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Site-Specific Mutations in a Minimal Voltage-Dependent K+ Channel Alter Ion Selectivity and Open-Channel Block

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Summary

MinK is a small membrane protein of 130 amino acids with a single potential membrane-spanning α-helical domain. Its expression in Xenopus oocytes induces voltage-dependent, K^+-selective channels. Using site- directed mutagenesis of a synthetic gene, we have identified residues in the hydrophobic region of minK that influence both ion selectivity and open-channel block. Single amino acid changes increase the channel's relative permeability for NH_4^+ and Cs^+ without affecting its ability to exclude Na^+ and Li^+. Blockade by two common K^+ channel pore blockers, tetraethylammoniumand Cs^+, was also modified. These results suggest that an ion selectivity region and binding sites for the pore blockers within the conduction pathway have been modified. We conclude that the gene encoding minK is a structural gene for a K^+ channel protein.

Introduction

Voltage-gated K^+ channels are essential to the integrated electrical activity of excitable cells (Hille, 1984). Responding to changes in membrane potential, these molecules open a water-filled transmembrane pore through which ions rapidly diffuse. Once open, K^+ channels exhibit high selectivity among similar inorganic cations, allowing K^+ but not Na^+ to permeate. Two types of voltage-dependent K^+ channels have been distinguished on the basis of molecular characteristics. "*Shaker*-type" K^+ channels, originally cloned from Drosophila (Papazian et al., 1987; Pongs et al., 1988; Kamb et al., 1988), have been the subject of intense study by site-directed mutagenesis (Miller, 1991). These K^+ channel proteins are rather large (~50-100 kd), span the membrane at least eight times (Yellen et al., 1991), and form functional channels through tetrameric association (Jan and Jan, 1989; Catterall, 1988; MacKinnon, 1991).

We are studying the first representative of a second type of K+ channel. The gene for this pore was originally cloned from rat kidney (Takumi et al., 1988), but is also present in mammalian heart, uterus, and sub- mandibular gland (Folander et al., 1990: Pragnell et al., 1990; Sugimoto et al., 1990.) The product of this gene is a protein called minK (Hausdorff et al., 1991), and its molecular character is unprecedented among eukaryotic ion channels. The protein has only 130 amino acids (~15 kd) and a single potential membrane- spanning α-helical domain by hydropathy analysis (Takumi et al., 1988). When expressed in Xenopus oocytes, this small protein induces slowly activating, voltage-dependent, K^+-selective currents (Takumi et al., 1988).

The small size of minK suggests that it might be extremely well suited for investigations into basic mechanisms of ion channel function. However, the induction of K+-selective currents in Xenopus oocytes is not evidence that the minK molecule, so unlike other ion channels, is in fact an ion channel protein. Recently, we argued that minK cRNA-induced currents are mediated by a channel rather than by a carrier-type transport mechanism (Hausdorff et al., 1991). A circumstantial case was based on the functional characteristics of minK-induced macroscopic currents, since single channels have not been observed. MinK currents are similar to K^+ channels and unlike carrier-type transporters in their voltage dependence, reversible block by tetraethylammonium (TEA), Cs^+, and Ba^{2+}, and strong selectivity for K^+ and its close analogs (Hausdorff et al., 1991).

In this paper, we ask whether minK is a K^+ channel protein itself or a "regulator" of a K^+ channel endogenous to oocytes but normally silent. We introduced site-specific changes in the minK gene and observed alterations in ion selectivity and open-channel block in K^+ currents expressed in Xenopus oocytes. The results strongly support the thesis that minK is a structural gene for a protein that forms the pore of a K^+ channel.

Results

Expression of Wild-Type and Mutant MinK-lnduced Channels

We studied the effects of point mutations in the minK gene on the function of the K^+ channels induced by this gene in Xenopus oocytes. Changes were introduced into the single extended hydrophobic domain of minK identified by hydropathy analysis (Takumi et al., 1988), shown in Figure 1. We focused on this region of 23 uncharged amino acids, since it seemed plausible that these residues might contribute to a pore if minK were a channel protein.

Site-directed mutants were constructed by the cassette technique employing a synthetic minK gene (Hausdorff et al., 1991). The coding sequence for the hydrophobic domain was removed using two unique restriction enzyme sites and replaced with a variety of synthetic DNA duplexes bearing desired base changes. Xenopus oocytes were injected with cRNA synthesized in vitro and assessed for voltage-dependent K+ currents by two-electrode voltage clamp 1-5 days later.

Wild-type minK cRNA induces a slowly activating, voltage-dependent, K^+-selective outward current (Figure 2) not present in uninjected oocytes (Hausdorff et al., 1991). These currents are identical to those seen in oocytes injected with native uterine mRNA or cRNA from other sources (Boyle et al., 1987; Folander et al., 1990; Takumi et al., 1988). To demonstrate wild-type minK-induced currents, oocytes were held at -80 mV and then depolarized to various test potentials for 10 s (Figure 2). The conductance increases e-fold per 13 mV depolarization, as previously reported (Hausdorff et al., 1991).

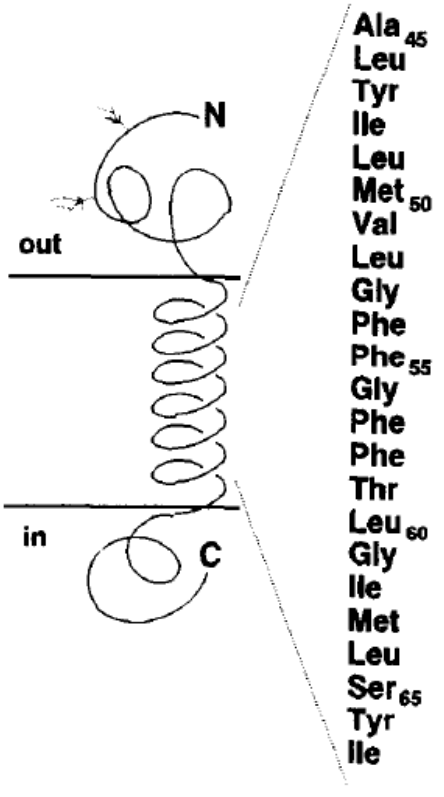

Figure 1. Polypeptide Sequence of the Extended Hydrophobic Domain of MinK
This region has 23 amino acids and 4 hydroxyl-bearing residues. Residues are numbered from the N-terminus. The protein has 130 amino acids, two external N-1inked glycosylation sites as indicated, and 1 internal cysteine (Takumi et al., 1988).

Point mutations in the hydrophobic domain of minK did not disturb expression of these K^+-selective, voltage-gated currents. All mutations described here induced slowly activating currents whose voltage dependence was indistinguishable from wild-type currents. To focus this analysis, the effects of one such mutation will be documented in detail. In this mutation, a native phenylalanine residue at position 55 (Figure 1) was changed to a threonine (F55T). Oocytes expressing this altered minK were held at -80 mV and then depolarized to various test potentials for 10 s (Figure 2). As with wild-type minK, the slowly activating conductance induced by this cRNA increases e-fold per 13 mV membrane depolarization.

The gating kinetics of both wild-type and mutant currents are complex. Slow activation proceeds for

minutes if a depolarizing test pulse is maintained and is strongly influenced by prepulse holding potential (Boyle et al., 1987; Hausdorff et al., 1991). A 10 s conductance measurement is therefore a crude approximation of the steady-state value at best. To focus on properties associated with ion conduction through open channels such as ion selectivity and open-channel block, we employed identical test-pulse and interpulse periods and a single holding potential in all experiments.

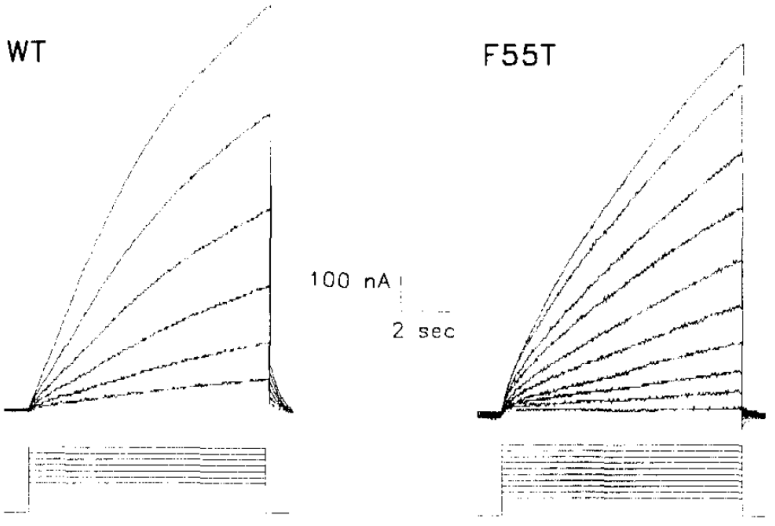

Figure 2. Expression of MinK-lnduced Channels in Xenopus Oocytes
Currents were elicited by10 s depolarizing pulses from a holding voltage of -80 mV; pulses were from -40 to +40 mV. Currents are displayed after leak subtraction for oocytes injected with wild-type (WT) or F55T minK cRNA.

K+ Selectivity of Wild-Type and Mutant MinK-lnduced Channels

The hallmark attributes of wild-type minK-induced channels are slow activation, voltage-dependent gating, and K^+ selectivity. Channels induced by expression of F55T minK are identical to wild-type channels in their high selectivity for K^+ over both Na^+ and Cl^-. This was shown with a tail current protocol (Figure 3A). Oocytes were held at -80 mV, pulsed to +20 mV to open the channels, and then repolarized to various test potentials. The initial slopes of tail currents were examined to determine the reversal potential (V_{rev}). In both wild-type and mutant channels, reversal potentials followed external K^+ concentration in a strictly Nernstian manner (Figure 3B), as expected for channels ideally selective for K^+ over both Na^+ and Cl^-.

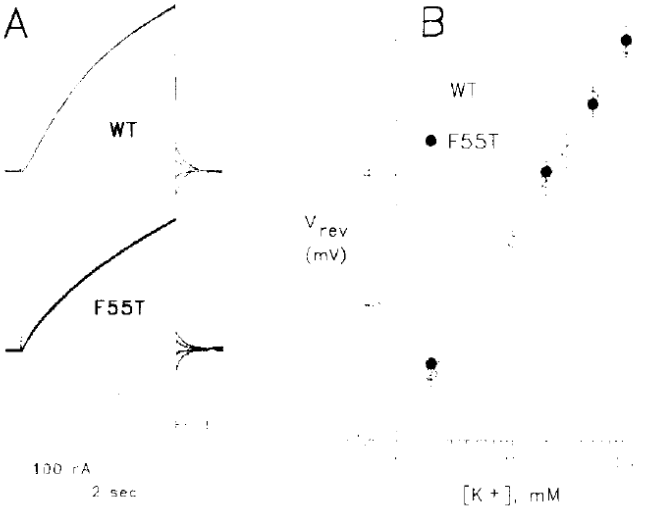

Figure 3. Reversal Potentials of MinK-lnduced Channels in Varying Concentrations of External K^+
Oocytes injected with either wild-type (WT) or F55T mutant cRNA were depolarized to +20 mV for 10 s, and tail currents were observed by repolarizing to voltages near the reversal potential, here in an external solution containing 2mM KCI(see Experimental Procedures). Currents are leak subtracted. (B) Both channel types were studied in external solutions containing the indicated K^+ concentrations (adjusted by replacement for Na^+). Wild-type (open circles); F55T minK (closed circles). The slope of the solid line is 58 mV per decade.

Ion Selectivity by Wild-Type and Mutant Mink-Induced Channels

Although F55T minK-induced channels, like the wild-type channels, are ideally selective for K^+ over Na^+, they display altered discrimination among close K^+ analogs. To assess relative permeability, a test cation, X^+, was isotonically substituted for Na^+ in the bath solution and tail current reversal potentials were analyzed in terms of the Goldman-Hodgkin-Katz relation (Hille, 1984):

$$V_{rev} = (RT/zF)\ln\left(\frac{P_X[X^+]_o + P_K[K^+]_o + P_{Na}[Na^+]_o + P_{Cl}[Cl^-]_i}{P_X[X^+]_i + P_K[K^+]_i + P_{Na}[Na^+]_o + P_{Cl}[Cl^-]_o}\right) \quad (1)$$

where V_{rev} is the measured reversal potential; P_X, P_K, P_{Na}, and P_{Cl} are the ion permeabilities; and z, F, R, and T have their usual meanings. If K^+ is the only permeant ion inside the oocyte and the test cation is the only permeant ion in the bath solution, V_{rev} reflects the relative permeability of the external test cation, X^+, against K^+, and Equation 1 simplifies to

$$V_{rev} = (RT/zF)\ln(P_X[X^+]_o/P_K[K^+]_i) \quad (2)$$

Equation 2 predicts the dependence of V_{rev} on the external test cation concentration $[X^+]_o$, We compared these predictions with the behavior of wild-type, F55Y, and F55T minK-induced channels with Cs^+ as the only permeant cation in the bath solution (Figure 4A). Cs^+ data are shown here because they are the most difficult to obtain; this ion is an open-pore blocker of minK (Hausdorff et al., 1991) and as such diminishes currents when present in the external solution. Nonetheless, clear inward Cs^+ tail currents were detectable and allowed measurement of minK reversal potentials (Figure 4A). F55T minK reversal potentials shift by 58 mV per 10-fold change in external Cs^+ (Figure 4B), as demanded (Equation 2) for a channel ideally selective for Cs^+ over both Na^+ and Cl^-. This result gives us confidence in the validity of reversal potentials measured in the presence of poorly permeating K+ analogs.

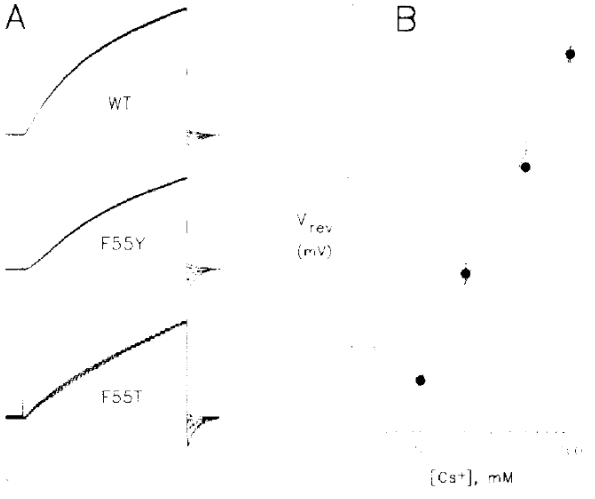

Figure 4. Reversal Potentials of MinK-lnduced Channels in Varying Concentrations of External Cs^+
Oocytes injected with wild-type (WT), F55Y, or F55T mutant cRNA were depolarized to +20 mV for 10 s, and tail currents were observed by repolarizing to voltages near the reversal potential, here in external solutions contained no K^+ and either 98 mM CsCl (WT, F55Y) or 50 mM CsCl with 48 mM NaCl (F55T). Currents are leak subtracted. Scale bars represent 100 nA and 2 s. (Bl F55T minK-induced channels were studied in external solutions containing the indicated Cs^+ concentrations (adjusted by replacement for Na^+). The slope of the solid line is 58 mV per decade.

Solution of Equation 2 for the bi-ionic condition, $[K^+]_i = [X^+]_o$, allows definition of a permeability ratio for K^+ versus the test cation X^+, P_K/P_X (Hille, 1984). Tail currents were measured for both wild-type and F55T minK-induced channels in the presence of 98 mM external test cation, a nearly bi-ionic condition. Wild- type and mutant channels both exhibit a permeability sequence (Figure 5) common to virtually all known K^+ channels: $K^+ > Rb^+ > NH_4^+ > Cs^+ >> Na^+, Li^+$ (Latorre and Miller, 1983; Yellen, 1987). The mutant remains readily permeable to K^+ and Rb^+ and essentially impermeable to Na^+ and Li^+. The mutant is, however, 3-fold more permeable to NH_4^+ than the wild-type channel and 3.5-fold more permeable to Cs^+. The fine ionic discrimination of the channel has been altered by this change in position 55.

Not all changes in position 55 affected the channel's selectivity. Mutation F55Y adds a hydroxyl moiety at this position, as does F55T, but preserves the size of the native residue; this mutant exhibits wild-type behavior (Table 1; Figure 4A). On the other hand, mutation F55A, which decreases the size of residue 55 but does not introduce a hydroxyl group, is more permeable to Cs^+ than the wild-type channel (Table 1). Not all residues in this region of the minK sequence are involved in determining the channel's ion selectivity. Residue 54 is also phenylalanine (Figure 1), but the mutant F54T exhibits wild-type selectivity (Table 1). These results might seem to argue that the channel's selectivity is specifically sensitive to the size of the residue at position 55. This is not strictly the case, however. The permeability changes resulting from the F55T mutation can be reversed by a mutation at residue 59, which is normally a threonine. The double mutant F55T/T59F fully restores wild-type ionic selectivity (Table 1).

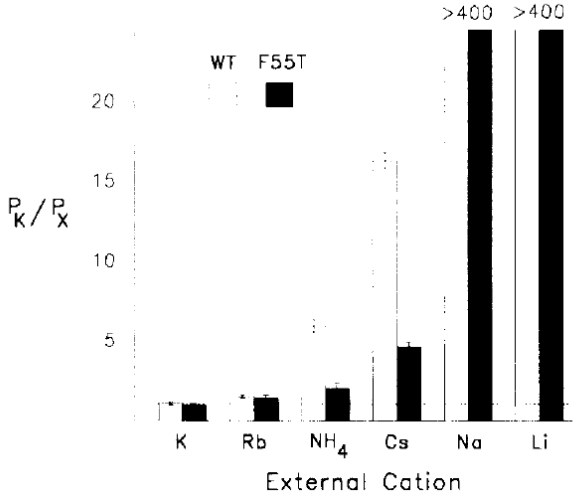

Figure 5. Ionic Selectivity of MinK-Induced Channels
Reversal potentials were measured under nearly bi-ionic conditions, with 98 mM of the indicated cation in the external solution. Values represent the mean ± SEM of 4- 8 oocytes. Permeability ratios are defined as $P_K/P_X = \exp(-FV_{rev}/RT)$ (Hille, 1984). Wild- type (open bars); F55T (closed bars).

Effects of Mutations on Open-Channel Block

MinK-induced channels are reversibly inhibited by the familiar K^+ channel blockers TEA, Cs^+, and Ba^{2+} applied in the bath solution (Hausdorff et al., 1991). Both TEA and Cs^+ act as classic open-channel pore blockers, whereas Ba^{2+} inhibits by a more complicated mechanism involving the gating of the minK channel (Hausdorff et al., 1991). We investigated the effects of these channel blockers on the ion selectivity mutant F55T.

Table 1. Ionic Selectivity of Wild-Type and Mutant MinK-Induced Channels

Mutation	V_{rev} (mV)	P_K/P_{Cs}
Wild type (6)	−70 ± 1	16.4 ± 0.1
F55T (6)	−38 ± 1	4.6 ± 0.2
F55Y (2)	−68 ± 1	15 ± 1
F55A (1)	−57	7 ± 1
F54T (3)	−70 ± 1	17 ± 1
F55T/T59F (2)	−66 ± 2	14 ± 1

Tail currents were studied with Cs^+ as the only permeant ion in the external solution. Reversal potentials are reported for nearly bi-ionic conditions with 98 mM external CsCl (for the number of oocytes indicated). Values represent the mean ± SEM for 3 or more oocytes. Permeability ratios were defined as $P_K/P_X = \exp(-FV_{rev}/RT)$ (Hille, 1984). The permeability ratio for F55A was confirmed in 3 additional oocytes with lower $[Cs^+]_o$ using Equation 2.

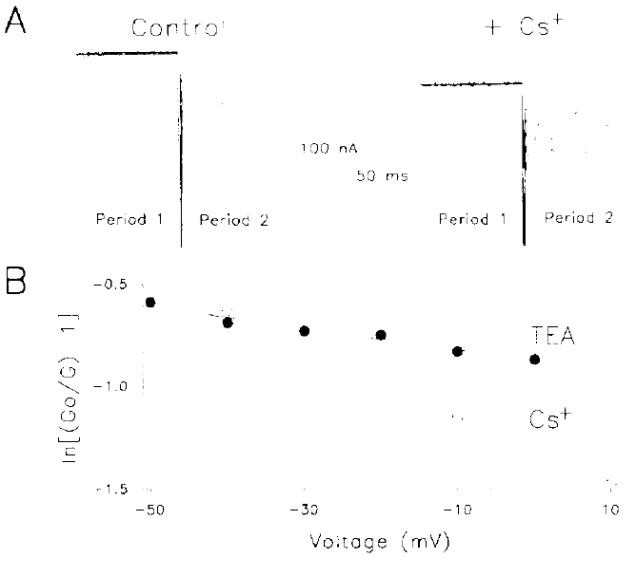

Figure 6. Blockade of MinK-lnduced Currents
(A) Raw records in the presence or absence of 12.5 mM Cs^+ added to standard external buffer are shown. Oocytes were held at -80 mV, depolarized to +20 mV for 5 s (period 1, final 150 ms displayed), repolarized to various test potential s (-50 to +10 mV in 10 mV steps) for 300 ms (period 2, final 150 ms displayed), and then returned to -80 mV. Oocytes shown were injected with wild-type minK cRNA. Currents are displayed for periods 1 and 2 only. Dashed line represents zero-current level. (B) Voltage dependence of Cs^+ block. Measurements of conductance were made from - SO to +10 mV with 12.5 mM Cs^+ or 95 mM TEA in the bath solution. Data are from a single oocyte injected with wild-type minK cRNA, but are representative of several experiments.

In the absence and presence of varying blocker concentrations, the oocytes were depolarized to +20 mV to open the channels and then repolarized to various test potentials. Current was measured 25 ms after the voltage shift, a time too short for channel gating to occur (Figure 6A). Both the zero-voltage inhibition constant $K_i(0)$ and the effect of voltage on block were studied (Figure 6B). Table 2 shows that both open-channel blockers, TEA and Cs^+, are more effective in F55T than in wild-type minK channels, whereas Ba^{2+} inhibition is unaffected by the mutation. Mutant channels were twice as sensitive to blockade by Cs^+ as wild-type channels and at least 3-fold more sensitive to blockade by TEA. Since only the zero-voltage dissociation constants for TEA and Cs^+ are altered in F55T, and not the voltage dependence of block (Table 2), we argue that the mutation does not alter the positions in the pore at which these blockers bind.

Table 2. Blocking Parameters for Wild-Type and Mutant MinK-Induced Channels

	Wild Type		F55T	
	$K_i(0)$ (mM)	δ	$K_i(0)$ (mM)	δ
Cs^+	41 ± 3	0.37 ± 0.02	17 ± 4	0.30 ± 0.01
TEA	>150	0.13 ± 0.03	42 ± 2	0.08 ± 0.03
Ba^{2+}	3.6 ± 0.1	0.09 ± 0.01	5 ± 1	0.14 ± 0.02

Inhibition of minK-induced currents was measured with various concentrations of Cs^+, TEA, or Ba^{2+} in the external solution. Channels were opened for 5 s at +20 mV, and then currents were measured 25 ms after repolarizing to test potentials between −50 and +20 mV. Block was calculated by normalization to conductance in the absence of blocker, after correction for leak currents; reversal potentials were measured in each experiment. The inhibition constant $K_i(0)$ and the electrical distance (δ) are reported for each blocker as calculated from $K_i(V) = K_i(0)\exp(z\delta FV/RT)$, where $K_i(0)$ is the zero-voltage inhibition constant, z is the valence of the blocking ion, and δ is the fraction of the applied voltage drop experienced at the blocker's binding site (Woodhull, 1973; Coronado and Miller, 1979). Each value represents the mean ± SEM of 3–6 oocytes.

Discussion

We have shown that point mutations in the extended hydrophobic domain of minK subtly change functional attributes of the conduction pathway in minK- induced channels. Two mutations that reduce the size of the residue at position 55 alter the channel's fine discrimination among permeating ions and its

affinity for two open-channel blockers. F55T minK channels are 3-fold more permeable to NH_4^+ and Cs^+ and at least twice as sensitive to TEA and Cs^+ blockade than wild-type channels. Broadly speaking, the F55T mutation acts to lower the overall free energy profile for Cs^+; increased permeability (as measured by bi-ionic potential) is consistent with a lowering of a "peak energy," while higher binding affinity and diminished conductance implies a deeper energy well (Hille, 1975).

While mutation F55T clearly affects the channel's pore-associated behavior, it leaves other essential characteristics of the channel undisturbed. Slow voltage-dependent activation remains qualitatively unaltered. The channel remained ideally selective for K^+ over both Na^+ and Cl^-, and the permeability order among monovalent cations ($K^+ > Rb^+ > NH_4^+ > Cs^+ >> Na^+, Li^+$) was not modified. The voltage dependence of block by TEA and Cs^+ and the "gating inhibition" by Ba^{2+} were both unchanged by the selectivity mutation. These results argue that mutation at position 55 does not grossly distort the structural and functional integrity of these channels. Rather, the observed changes involve small energies that influence a localized region of the protein intimately associated with the K^+ conduction pathway.

We emphasize that our results have no explicit structural meaning. They give no information about the mechanism by which the mutations affect the channel's ion selectivity. They do not provide evidence that the hydrophobic domain of minK forms the conduction pathway, nor that position 55 directly interacts with ions permeating minK channels. The functional subtlety of the effects of these mutations does not imply their structural locality. Although the observations are consistent with the idea that residues at positions 55 and 59 directly interact with ions in the minK conduction pore, they do not by themselves lead to this conclusion. To make such a conclusion from ion permeation mutations, as has been recently achieved in a *Shaker* K^+ channel (Yool and Schwarz, 1991), requires that the case be buttressed by prior knowledge that the region being mutated does in fact form the pore, as in *Shaker*-type K^+ channels (Yellen et al., 1991; Hartmann et al., 1991).

Our results do allow us to answer the question originally motivating this study. We conclude that the minK gene encodes an ion channel protein. The specific effects of point mutations on ionic interactions within the channel's conduction pathway are not consistent with the idea that minK is a regulator protein which activates a channel endogenous to the oocyte but not normally active. The minK gene was originally identifed by expression cloning in oocytes, and the minK protein has not been isolated and functionally reconstituted; therefore, the regulator hypothesis has been a concern for those hoping to use the molecular simplicity of minK as an advantageous focus for future structure-function analysis of basic K^+ channel mechanisms. We consider that this worry is now put to rest.

Experimental Procedures

Biochemical Methods

Cassette mutagenesis of minK transcription plasmid pSF101 (Hausdorff et al., 1991) was performed by standard techniques. Double restriction enzyme digestion with Xhol and Bglll removes a 75 bp segment covering the hydrophobic domain under study. Oligonucleotide duplexes with the desired sequences were phosphorylated and annealed prior to unidirectional ligation into the plasmid. Mutations were confirmed by sequencing both strands, and cRNA was prepared using the SP6 polymerase riboprobe kit (Promega) supplemented with capping nucleotide as previously described (Hausdorff et al., 1991).

Electrophysiology

Xenopus oocytes (NASCO) were prepared and injected with 0.4 ng of cRNA as described (Hausdorff et al., 1991). Currents were recorded 1-5 days postinjection using a computer-controlled, two-electrode voltage clamp (Axoclamp-2A, Axon Instruments, Burlingame, CA). The bath solution contained 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 0.3 mM $CaCl_2$, and 5 mM HEPES (pH 7.6) and was perfused continuously throughout the experiment. For ion replacement experiments, K^+ was removed and other monovalent cations were isotonically substituted for Na^+. Blockers were also added by isotonic substitution for Na^+. To maintain good voltage clamp and reproducible activation kinetics, all experiments were performed at 21°C-23°C with the oocyte mem- brane potential held at -80 mV and a 10 s interpulse "resting" period.

Leak correction was performed after data collection by subtracting the current value measured 25 ms after switching to a test potential (a time too short for channel gating to occur), or that measured at the end of a repolarization period. Without leak correction, certain external ionic conditions (i.e., high K^+) were found

to produce a minK-induced current that was not time dependent and thus formally a "leak" current. This current, however, exhibits K^+ selectivity, monovalent cation discrimination, and blockade by Ba^{2+}, Cs^+, and TEA, and it is the subject of ongoing study. All experiments here report properties only of the slowly activating, voltage-dependent part of the minK-induced currents.

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