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Interaction between Tetraethylammonium and Amino Acid Residues in the Pore of Cloned Voltage-dependent Potassium Channels*

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Extracellular tetraethylammonium (TEA) inhibits currents in *Xenopus* oocytes that have been injected with mRNAs encoding voltage-dependent potassium channels. Concentration-response curves were used to measure the affinity of TEA; this differed up to 700-fold among channels RBK1 (K_D 0.3 mM), RGK5 (K_D 11 mM), and RBK2 (K_D > 200 mM). Studies in which chimeric channels were expressed localized TEA binding to the putative extracellular loop between transmembrane domains S5 and S6. Site-directed mutagenesis of residues in this region identified the residue Tyr^{379} of RBK1 as a crucial determinant of TEA sensitivity; substitution of Tyr in the equivalent positions of RBK2 (Val^{381}) and RGK5 (His^{401}) made these channels as sensitive to TEA as RBK1. Nonionic forces are involved in TEA binding because (i) substitution of the Phe for Tyr^{379} in RBK1 increased its affinity, (ii) protonation of His^{401} in RGK5 selectively reduced its affinity, and (iii) the affinity of TEA was unaffected by changes in ionic strength. The results suggest an explanation for the marked differences in TEA sensitivity that have been observed among naturally occurring and cloned potassium channels and indicate that the amino acid corresponding to residue 379 in RBK1 lies within the external mouth of the ion channel.

Ionic currents through voltage-gated potassium channels are blocked by tetraethylammonium ions (TEA)[1] (Stanfield, 1983; Hille, 1984; Armstrong and Binstock, 1965; Rudy, 1988). There are both internal (Armstrong and Binstock, 1965; Tasaki and Hagiwara, 1957) and external (Hille, 1984; Rudy, 1988; Hille, 1967) blocking sites; block at the external site has been widely used for the experimental isolation of potassium currents (Stanfield, 1983; Hille, 1984; Rudy, 1988). When applied externally, TEA binds with 1:1 stoichiometry to a site within the channel mouth but outside the membrane electric field (Hille, 1967). Likewise, voltage-dependent potassium currents expressed in oocytes from cloned DNA are blocked by external TEA; the block shows 1:1 stoichiometry and also has little voltage dependence. However, individual potassium channels differ markedly in their affinity for TEA, either as they occur normally in cells (Stanfield, 1983; Hille, 1984; Rudy, 1984) or when expressed from cloned subunits in *Xenopus* oocytes (Christie *et al.*, 1989, 1990; Douglass *et al.*, 1990; Stühmer *et al.*, 1988; Grupe *et al.*, 1990; Tempel *et al.*, 1987; Frech *et al.*, 1989; McKinnon, 1989; Stuhmer *et al.*, 1989; Yokoyama *et al.*, 1989).

Comparison of primary sequence data among voltage-dependent cation channels shows that potassium channel subunits are analogous to one of the four internally homologous domains of sodium or calcium channel α-subunits. Therefore, it is likely that four potassium channel subunits assemble to form a functional channel (Catterall, 1988; Montal, 1990; Guy and Conti, 1990). Hydropathy analysis suggests that the polypeptide backbone of a single voltage-dependent potassium channel subunit spans the membrane six times (S1 through S6); both N- and C-terminals are thought to be intracellular, so there are three extracellular loops (Tempel *et al.*, 1987; Stühmer *et al.*, 1988). Controversy remains regarding which portion actually forms the lining of the pore through which potassium ions pass. One possibility is that the third membrane-spanning domain lines the pore (S3; Montal, 1990), but another suggestion is that the short hydrophobic segments SS1 and SS2 (between S5 and S6) line the pore by forming an invagination into the membrane (Guy and Seetharalamu, 1986; Guy and Conti, 1990). TEA is a relatively small, symmetrical ion (diameter about 0.8 nm) bearing a single positive charge which, at least in the case of one class of potassium channel, physically enters and blocks the conducting pore (Vergara *et al.*, 1984; Villaroel *et al.*, 1988). Therefore, information about the mechanism and site of interaction between TEA and the ion channel at the molecular level may provide insight into the structure of the channel itself.

EXPERIMENTAL PROCEDURES

Chimera Channel Construction and Site-directed Mutagenesis—RBK1 and RBK2 were subcloned in the phagemid pTZ-18 (Bio-Rad). Chimeric molecules were constructed by digestion, fragment isolation, and ligation using a conserved *Msc*I site. Site-directed mutagenesis was performed on single-stranded phagemid DNA following helper phage-mediated rescue (M13K07) from the ung^-/dut^- strain CJ236 as described (Kunkel, 1985). Oligonucleotides were synthesized on an Applied Biosystems 380A DNA synthesizer, and DNA sequence analysis was performed using the dideoxynucleotide chain termination method. Capped RNA was transcribed using T7 RNA polymerase as previously described (Christie *et al.*, 1989).

Electrophysiology—Oocytes were injected with 0.5 ng of RNA 24 to 96 h prior to recording membrane currents with a two-electrode voltage-clamp method (Christie *et al.*, 1989). The basic extracellular recording solution contained 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, and 1 mM $MgCl_2$: in some experiments, the sodium concentration was reduced to 48 mM with iso-osmotic substitution of sucrose. When

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[1] The abbreviations used are: TEA, tetraethylammonium; RBK1, potassium channel subunit clone from rat brain (same as RCK1); RBK2, potassium channel subunit clone from rat brain (same as BK2, two amino acids different from RCK5); RGK5, potassium channel subunit clone from rat genomic DNA (nine amino acids different from RCK3); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid.

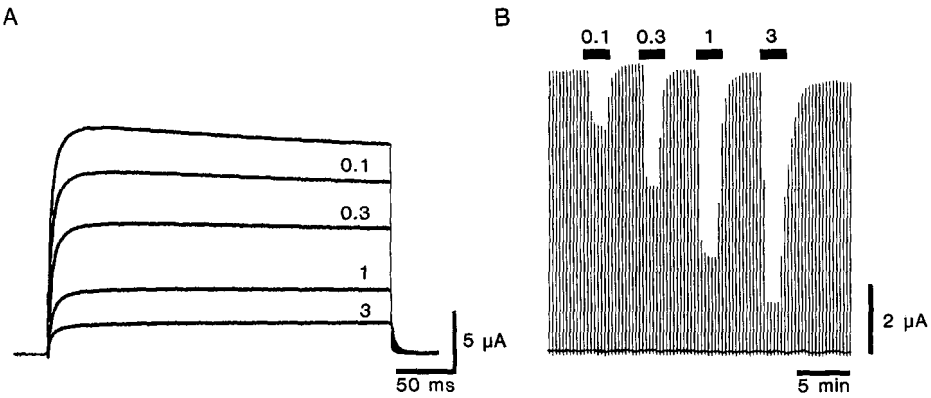

FIG. 1. **Block by TEA of current through expressed RBK1 potassium channels.** *A*, outward currents evoked by depolarizing the oocyte from −80 to 0 mV. The concentration of TEA present is indicated by each trace. *B*, the depolarizing pulse was repeated at intervals of 20 s. Note that the action of TEA had a rapid onset and reversed readily when the application was discontinued.

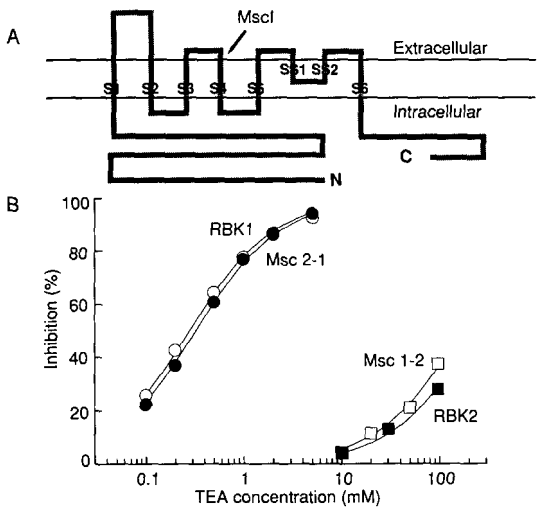

FIG. 2. **Chimeric channels indicate region involved in TEA action.** *A*, diagram to show likely orientation of potassium channels in membrane. *B*, inhibition by TEA of potassium current through RBK1, RBK2, and chimeric channels. *Msc 2-1* is RBK2(Met^{1}-Leu^{290})/RBK1(Ala^{289}-Val^{496}); *Msc 1-2* is RBK1(Met^{1}-Leu^{288})/RBK2(Ala^{291}-Val^{499}).

TEA was added, the concentration of NaCl was reduced by the same amount. Solutions of differing pH were made using HEPES (5 mM, pH 7.0–8.0) or PIPES (5 mM, pH 6.5). Currents were evoked by depolarizing commands from −80 mV to 0 or +10 mV, 200- or 300-ms duration, repeated every 20 s. TEA dose-response curves were determined as previously described (Christie *et al.*, 1990). Four to six concentrations of TEA were applied to each oocyte, the inhibition (%) was fitted to $100 \times [TEA]^n/(K_D^n + [TEA]^n)$ by minimizing the residual sum of the squares, and the K_D was estimated. n was not significantly different from 1. Numbers given (K_D values) are mean ± S.E. for the number of oocytes indicated.

RESULTS

Chimeric Channels—Potassium channels expressed from cDNA clone RBK1 were blocked by TEA with a K_D of 0.33 mM (Fig. 1) whereas channels expressed from clone RBK2 had a much lower affinity (>200 mM); current through RGK5 channels was blocked by TEA with a K_D of about 11 mM (see Christie *et al.*, 1990). To localize regions involved in TEA binding, RBK1/RBK2 chimeric channels were constructed by utilizing a conserved *Msc*I restriction site located immediately 5′ to the region coding for the S4 transmembrane segment (Fig. 2*A*). *In vitro* transcripts from the chimeric sequences were expressed in oocytes, and the action of TEA was compared with its effect on currents expressed from wild-type RBK1 and RBK2. The results of these experiments are shown in Fig. 2. They indicated that sensitivity to TEA was conferred by the C-terminal portion of the molecule with respect to Leu^{288} in RBK1 (Leu^{290} in RBK2).

Point Mutations in the S5-S6 Region—The rapid onset and offset of the blocking action of TEA indicates that it acts from the extracellular membrane surface. The only part of the molecule C-terminal to the chimera junction site that is believed to lie outside the cell is the region between transmembrane segments S5 and S6; therefore, this region was chosen for site-directed mutagenesis. All the Shaker-related mammalian channels cloned to date have about 40 amino acids in this region, and, in the case of RBK1 and RBK2, all but seven of these are identical (Table I). Near the N terminus of this segment (between S5 and SS1) is a well conserved cluster of acidic amino acids. It has been shown that a Glu residue in the homologous region of the Shaker channel ShB1 influences the binding of the channel blocking peptide charybdotoxin (MacKinnon and Miller, 1989). There is a charge difference between RBK1 and RBK2 in this region; Ala^{352} in RBK1 is substituted by Arg in RBK2 (Fig. 3*A*). If electrostatic interactions occur between this residue and the positively charged TEA ion, then such a charge difference could contribute to the reduced affinity of RBK2 for TEA. However, replacement of Ala^{352} by Arg in RBK1 did not result in any change in TEA affinity. Fig. 3 shows the K_D for TEA determined for several mutants in the S5-S6 region close to S5. Even when all five divergent amino acids in this region of RBK1 were mutated to their counterparts in RBK2, the resultant channels had an affinity for TEA which was the same as wild-type RBK1. These results indicate that sequence differences in this region of the molecule are not responsible for the difference in TEA affinity between RBK1 and RBK2.

There are two further amino acid differences between RBK1 and RBK2 at the C-terminal end of SS2; they are on either side of a highly conserved Pro (Tyr^{379} and Val^{381} in RBK1, Val^{381} and Thr^{383} in RBK2; Fig. 3). Mutation of Val^{381} to Thr did not change the effect of TEA, but mutation of Tyr^{379} to Val caused a 30-fold reduction in TEA affinity. The double mutant (RBK1(Y379V,V381T)) was more than 200-fold less sensitive to TEA than wild-type RBK1. Conversely, the single point mutation in RBK2 (Val^{381} to Tyr) increased the potency of TEA to block the current by 3 orders of magnitude (Fig. 3).

Similar results were observed with clone RGK5. Currents through wild-type RGK5 are blocked by TEA with a K_D of about 11 mM (Christie *et al.*, 1990). RGK5 has His in the position equivalent to Tyr^{379} of RBK1. Substitution of His^{401} by Tyr (RGK5(H401Y)) reduced the K_D for TEA from 11.1 ± 1.6 mM (n = 3) to 0.55 ± 0.05 mM (n = 3).

Forces Involved in TEA Binding—Through space electrostatic forces would be expected to be increased by reducing

TABLE I

Sequence of S5-S6 region and TEA sensitivity of cloned potassium channels

▼ marks the position of Tyr^{379} in RBK1.

	Sequence	Channel	TEA EC_{50}	Reference
		▼		
NGK2	365 YAERIGAQPNDPSASEHTHFKNIPIGFWWAVVTMTTLGYGDMYPQTWSGM		0.1	Yokoyama *et al.*, 1989
RBK1	347 FAEA EEAESHFSSIPDAFWWAVVSMTTVGYGDMYPVTIGGK		0.3	Christie *et al.*, 1989
RKShIIIA	402 YAERVGAQPNDPSASEHTQFKNIPIGFWWAVVTMTTLGYGDMYPQTWSGM		0.3	McCormack *et al.*, 1990
KV2[a]	397 FAEA DDVDSLFPSIPDAFWWAVVTMTTVGYGDMYPMTVGGK		4	Swanson *et al.*, 1990
RCK2[a]	396 FAEA DDVDSLFPSIPDAFWWAVVTMTTVGYGDMYPMTVGGK		7	Grupe *et al.*, 1990
drkl	347 FAEK DEDDTKFKSIPASFWWATITMTTVGYGDIYPKTLLGK		10	Frech *et al.*, 1989
Shab	629 FAEK DEKDTKFVSIPEAFWWAGITMTTVGYGDICPTTALGK		10	Wei *et al.*, 1990
RGK5[a]	368 FAEA DDPSSGFNSIPDAFWWAVVTMTTVGYGDMHPVTIGGK		11	Douglass *et al.*, 1990
ShB	416 FAEA GSENSFFKSIPDAFWWAVVTMTTVGYGDMTPVGVWGK		30	Isacoff *et al.*, 1990
RCK3[a]	367 FAEA DDPSSGFNSIPDAFWWAVVTMTTVGYGDMHPVTIGGK		50	Stühmer *et al.*, 1989
KV3[a]	367 FAEA DDPSSGFNSIPDAFWWAVVTMTTVGYGDMHPVTIGGK		>40	Swanson *et al.*, 1990
KV1	443 FAEA DNHGSHFSSIPDAFWWAVVTMTTVGYGDMRPITVGGK		>40	Swanson *et al.*, 1990
Shaw	349 YAERI QPN P HNDFNSIPLGLWWALVTMTTVGYGDMAPKTYIGM		100	Wei *et al.*, 1990
RCK4	500 FAEA DEPTTHFQSIPDAFWWAVVTMTTVGYGDMKPITVGGK		>100	Stühmer *et al.*, 1989
AK01a	373 FAEA DADQTHFKSIPDAFWWAVVTMTTVGYGDMRPIGVWGK		>100	Pfaffinger[2]
RCK5[a]	347 FAEA DERDSQFPSIPDAFWWAVVSMTTVGYGDMVPTTIGGK		>100	Stühmer *et al.*, 1989
RBK2[a]	348 FAEA DERDSQFPSIPDAFWWAVVSMTTVGYGDMVPTTIGGK		>100	Christie *et al.*, 1990
Shal	344 YAEK N V NGTNFTSIPAAFWYTIVTMTTLGYGDMVPETIAGK		>100	Wei *et al.*, 1990

[a] Channels that are identical in this region, but TEA sensitivities have been reported by different workers.

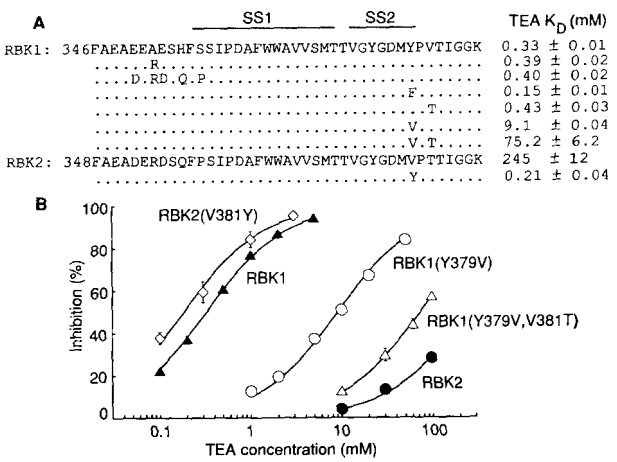

FIG. 3. **Sensitivity to TEA of some mutant potassium channels.** *A*, sequence of the S5/S6 region, with the K_D for TEA (mM). Short hydrophobic segments SS1 and SS2 are indicated. Estimates of K_D values are derived from 4–6 oocytes in each case. *B*, concentration-response curves for the inhibition by TEA of current through three mutant channels, compared with wild-type RBK1 and RBK2. Points are means of 3–7 oocytes; *vertical bar* is mean ± S.E. where this exceeds the size of the symbol.

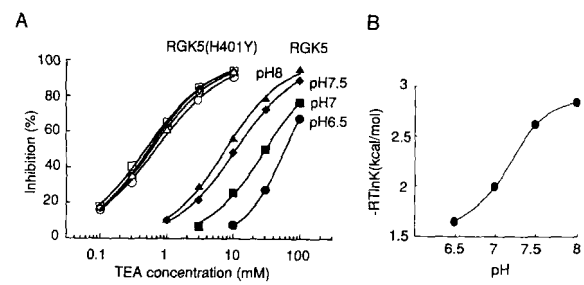

FIG. 4. **Effect of pH on the action of TEA to inhibit current through RGK5 channels.** *A, filled symbols* show the concentration-response curves for TEA in wild-type RBK1 at 4 pH values. *Open symbols* show that there was no effect of pH on current through channels in which His^{401} had been replaced by Tyr. *Error bars* have been omitted for clarity but did not exceed two times the size of the symbols. *B*, relation between pH and the apparent dissociation constant for block by TEA. Ordinate is expressed as $-RT\ln K_D$.

the ionic strength of the solution, as was seen by MacKinnon and Miller (1989) for the binding of charybdotoxin to Shaker ShB1 and by ourselves for the binding of dendrotoxin to RBK1.[3] When the ionic strength of the solution was reduced to one-half, there was no increase in the effectiveness of TEA, consistent with the previous conclusion that differences in charge between RBK1 and RBK2 in this region do not contribute to the difference in TEA affinity.

The experiments described above indicated that the residue at position 379 of RBK1 (381 in RBK2, 401 in RGK5 by alignment) was a crucial determinant of sensitivity to TEA. Substitution of tyrosine in this position by Val^{381} in RBK2 or His^{401} in RGK5 resulted in expression of channels with TEA sensitivity equal to RBK1. Possible mechanisms of interaction between Tyr and TEA include electrostatic forces between the quaternary nitrogen and the phenolate or hydrophobic interaction between ethyl groups and the benzene ring. This question was addressed by constructing and expressing the mutant in which tyrosine was replaced by phenylalanine, RBK1(Y379F): the K_D of TEA was decreased 2.5-fold by comparison with RBK1 (Fig. 3*A*).

Because RGK5 has a His residue at the position occupied by Tyr^{379} in RBK1, this provided the opportunity to examine the effect of varying the degree of ionization of this residue on TEA affinity. TEA was less effective at inhibiting current through RGK5 channels at lower pH and more effective at higher pH (Fig. 4). Substitution of His^{401} with Tyr both increased the affinity for TEA and abolished the pH dependence of the TEA action, suggesting that His^{401} was responsible (Fig. 4). (Total current was also reduced by lowering pH; the apparent pK for this action was 6.6. However, changes in current amplitude were clearly independent of titration of His^{401} because the mutant RGK5(H401Y) showed the same effect.)

DISCUSSION

Recent models of the tertiary and quaternary structure of voltage-dependent cation channels suggest that the C-termi-

[2] P. Pfaffinger, personal communication.

[3] R. S. Hurst, A. E. Busch, M. P. Kavanaugh, P. B. Osborne, R. A. North, and J. P. Adelman, manuscript submitted for publication.

nal portion of the extracellular domain between S5 and S6 (termed *SS2*, Fig. 3) is folded back into the membrane where it can participate in forming a β-sheet structure with a portion of the voltage-sensing domain (Guy and Conti, 1990). This SS2 region has been proposed to form part of the ion selectivity filter and channel lining. S4 has regularly spaced positively charged residues, and voltage-dependent transitions could cause conformational changes leading to channel opening by disrupting the β-sheet between S4 and SS2 (Guy and Conti, 1990). Tyr^{379} of RBK1 is located toward the C-terminal end of SS2, which would place it near the outer mouth of the channel. Energy-minimized structural modeling predicts that the narrowest part of the channel is formed in part by residues Asp^{377} and Tyr^{375} further inside the pore. Our results are in good agreement with such a model, since they indicate a critical role of Tyr^{379} in stabilizing TEA bound to the mouth of the pore in RBK1.

Comparison of the sequences of several voltage-dependent potassium channels in the region joining S5 and S6 strongly supports the present finding that Tyr in this position endows high sensitivity to TEA (Table I). Some reports of TEA sensitivity are less reliable than others, being based on single concentrations of TEA, and the number of experiments performed as well as the test potential range considerably from study to study; nonetheless, the overall conclusion is that sensitivity to TEA is correlated with the presence of a Tyr residue in the position equivalent to Tyr^{379} of RBK1. In naturally occurring channels that are less sensitive to TEA, the residue in this position is quite variable, sometimes hydrophobic (Val, Ala), sometimes nonpolar (Cys, Thr), and sometimes positively charged (Arg, Lys). Although the identity of the residue at the position equivalent to Tyr^{379} of RBK1 is an important determinant of sensitivity, the residue on the C-terminal side of the conserved Pro (Pro^{380} in RBK1) is also capable of influencing affinity (Fig. 3 and Table I).

Possible mechanisms by which Tyr^{379} could influence binding of TEA include a direct hydrophobic interaction with the aromatic ring and an electrostatic interaction between the quaternary nitrogen and the phenolate. The mutation from Tyr to Phe addressed this question. This mutation resulted in a channel in which the sensitivity to TEA was increased about 2.5-fold. This result, taken together with the lack of effect of ionic strength on TEA binding, supports the interpretation that Tyr^{379} directly participates in a nonpolar interaction with TEA. One drawback of replacement of amino acids by site-directed mutagenesis is the possibility of changes in secondary or tertiary protein structure that could complicate the interpretation of the direct role of the amino acid in TEA binding. Because RGK5 has a titratable His residue in this position, it provided the opportunity to test the effects of ionization of a residue in this microenvironment while minimizing nonspecific structural perturbations. At reduced pH, the inhibition by TEA was much reduced; the absence of this effect in RGK5(H401Y) implies that it results specifically from protonation of His^{401}. The pK of this effect was about 7.1 (Fig. 4*B*). This result is consistent with the structural model that places His^{401} near the external channel mouth where it interacts directly with TEA in a manner which is inhibited by ionization of the imidazole ring.

Potassium channels are thought to be formed by polymerization of four subunits (Tempel *et al.*, 1987; Catterall, 1988). One might ask whether the binding of TEA to a single subunit is sufficient to block ion permeation or whether the binding is stabilized by interactions with more than one subunit. The difference in K_D between RBK2 and RBK2(V381Y) is more than 1000-fold (Fig. 3*A*), equivalent to about 4.2 kcal/mol; this would be about 1 kcal/mol per Tyr if one is contributed by each of four subunits, which is in the range of hydrophobic interactions. This interpretation is in agreement with our previous work in which mixtures of potassium channel subunits were expressed in oocytes (Christie *et al.*, 1990). Currents in oocytes that had been injected with both RBK1 and RBK2 RNAs showed an inhibition by TEA that could not be accounted for by the expression of any proportion of homopolymeric RBK1 and homopolymeric RBK2 channels, indicating that new channels having intermediate sensitivity to TEA must be formed by heteropolymerization. If the binding of TEA by a single RBK2 subunit conferred high sensitivity, then all heteropolymers containing an RBK1 subunit would have been indistinguishable and it would have appeared that the oocyte expressed only a combination of homopolymers of RBK1 and RBK2.

The peptide toxins charbydotoxin (MacKinnon and Miller, 1989) and dendrotoxin (Hurst *et al.*[3]) both block voltage-dependent potassium channels by interacting with amino acids in the S5-S6 region, as do tetrodotoxin (Noda *et al.*, 1990) and α-scorpion toxin (Tejedor and Catterall, 1988) in domain I of the voltage-dependent sodium channels. These toxins are relatively large molecules by comparison with TEA (for example, charbydotoxin is about 2.5 × 1.5 nm: Massefski *et al.*, 1990) and interact with multiple residues of the channel protein (MacKinnon and Miller, 1989; Hurst *et al.*[3]). In the case of a large conductance, calcium-activated potassium channel that is sensitive to both charbydotoxin and TEA, these two blockers appear to compete for the same or an overlapping binding site (Miller, 1988). The binding of the toxin blockers has been shown to involve interaction with several amino acid side chains around the channel mouth, resulting in very high affinity, while the smaller TEA ion appears to interact primarily with the residue identified in the present study.

A report by MacKinnon and Yellen (1990) appeared when this paper was in preparation; they also found that the amino acid in the equivalent position of Shaker H4 was an important contributor to TEA binding and thus concluded that it lay at the external mouth of the channel. Mutations in this region also affected the unit conductance and rectifying properties of the channel.

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Note Added in Proof—Our conclusion that TEA binds by nonionic forces is consistent with a recent report (Dougherty, D. A., and Stauffer, D. A. (1990) *Science* **250,** 1558–1560) that the quaternary ammonium group of acetylecholine binds to aromatic amino acids by a cation-π interaction.

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