeast expression library was constructed to contain all possible amino acid combinations at the Tyr263 Glu264 positions of Kat1. Mutant Kat1 channels from this YG library were first screened for those that retained function. Approximately 10,000 individual transformants of strain CY162 were allowed to develop into colonies on glucose medium containing a permissive concentration of potassium (100 mM KC1). This number of colonies was sufficient to ensure approximately a 10-fold coverage of the library combinations based on 32 nucleotide combinations at each codon. These colonies were replica-plated to galactose media containing either 7 mM or 0.2 mM K′.

The abbreviation used is: MES, 4-morpholineethanesulfonic acid.

| Amino acid | Expected frequency | Observed frequency (position 263) | Observed frequency (position 264) |
|------------|-------------------|-----------------------------------|-----------------------------------|
| Ala        | 0.062             | 0.037                             | 0.069                             |
| Cys        | 0.031             | 0.047                             | 0.033                             |
| Asp        | 0.031             | 0.028                             | 0.021                             |
| Glu        | 0.031             | 0.052                             | 0.048                             |
| Phe        | 0.031             | 0.020                             | 0.013                             |
| Gly        | 0.062             | 0.117                             | 0.228                             |
| His        | 0.031             | 0.017                             | 0.010                             |
| Ile        | 0.031             | 0.013                             | 0.007                             |
| Lys        | 0.031             | 0.040                             | 0.012                             |
| Leu        | 0.094             | 0.067                             | 0.072                             |
| Met        | 0.031             | 0.025                             | 0.015                             |
| Asn        | 0.031             | 0.021                             | 0.005                             |
| Pro        | 0.062             | 0.023                             | 0.033                             |
| Gln        | 0.031             | 0.032                             | 0.032                             |
| Arg        | 0.084             | 0.130                             | 0.146                             |
| Ser        | 0.094             | 0.074                             | 0.050                             |
| Thr        | 0.062             | 0.028                             | 0.017                             |
| Val        | 0.062             | 0.049                             | 0.069                             |
| Trp        | 0.031             | 0.088                             | 0.076                             |
| Tyr        | 0.031             | 0.032                             | 0.010                             |
Of the 10,000 potential mutants, only 230 exhibited growth on 7 mM KCl, 64 of which could also grow on media containing 0.2 mM KCl. This observation suggested that the majority of substitutions at the YG positions produced channels incapable of suppressing the potassium uptake-defective phenotype of trk1 Δ trk2 Δ cells. Plasmids from colonies capable of growing on 7 mM KCl *were* isolated, prepared for DNA sequencing, and retransformed into yeast to confirm the mutant phenotypes. Mutant Kat1 channels deemed functional by this test conferred a broad range of growth phenotypes ranging from weak growth on 7 mM KCl to strong growth on 0.2 mM KCl. Fig. 2A shows representative phenotypes of mutant Kat1 channels. A complete listing of mutant phenotypes is shown in Table II. If other mutants retained function, the rate of K⁺ uptake was too low to even partially suppress the trk1 Δ trk2 Δ phenotype. The 7 mM cut-off point was chosen because higher K⁺ concentrations result in semipermissive growth of the trk1 Δ trk2 Δ recipient cells and would thus prevent identification of weak mutants.

DNA sequence analysis of plasmid DNA from transformants that grew on medium containing 0.2 mM KCl identified only three amino acid combinations capable of allowing strong growth at this concentration of potassium: wild type (YG), FG, and PG (Fig. 2A). The conservative tyrosine to phenylalanine substitution conferred growth as strong as the wild-type channel on media containing 7 mM or 0.2 mM K⁺. The PG mutant, too, conferred strong growth on 0.2 mM K⁺, although growth was not quite as robust as that conferred by the wild-type or PG channel. Two other substitutions, IG and TG, sustained weaker growth on the media containing 0.2 mM K⁺.

The remaining mutant channels exhibited partial suppression of the trk1 Δ trk2 Δ phenotype by conferring growth on medium containing 7 mM KCl, but not on 0.2 mM KCl. DNA sequence analysis of these mutants revealed that most amino acid substitutions at Tyr²⁶³ did not abolish channel function. Although very high concentrations of extracellular sodium are toxic, *S. cerevisiae* cells tolerate growth on media containing as much as 1 M NaCl (38). Growth of trk1 Δ trk2 Δ cells expressing a wild-type KAT1 allele was essentially unaffected on medium containing 7 mM KCl/400 mM NaCl (Fig. 2A). From these observations we conclude that for cells expressing the wild-type K⁺-selective channel (i) K⁺ permeated the channel at levels sufficient to allow strong growth of trk1 Δ trk2 Δ cells, (ii) Na⁺ could not permeate the channel at levels sufficient to cause toxicity to the cell and, (iii) passage of K⁺ was not substantially blocked by sodium.

The collection of mutants showed a wide range in the ability of Na⁺ to inhibit rescue of the trk1 Δ trk2 Δ phenotype. At one extreme, the FG channel in the presence of high sodium appeared indistinguishable from the wild type, suggesting that it retained wild-type properties of selectivity (Fig. 2A). Rescue by the IG and TG mutants was also quite resistant to the presence of high sodium (Fig. 2A). These mutants are considered to be selective, although not as selective as the wild-type or FG channel. This class distinction became more obvious when higher amounts of sodium were added to the media, and growth was more inhibited compared to the wild-type channel (data not shown).

Rescue of the trk1 Δ trk2 Δ phenotype by the remaining mutants was severely inhibited by the presence of sodium, suggesting a decrease in channel selectivity. For example, mutants such as CG and DS, which conferred strong growth on 7 mM KCl, exhibited no growth when the same medium was supplemented with 400 mM NaCl (Fig. 2A). Even the PG mutant, which conferred growth nearly as strong as the wild-type channel on 0.2 mM KCl, was unable to rescue the K⁺ uptake defect of trk1 Δ trk2 Δ cells on the sodium media. Thus, the ability of a mutant channel to suppress the K⁺ requirement of trk1 Δ trk2 Δ cells can be independent of channel selectivity. Whether the Na⁺ sensitivity of these channels was due to actual permeation by Na⁺ or merely blockage or inactivation of the pore by Na⁺ could not be determined by this test but was revealed by an independent assay (see below).

**Phenotypic Test for Na⁺ Permeation in *S. cerevisiae***

The inability of most mutant Kat1 channels to confer growth to trk1 Δ trk2 Δ cells on media containing high levels of sodium...
could be attributed to several possibilities. For example, inhibition may be caused by blockage of potassium uptake, altered channel gating, toxicity due to the uptake of sodium ions, or a combination of factors that could result in inactivation of the channel. The ability of Na\(^+\) to permeate wild-type and mutant channel pores was assessed by expressing the channels in *S. cerevisiae* cells that also expressed the *TRK1* and *TRK2* genes. These cells are not dependent upon *KAT1* for growth on K\(^+\)-limiting media due to the activity of the endogenous K\(^+\) transporters. Thus, cells expressing either wild-type *KAT1* or the empty vector resulted in equally strong growth on low potassium media (Fig. 2B). Furthermore, cation uptake by *TRK1*-expressing cells is highly selective for K\(^+\) over Na\(^+\); *TRK1* cells expressing either wild-type *KAT1* or vector alone grew equally strongly on 2 mM KCl medium or 2 mM KCl supplemented with 700 mM NaCl (Fig. 2B). However, mutant Kat1 channels with increased sodium permeation conferred sodium sensitivity to these cells.

Most of the mutant channels identified as nonselective based on the ability of Na\(^+\) to inhibit their rescue of the *trk1Δ* *trk2Δ* phenotype conferred various degrees of Na\(^+\) sensitivity to wild-type cells (Table II). The most striking example was the PG mutant, which, when expressed in *TRK1* *TRK2* cells on medium supplemented with 700 mM Na\(^+\), severely inhibited growth. Other examples of nonselective mutants inferred to permeate sodium by this test included the DS and CG channels (Fig. 2B). Thus, a major component of the sodium sensitivity conferred to *S. cerevisiae* by the mutant Kat1 channels was likely through increased sodium permeability.

The FG mutant, which appeared to be as selective as the wild-type channel when expressed in *trk1Δ* *trk2Δ* cells, did not confer sodium sensitivity to the *TRK1* *TRK2* cells, suggesting again that very little sodium permeates this channel. Of note, the TG and IG substitutions, identified as modestly selective because of their Na\(^+\)-resistant rescue of *trk1Δ* *trk2Δ* cells, conferred sodium sensitivity to the wild-type cells. Thus, mutations of Tyr263 to Thr or Ile seem to confer increased permeation to Na\(^+\) and therefore decreased selectivity of the pore.

Several channels that were identified as nonselective due to their Na\(^+\)-sensitive rescue of the *trk1Δ* *trk2Δ* phenotype seemed to have little or no effect on the growth of wild-type cells on the high sodium medium. In these cases it is possible that the channels were primarily blocked by, but not permeable to, sodium. A fairly strong suppressor of the *trk1Δ* *trk2Δ* phenotype such as the VT channel may be a likely candidate for this hypothesis. Alternatively, if total conduction of ions through these channels is low, even mutant channels with increased sodium-potassium permeability might not substantially affect growth of wild-type yeast in the presence of potentially toxic concentrations of sodium. Weaker suppressors of the *trk1Δ* *trk2Δ* phenotype such as LG and LA are more consistent with this explanation.

### Electrophysiology of Wild-type Kat1 and Mutant Kat1 Channels

The selectivity of wild-type and mutant Kat1 channels identified from the genetic screen was also assessed by two-elec-

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| Summary of phenotypes of wild-type and mutant Kat1 channels expressed in *S. cerevisiae* |
|---------------------------------------------------------------|
| **Channel Selectivity** |
| **trk1Δ trk2Δ** | **7 mM K\(^+\)** | **0.2 mM K\(^+\)** | **400 mM Na\(^+\)** | **TRK1 TRK2** | **2 mM K\(^+\)** | **2 mM K\(^+\)** |
| Single substitutions | Wild type (YG) | + + | + + | + + | + + | + + |
| | Vector | – | – | – | + + | + + |
| | AG | + + | – | – | + + | + + |
| | CG | + + | – | – | + + | + + |
| | DG | + | – | – | + + | + + |
| | EG | + | – | + | + + | + + |
| | FG | + | + | + | + + | + + |
| | GG | + | + | – | + + | + + |
| | IG | + | + | – | + + | + + |
| | LG | + | – | + | + + | + + |
| | MG | + | – | + | + + | + + |
| | NG | + | – | + | + + | + + |
| | PG | + | – | + | + + | + + |
| | SG | + | – | + | + + | + + |
| | TG | + | + | – | + + | + + |
| | VG | + | + | + | + + | + + |
| Double substitutions | GA | + | – | – | + | + |
| | LA | + | – | – | + | + |
| | PA | + | – | – | + | + |
| | QA | + | – | – | + | + |
| | PN | + | + | – | + | + |
| | AS | + | + | – | + | + |
| | DS | + | – | + | + | + |
| | GS | + | – | + | + | + |
| | LS | + | – | + | + | + |
| | MS | + | – | + | + | + |
| | QS | + | – | + | + | + |
| | VS | + | – | + | + | + |
| | IT | + | – | + | + | + |
| | LT | + | – | + | + | + |
| | VT | + | – | + | + | + |
Structural Requirements for K⁺ Channel Selectivity

Channel selectivity was assessed under bi-ionic conditions when the bath solution was substituted with the test ions potassium, sodium, rubidium, and ammonium. Conductance ratios for the test ions were determined relative to the potassium conductance at the end of a 1-s pulse at a membrane potential of −150 mV. When possible, reversal potentials were used to determine permeability ratios as predicted by the Nernst-Planck electrodiffusion equation (37).

Wild-type and Wild-type-like Channels—The potassium currents elicited by the wild-type Kat1 channel upon hyperpolarization were essentially identical to those previously described for this channel (Fig. 3A). Significant KAT1-dependent currents were detected when the bath was switched to rubidium or ammonium (Fig. 3, A and D). Conductance of these ions relative to potassium when the cell was hyperpolarized to a potential of −150 mV was 18 ± 3% and 44 ± 17% (n = 3), respectively (conductance ratios are summarized in Table III). In addition, reversal potentials of tail currents were measured in response to hyperpolarization with potassium in the potassium, rubidium, and ammonium baths and determined to be −5 ± 2 mV, −29 ± 1 mV, and −75 ± 5 mV, respectively, representing permeability ratios of $P_{\text{Rb}^+}/P_{\text{K}^+} = 39\%$ and $P_{\text{NH}_4^+}/P_{\text{K}^+} = 6\%$. When the bath solution was exchanged for sodium, very little inward current was detected upon hyperpolarization (Fig. 3A). Tail current analysis in this bath solution was not performed, since the reversal potential fell below −160 mV. Potassium currents could be restored upon exchange back to the potassium bath (data not shown).

Consistent with findings in the yeast system, only one mutant channel, the YG to FG substitution, duplicated the selectivity properties of the wild-type channel (Fig. 3B). Currents measured in response to hyperpolarization with potassium in the bath solution were larger than those of ammonium and rubidium (Fig. 3, B and D; Table III). Sodium was highly impermeant, since no current was detected in the sodium bath. Reversal potentials measured in the potassium, rubidium, and ammonium baths were −2 ± 3 mV, −28 ± 3 mV, and −62 ± 10 mV, representing permeability ratios of $P_{\text{Rb}^+}/P_{\text{K}^+} = 36\%$ and $P_{\text{NH}_4^+}/P_{\text{K}^+} = 9\%$.

Mutants with Decreased Selectivity—Based on the phenotypes exhibited in S. cerevisiae, the TG mutant was inferred to be highly selective for K⁺ over Na⁺ but not as selective as the wild type or FG mutant. Expressed in oocytes, the TG mutant elicited slowly activating potassium currents under hyperpolarization, similar to the wild-type channel (Fig. 3C). However, currents conducted by ammonium and rubidium ions were equal or greater in magnitude to the potassium currents (Fig. 3, C and D). Conductance ratios measured at −150 mV for Rb⁺ and NH₄⁺ were 130 ± 32% and 123 ± 15% (n = 4), respectively. The reversal potential measurements taken in potassium, rubidium, and ammonium baths were −1 ± 2 mV, −15 ± 5 mV,
and $-60 \pm 2 \text{ mV}$, respectively, representing permeability ratios of $P_{\text{Rb}}/P_{K^+} = 57\%$ and $P_{\text{NH}_4}/P_{K^+} = 10\%$. Despite the alteration in channel selectivity, currents were not detected in the sodium bath, consistent with this channel being selective for potassium over sodium. Thus, a single amino acid substitution at this highly conserved site (Y263T) can increase the magnitude of currents carried by Rb or NH$_4$ relative to K without a major decrease in selectivity for K$^+$ over Na$^+$.

**Mutants with Loss of Selectivity**—The majority of mutant Kat1 channels identified in the yeast screen were inferred to be nonselective by inhibition of growth on media containing high sodium. Two examples of this class of mutant channels, DS and CG, conferred detectable currents when expressed in oocytes. The DS substitution lost all wild-type properties of selectivity. Potassium currents conducted by this channel were clearly nonselective by inhibition of growth on media containing high potassium. Two examples of this class of mutant channels, DS and CG Kat1 mutants. A and B, currents elicited by the DS and CG channels in baths containing 100 mM K$^+$, Rb$^+$, NH$_4^+$, or Na$^+$. Oocytes were held at $-40 \text{ mV}$ and hyperpolarized to $-160 \text{ mV}$ in 10-mV steps. C, current-voltage relationships at 1 s for the traces shown in bath solutions containing 100 mM K$^+$ (square), 100 mM Rb$^+$ (circle), 100 mM NH$_4^+$ (triangle), and 100 mM Na$^+$ (diamond).

Tail current analysis for the nonselective channels revealed complex inactivation kinetics possibly due to activity of endogenous channels. These currents may be attributed to Ca$^{2+}$-activated Cl$^-$ channels similar to those observed in recordings of chimeric channels of Kat1 and the Shaker potassium channel (39). Another possibility is that this mutant channel can be blocked in a voltage-dependent manner by a divalent cation, resulting in the “hump” in the tail currents. However, we are confident that the inward currents measured in response to hyperpolarization in the various test baths are mostly carried by the bath cation, because the kinetics of activation and saturation are similar to those of the wild-type Kat1 channel.

**pH-sensitive RG Channel**

Mutants in which a basic amino acid occupied the Tyr$^{263}$ site did not form channels capable of rescuing growth of trk1Δ trk2Δ cells under the conditions of the screen (data not shown). Given that most other substitutions formed functional channels, we suspected that potassium conduction might be blocked by protonated side groups.

Although the growth medium used in the screen was prepared to pH 5.9, after several days of growth it became acidic (data not shown). Medium buffered with MES and Tris-base maintained its pH after several days. On low potassium medium buffered to pH 7.5, the RG channel conferred strong growth of trk1Δ trk2Δ cells, suggesting that this channel can be titrated by a shift in pH of the medium (Fig. 5A). A similar effect was seen for the RG channel expressed in oocytes. The current amplitudes approached zero as the pH of the bath was lowered from 7.4 to 4.7 (Fig. 5, B and C). This result is striking because the current amplitudes of the wild-type channel following the same pH shift actually increase 10-fold (Fig. 5, B
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DISCUSSION

We have presented the results of a combinatorial saturation mutagenesis of two highly conserved sites, Tyr²⁶³ and Gly²⁶⁴, in the pore of Kat1. At these positions, almost any substitution alters channel selectivity. Of the 400 possible amino acid combinations at these positions, only the conservative tyrosine to phenylalanine mutation at position 263 resulted in a channel with completely wild-type permeation properties assayed in the S. cerevisiae and Xenopus systems. All other mutants isolated in the screen were phenotypically distinct from cells expressing the wild-type Kat1 channel. Growth of trk1Δ trk2Δ cells expressing these mutants on low K⁺ was always weaker than growth conferred by the wild-type channel. More importantly, on media containing high concentrations of sodium, all of the mutant channels except FG exhibited phenotypes strongly suggestive of a decrease in ion selectivity.

The phenotypes of Kat1 channels expressed in both the yeast and oocyte systems were similar. The YG to FG substitution, which retained all selective properties of the wild-type channel in two separate tests in yeast, also maintained wild-type selectivity when expressed in oocytes. Two channels that appeared nonselective in the yeast system, the CG and DS mutants, were shown in oocytes to have completely lost wild-type properties of ion selectivity. Both mutants were highly permeable to sodium and exhibited alterations in permeability with other test ions.

The TG substitution, which was inferred to be highly selective for K⁺ over Na⁺ in S. cerevisiae was impermeable to sodium when expressed in oocytes. Alterations in the selectivity of the semipermeant ions rubidium and ammonium suggested that the mutation caused only a minor change in the ability of the pore to discriminate between monovalent cations. The Na⁺ sensitivity of wild-type TRK1 TRK2 cells expressing the TG channel suggested that sodium, too, permeated this channel in S. cerevisiae. We believe that for this channel the apparent difference in sodium phenotypes observed in Xenopus oocytes and S. cerevisiae is due to a fundamental difference between the two systems of analysis. The most extreme hyperpolarization episode of oocytes lasted 1 s at a membrane potential of ~160 mV, whereas yeast cells are likely to maintain potentials at least this large over periods of time several orders of magnitude longer. Thus, the S. cerevisiae system seems to be more sensitive with regard to the ability to detect increases in uptake of Na⁺. A change in channel selectivity that results in a very low velocity of Na⁺ uptake may nevertheless be sufficient to confer toxicity when uptake of the toxic ion is accumulated over the time scale of cell division.

A recent model of the pore of the Shaker channel has positioned the side chain at the tyrosine position away from the center of the pore (41). However, our results indicate a strict requirement for either tyrosine or phenylalanine, providing strong support for hypotheses that aromatic rings form a key component of the K⁺ channel selectivity filter through cation–π interactions (20, 42, 43). The solvent accessibility of the titratable group in the RG mutant further supports this model. Although we can only speculate about the mechanism of the pH sensitivity for this mutant, a compelling model positions the side chain of the arginine residue into the lining of the pore regulating potassium flux directly by the presence or absence of a proton.

Our results also support predictions that the tyrosine residue is positioned at the narrowest region of the pore loop (24–26, 41, 42, 44). Substitutions to smaller side chains, which would be predicted to directly increase the diameter of the pore, decreased selectivity, again with the notable exception of phenylalanine. Conversely, the tyrosine substitution to the larger tryptophan did not form a functional channel, possibly due to a decreased diameter of the pore.

The ability of the genetic screen to test the effect of simultaneous mutations at both Tyr²⁶³ and Gly²⁶⁴ revealed a highly interdependent relationship between these sites. Single amino acid substitutions at Gly²⁶⁴ apparently do not result in functional channels. However, substitutions at this site can be accommodated if a second, compensatory mutation, occurs at Tyr²⁶³. Although we cannot rule out the possibility that single mutations at this site prevent expression or reduce stability of the channel, the fact that double mutations at YG sometimes result in functional channels indicates that a glycine at position 263 is not an absolute requirement for expression or stability.

We suggest two possibilities to explain why channels containing single mutations at Gly²⁶⁴ are nonfunctional. The increase in size of the amino acid side chain at this position could either occlude the pore directly or could cause the adjacent tyrosine residue to move further into the pore and thereby block current. Either model is consistent with the observation that, for the most part, the amino acids that can be accommodated at Gly²⁶⁴ (when accompanied by a change at Tyr²⁶³), are those that contain the smallest side groups, i.e. alanine, serine, and threonine. Notably, one larger side group, that of asparagine, can also be accommodated at Gly²⁶⁴ in combination with a proline at Tyr²⁶³. The potential of prolines to confer turns along the peptide may be necessary to compensate for the occlusion that would otherwise result from the large side group of asparagine. Interestingly, in Shaker, mutations are tolerated at the analogous glycine position (20). Nevertheless, the observation that these channels are highly nonselective is consistent with the requirement of an aromatic amino acid paired with glycine in formation of the optimally selective pore.

In apparent contradiction to our conclusions, the Y445V mutation in Shaker was deemed selective for K⁺ over Na⁺, based on a low Na⁺:K⁺ permeability ratio (<0.20) (20). However, as shown for the TG mutant, the sensitivity of the yeast system allows the identification of changes in ion selectivity that are exceedingly small or even undetected by conventional electrophysiological analyses. The Y263V mutation of Kat1 resulted in decreased selectivity but failed to express currents in Xenopus oocytes.

Clearly, channels that are selective for potassium over sodium can be formed with nonaromatic substitutions at the tyrosine position. However, given the comprehensive analysis of this position described here and the sensitivity of the yeast growth assays, maximal potassium selectivity in Kat1 was achieved only by the tyrosine/phenylalanine-glycine pair.

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