

Patterns of Internal and External Tetraethylammonium Block in Four Homologous K⁺ Channels

M. TAGLIALATELA, A. M. J. VANDONGEN, J. A. DREWE, R. H. JOHO, A. M. BROWN, and G. E. KIRSCH

Departments of Molecular Physiology and Biophysics (M.T., A.M.J.V., J.A.D., R.H.J., A.M.B., G.E.K.) and Anesthesiology (G.E.K.), Baylor College of Medicine, Houston, Texas 77030

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SUMMARY

Tetraethylammonium (TEA) is a small ion that is thought to block open K⁺ channels by binding either to an internal or to an external site. For this reason, it has been used to probe the ion conduction pathway or pore of K⁺ channel mutants and a K⁺ channel chimera. The results suggested that the region between transmembrane segments 5 and 6 (S5-S6 linker) was involved in the formation of both the internal and the external TEA binding sites and the K⁺ conduction pathway. Therefore, we compared internal and external TEA block of the currents expressed in *Xenopus* oocytes injected with RNAs from four related K⁺ channel clones, DRK1, RCK1, RCK2, and r-NGK2, which have only subtle structural differences in the S5-S6 linker. r-NGK2 was the most sensitive to external TEA and the least sensitive to internal TEA application. For DRK1 the profile was reversed. RCK1 was blocked equally well from either side, whereas RCK2 was more strongly blocked by internal TEA. The internal block was voltage dependent, whereas the external block was virtually voltage

independent. As predicted from block of whole-oocyte currents, internal TEA produced a slow block of DRK1 and RCK2 single-channel currents but had almost no effect on r-NGK2 single-channel currents. Tetrapentylammonium produced a stronger block than TEA at the internal site, and the block was relieved by inward K⁺ currents, therefore suggesting that the internal TEA binding site is located within the K⁺ conduction pathway. These results, together with the TEA block of single-channel currents, establish what has until now been inferred by extrapolation from other studies, i.e., that TEA is an open-channel blocker in K⁺ channel clones. DRK1 mutants with extensive amino- and carboxyl-terminal deletions showed the same blocking profile as the parent DRK1. We conclude that TEA blocks these K⁺ channels at two sites, which define the inner and outer mouths of the channel pores. Comparison of the primary amino acid sequences in the S5-S6 linker suggests which residues may be responsible for the different patterns of TEA block.

Voltage-dependent K⁺ channels are membrane proteins whose function in nerve and muscle cells is to repolarize the action potential (1) and to modulate electrical excitability (2). Based on differences in gating, voltage-dependent K⁺ channels have been classified as either noninactivating or slowly inactivating DRs or rapidly inactivating transient A-type channels. Since the isolation of SHA from *Drosophila melanogaster* (3-5), other K⁺ channels bearing extensive sequence homology with SHA have been cloned from mammalian tissue and expressed in *Xenopus* oocytes (6-13). Unlike the rapidly inactivating SHA, most of these mammalian channels are of the slowly inactivating DR type.

Although the amino acid sequences of cloned mammalian K⁺ channels show extensive similarities, clear differences exist in

their pharmacological profiles, e.g., their sensitivities to various blockers such as TEA, 4-aminopyridine, and peptide toxins (7, 8, 12-14). With respect to TEA, it is known that this quaternary ammonium ion can block K⁺ conductance in various preparations by binding to two distinct sites, which are accessible either from the extracellular or from the intracellular space (15). We were particularly interested in identifying variants that differ in their sensitivities to TEA, because external (16) and internal (17) TEA binding sites may be part of the ion conduction pathway. The aims of the present study were 1) to compare the sensitivity to internal and external application of TEA among four different cloned mammalian DR K⁺ channels, DRK1 (9), r-NGK2 (10), RCK1 (7, 8), and RCK2 (14, 18); 2) to gain information concerning the biophysical characteristics of TEA interaction with the different channels, which, together with structural information, may provide clues to define functionally important channel regions, and 3) to investigate, in one of the DR channel variants, the possible involvement of the amino and carboxyl termini in modulating TEA binding to the channel.

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ABBREVIATIONS: DR, delayed rectifier; SHA, (Shaker K⁺ channels); TEA, tetraethylammonium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; MES, methanesulfonic acid; TPeA, tetrapentylammonium.

Materials and Methods

Oocyte expression of mammalian DR K⁺ channels. A cDNA encoding DRK1 was isolated from a directional rat brain cDNA library in λ ZAP by expression cloning in *Xenopus* oocytes (9). r-NGK2, RCK1, and RCK2 cDNAs were isolated from rat brain λ ZAP cDNA libraries by screening with a 128-fold degenerate oligonucleotide coding for seven conserved amino acids (NEYFFDR) found in the amino terminus of cloned K⁺ channels. The RCK1 and RCK2 nucleotide sequences are identical to those published. The rat NGK2 clone utilized here has a different carboxyl terminus from the one described by Yokoyama *et al.* (10). The last 10 amino acids of the published sequence (GRKPLRGMSI) are replaced by an 84-amino acid stretch (DSKLN GEVAKAALANEDCPHIDQALTPDEGLPFTRSGTRER-YGPCFLLSTGEYACPPGGGMRKDLCKESPVIKYMPTAEV-RVT). Because our NGK2 was cloned from a rat brain library, whereas the previously described NGK2 clone was from a cDNA library from rat-mouse neuroblastoma-glioma hybrid cells, we will refer to our clone as r-NGK2 (rat-NGK2). The design of the DRK1 deletion mutants Δ C318 and Δ N139 Δ C318 is described elsewhere (26). Deletion mutant Δ C416 was constructed by digesting DRK1 with *Bsm*I (position 2218). The DNA was then digested back to Δ C416 (position +1312) and 659 nucleotides were also removed from the 3' untranslated region with exonuclease III. After digestion with mung bean nuclease, blunt ends were formed with T4 DNA polymerase. The long fragment (4.5 kilobases) was isolated by agarose gel electrophoresis, ligated, and used to transform *Escherichia coli* strain XL1-Blue. The nucleotide sequence at the 3' end of the coding region of Δ C416 was determined. Four hundred-sixteen amino acids were removed from the 3' end of DRK1, and 22 amino acids were derived from the 3' untranslated region (TEKIRRRQICKRDLFKETVCD).

cRNAs were transcribed from linearized cDNAs to obtain run-off transcripts. Stage V–VI *Xenopus* oocytes were injected with 75 nl of 2–100 ng/ μ l cRNA encoding DRK1 or its deletion mutants, r-NGK2, RCK1, or RCK2, in 0.1 M KCl. After the injection, oocytes were kept at 19° in modified Barth's solution.

Electrophysiology. Two to 5 days after the injection, the follicular cell layer was manually removed and oocytes were voltage-clamped using a commercial two-electrode voltage-clamp amplifier (Dagan 8500; Dagan Corp.). The current electrode was filled with 3 M KCl, 10 mM HEPES (pH 7.4), whereas the voltage electrode was filled with 120 mM KOH, 120 mM MES, 2 mM MgCl₂, 10 mM HEPES, 50 mM TEA-Cl (pH 7.2). TEA injection was performed using a pneumatic pressure ejection system (Picospritzer II; General Valve Corp.) connected to the voltage electrode of the two-electrode voltage-clamp. The amount of TEA injected by each pressure pulse was determined by estimating the volume of the fluid droplet expelled with the pipette tip in the air, both before and after the oocyte membrane penetration (20). The amount of solution injected into the oocyte during the experiment never exceeded 2% of the total oocyte volume. The injection of the same volume of TEA-free vehicle did not modify the oocyte-expressed currents. In order to reduce outward Cl[−] currents, the external solution contained 100 mM *N*-methyl-D-glucamine, 100 mM MES, 2.5 mM KOH, 2 mM MgOH, and 10 mM HEPES (pH 7.4), except in the experiments of Figs. 5 and 8 (see the corresponding figure legends). The oocytes were superfused with this solution in the recording chamber at a flow rate of 2 ml/min. The resistance of the current electrode was 1–2 M Ω and that of the voltage electrode varied between 10 and 50 M Ω . The experiments were performed at room temperature (21°). No compensation for the oocyte series resistance was performed. The PClamp system (Axon Instruments) was used for the generation of the voltage pulse protocols and for data acquisition. Linear leakage and capacity currents were corrected on-line using the *P*/4 subtraction method or by using an analog subtraction bridge circuit.

Single-channel recording from inside-out membrane patches was performed in oocytes dissected free of the vitelline envelope and patch-clamped using fire-polished, Sylgard-coated micropipettes of 2–5-M Ω resistance when filled with recording solution. The solution was 120

mM NaCl, 2.5 mM KCl, 2 mM MgCl₂, 10 mM HEPES, pH 7.2. During recording, the resting membrane potential was zeroed with an isotonic KCl depolarizing solution (100 mM KCl, 10 mM EGTA, 10 mM HEPES, pH 7.2). Data were low-pass filtered at either 500 Hz or 1 kHz (−3 dB, four-pole Bessel filter) before digitization at 2 or 4 kHz, respectively. Records were corrected for capacitive and leakage currents by subtraction of the smoothed average of records lacking channel activity.

Drugs. TEA and TPcA were obtained as chloride and bromide salts, respectively, from Sigma Chemical Co. (St. Louis, MO) and Eastman Kodak (Rochester, NY).

Results

Effects of external and internal TEA on four different DR K⁺ channels. *Xenopus* oocytes injected with cRNAs encoding each of the four DR K⁺ channels expressed K⁺ currents with DR properties. Under voltage clamp, depolarizing test potentials produced outward currents that did not inactivate during 400-msec pulses (Fig. 1, *left*). The amplitude of the currents varied between 1 and 4 μ A at the more depolarized test potentials (+50/+70 mV) and were 5 to 20 times higher than outward currents in uninjected oocytes of the same batch (typically 100–200 nA). Endogenous outward currents, which are mainly carried by Cl[−] (21), were insensitive to internal or external TEA application (data not shown). The thresholds for activation of the expressed K⁺ current were different, being −30, −20, −10, and 0 mV for RCK1, RCK2, DRK1, and r-NGK2, respectively. Block by external TEA was complete within 2 min after start of the superfusion and was reversible upon washout. Sensitivity to external TEA differed among the various K⁺ channels (Fig. 1, *middle left*). r-NGK2 showed the highest sensitivity, whereas DRK1 was the most resistant. After washout of external TEA (Fig. 1, *middle right*), the injection of micromolar concentrations of TEA into the oocyte produced a blockade of the K⁺ currents with, again, different sensitivities in the four K⁺ channels (Fig. 1, *right*). Whereas TEA in concentrations ranging between 10 and 300 μ M was equipotent in blocking DRK1, RCK1, and RCK2, concentrations of internal TEA up to 1 mM blocked r-NGK2 by 25% or less. In all cases, the block by internal TEA could not be washed out by superfusion with drug-free solution. However, it is unlikely that this inhibition was due to rundown, because injection of drug-free solutions had no effect in control experiments.

The dose-response relationships for internal and external TEA block of the four K⁺ channels studied at the end of a 350-msec depolarizing pulse at +50 mV are shown in Fig. 2. DRK1 and RCK2 were blocked weakly by external TEA, whereas r-NGK2 and RCK1 were more effectively blocked. On the other hand, DRK1, RCK1, and RCK2 were all blocked with the same potency by internal TEA, whereas r-NGK2 was at least 30 times less sensitive than the other K⁺ channel clones.

The IC₅₀ values for the blocking effects of external and internal TEA in the four K⁺ channels, calculated according to a model that assumes a one-to-one TEA-to-channel binding scheme (12, 22) and without subtraction of the contribution of endogenous outward currents, are reported in Table 1.

TEA blockage of single-channel currents. When applied to the cytoplasmic surface of excised inside-out membrane patches, TEA block varied among different K⁺ channel subtypes. Fig. 3 shows control single-channel records from oocytes injected with a single species of RNA encoding either DRK1 (Fig. 3A), RCK2 (Fig. 3C), or r-NGK2 (Fig. 3E). The three channel types are easily distinguished by the amplitude of their

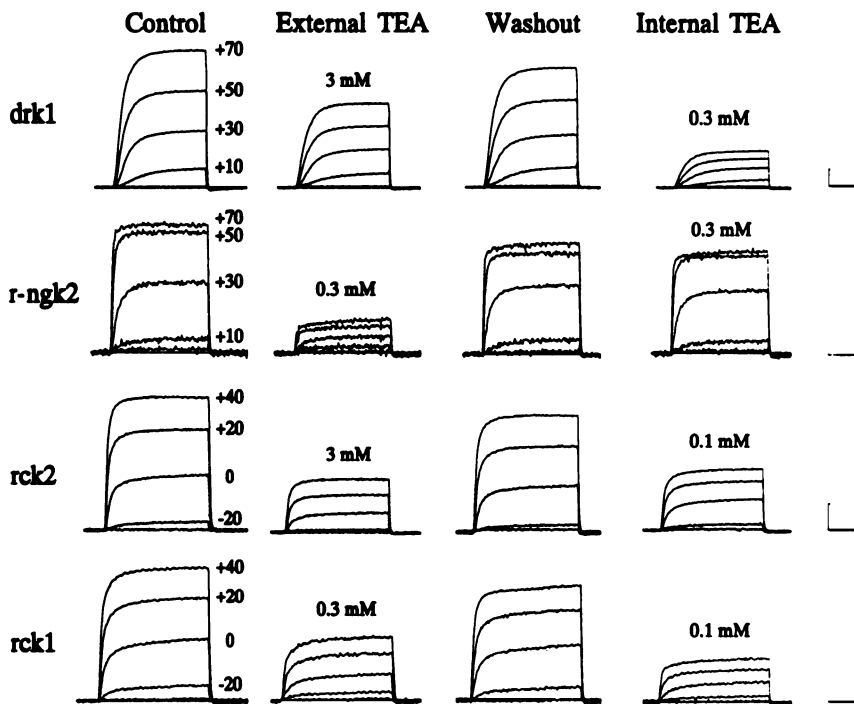


Fig. 1. Different sensitivities to external and internal TEA block in mammalian DR K⁺ channels. A family of currents elicited by a 350-msec depolarizing pulse to the potentials indicated from voltage-clamped oocytes injected with cRNAs encoding DRK1, r-NGK2, RCK2, and RCK1, from top to bottom, respectively. Representative current traces in the following order were obtained from the same oocyte: control, external TEA, washout of external TEA, and internal TEA (from left to right, respectively). Holding potential was -80 mV. Linear capacitive and leakage currents were subtracted by the *P/4* method. Scale bars, 500 nA and 100 msec in DRK1, RCK2, and RCK1; 100 nA and 100 msec in r-NGK2.

single-channel currents. r-NGK2 currents were 3 times larger than those of DRK1 at a test potential of 0 mV, whereas RCK2 currents were of intermediate amplitude. The channel types also are distinguishable on the basis of TEA sensitivity. As shown in Fig. 3B, DRK1 showed a "slow block" in response to 1 mM internal TEA. Long openings under control conditions (Fig. 3A) were converted to bursts of rapid open and blocked intervals, which may represent the repetitive association (on) and dissociation (off) reactions of the drug with the open channel (Fig. 3B). A qualitatively similar effect was apparent with RCK2 (Fig. 3D), where the on rate was faster than the frequency response of the recording system (1 kHz), resulting in a truncation of the amplitude of single-channel events. r-NGK2 (Fig. 3F), in contrast, was nearly unaffected by 1 mM TEA. A reversible 6% reduction in mean amplitude at a test potential of 0 mV was obtained in three patches. As shown in Fig. 3F, 1 mM TEA did not increase open-channel noise (in the frequency range of <1 kHz), consistent with fast block rates. It should be emphasized that, under the recording conditions utilized in the present experiments (filtering at 1 kHz, sampling at 2–4 kHz), we could have missed only very short-lived (<300 μ sec) closures and openings introduced by TEA.

Voltage dependence of the block by external or internal TEA. The effect of membrane potential on the block by external and internal TEA is shown by plotting of IC_{50} for blockade of the steady state K⁺ current as a function of pulse potential (Fig. 4). The IC_{50} for external TEA (Fig. 4A) did not show any dependence on membrane potential for r-NGK2 and RCK1, whereas for RCK2 a small degree of voltage dependence was found. In this K⁺ channel, the IC_{50} increased at more depolarized membrane potentials. This finding can be interpreted by assuming that, in RCK2, the site of the channel at which TEA binds is within the membrane electric field. The fraction of membrane field that is experienced by the TEA binding site can be determined according to the Woodhull model (23), using the following equation:

$$K_d = K_d(0) \exp(z\delta F/RTV)$$

where $K_d(0)$ is the dissociation constant at 0 membrane potential, z is the blocking ion valence (+1), δ is the fraction of the membrane voltage experienced by the blocking ion, V is the membrane voltage, and R , T , and F have their usual meanings. In our experiments, IC_{50} was substituted for K_d in the Woodhull model. Because the model itself is valid only when all the channels are open, we restricted our analysis to the range of test potentials that maximally activated K⁺ conductance. The values obtained from the linear regression analysis of the points in Fig. 4A show that membrane depolarization caused an e -fold increase in the IC_{50} for a 213-mV change in voltage for RCK2, which corresponds to a δ value of 0.11 ± 0.03 ($n = 4$); δ values for r-NGK2 and RCK1 were 0.02 ± 0.01 ($n = 3$) and 0.01 ± 0.01 ($n = 3$), respectively. The electrical distance would, therefore, correspond to about 10% from the outer margin of the membrane electric field in RCK2 and to 1 and 2% in RCK1 and r-NGK2, respectively. The same analysis was not performed on DRK1, because the poor block obtained within the concentration range of external TEA shown in Fig. 2A prevented accurate determination of the IC_{50} .

In contrast to the external TEA binding site, the internal TEA binding site showed voltage dependence in DRK1, RCK1, and RCK2 (Fig. 4B). The voltage dependence was in the expected direction for an internal blocking cation, with the IC_{50} becoming smaller at more positive test potentials. Membrane depolarization caused an e -fold decrease in the IC_{50} for 128 ± 17 ($n = 4$), 167 ± 32 ($n = 4$), and 178 ± 60 mV ($n = 4$), with corresponding values of δ of 0.23 ± 0.02 , 0.19 ± 0.02 , and 0.16 ± 0.03 for DRK1, RCK1 and RCK2, respectively. The electrical distance would, therefore, correspond to about 20% of the membrane electric field from its inner margin. This value closely matches the δ value (0.25) obtained from single-channel measurements of the voltage dependence of the TEA on and off rates in RCK2 inside-out patches.¹ The values for r-NGK2

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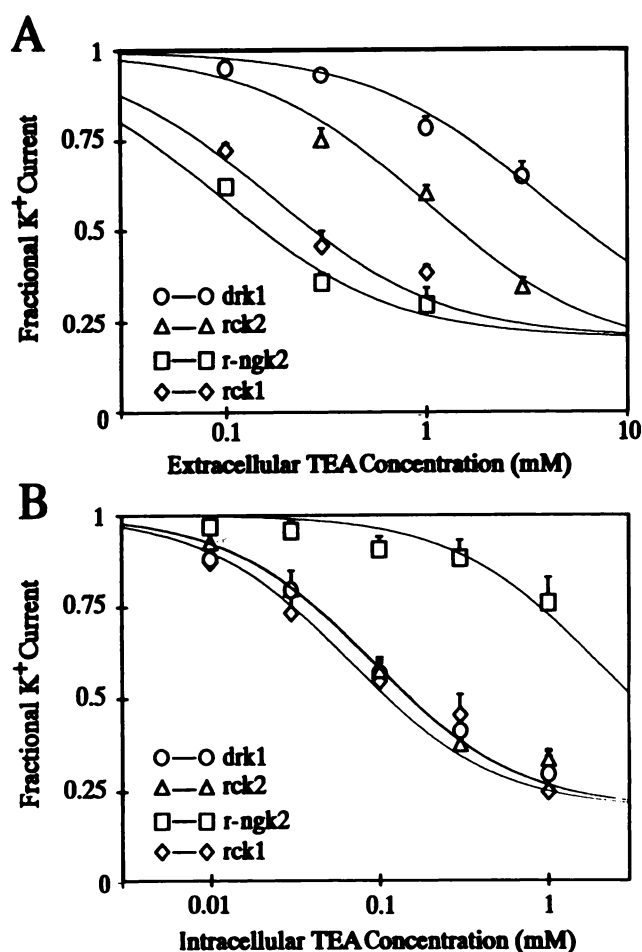


Fig. 2. Dose-response curve for the inhibition of DR K⁺ channels by external and internal TEA. The inhibition of the K⁺ current by external (A) and internal (B) TEA is expressed as fractional K⁺ current, which was calculated as $I_{TEA}/I_{control}$, both measured at the end of a 350-msec test pulse to +50 mV. Solid line, best fit of the experimental points for a one-to-one drug-to-receptor binding scheme. The contribution of endogenous outward currents was not subtracted in the calculation. Each value represents the mean \pm standard error of five to eight determinations, each obtained in different oocytes of at least three different batches.

TABLE 1
IC₅₀ for internal and external TEA on DR K⁺ channel clones

DR clone	IC ₅₀ , external TEA mM	n
DRK1	5.5 \pm 1.3	5
RCK1	0.26 \pm 0.06	5
RCK2	1.5 \pm 0.18	5
r-NGK2	0.18 \pm 0.02	5
DR clone	IC ₅₀ , internal TEA mM	n
DRK1	0.16 \pm 0.04	7
RCK1	0.18 \pm 0.05	5
RCK2	0.15 \pm 0.02	6
r-NGK2	4.224 \pm 1.9	5

are not reported due to the poor ability of internal TEA to inhibit this channel, thus preventing accurate determinations of the IC₅₀.

Effects of internal TEA on the DRK1 current-voltage relationship in a high external K⁺ solution. A strong test for occlusion of the K⁺ channel pore by a blocking ion at an

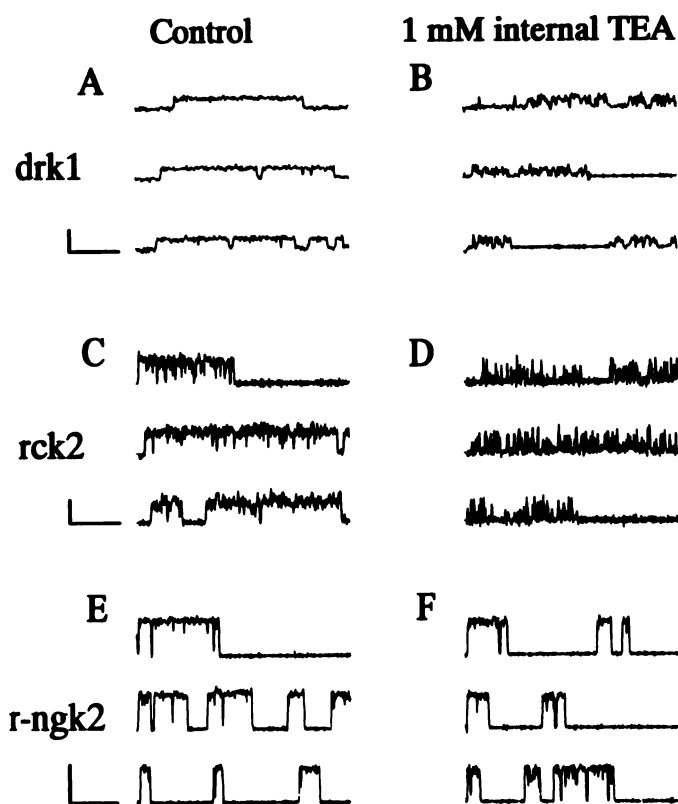


Fig. 3. Effects of internal TEA on single-channel inside-out patches from *Xenopus* oocytes injected with cRNAs encoding for DRK1, RCK2, and r-NGK2. Typical single-channel records from excised inside-out patches from oocytes injected with RNA transcribed from DRK1 (A and B), RCK2 (C and D), or r-NGK2 (E and F) K⁺ channel DNA clones. Test potential, 0 mV; holding potential, -80 to -100 mV. Records in A and B were filtered at 500 Hz; records in C to F were filtered at 1 kHz. A to D are at the same gain but E and F are at reduced gain because of the differences in single-channel amplitudes. Scale bar, 1 pA and 50 msec (A-D) and 2 pA and 50 msec (E-F).

internal site is whether external (*trans*) K⁺ can relieve the block (24). In high external K⁺, internal application of TEA in squid giant axon blocked outward K⁺ currents more effectively than inward K⁺ currents (19, 25). We examined whether DRK1 channels exhibited similar behavior. For this purpose, we measured DRK1 tail currents elicited upon repolarization to different test potentials after a 300-msec conditioning pulse to +30 mV from a holding potential of -80 mV, in a solution containing 100 mM K⁺, both in the absence and in the presence of 300 μ M internal TEA. The injection of TEA into the oocyte caused an equivalent inhibition of both inward and outward K⁺ currents between +30 and -100 mV, when the current values were extrapolated at the beginning of the tails fitted to a single exponential function (instantaneous *I/V*) (Fig. 5A). However, the absence of any rectification produced by internal TEA in the instantaneous *I/V* could be due to the relatively slow time course of the relief from TEA block by inward K⁺ currents. Such an effect should, therefore, be manifested during the time course of the tail deactivation, resulting in a prolongation of the deactivation of the channel (19). Fig. 5B shows a comparison of the deactivation time constants, calculated by fitting the tails to a single exponential function, in the absence and presence of a blocking concentration (300 μ M) of internal TEA. The data clearly show that, in the presence of internal TEA, the time course of the inward K⁺ currents is consistently slowed

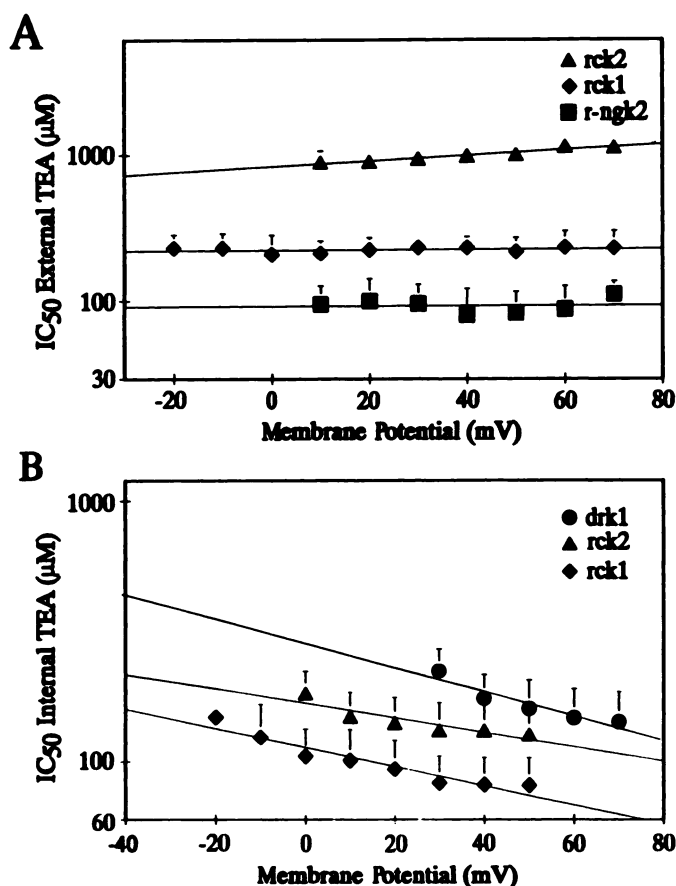


Fig. 4. Voltage dependence of external and internal TEA block of DR K⁺ channels. The IC₅₀ for the inhibition of different K⁺ channels by external (A) and internal (B) TEA at different potentials, calculated according to a model that assumes a single binding site for external or internal TEA, was plotted as a function of membrane potential. Each value represents the mean \pm standard error of four determinations. Solid line, linear regression analysis of the data.

down. This result can be interpreted as a consequence of the removal of internal TEA from its binding site by inward K⁺ currents, suggesting that the site itself may be located within the K⁺ conduction pathway. However, it should also be noted that in the present experiments we cannot exclude the possibility that the prolongation of the tails observed in the presence of internal TEA can arise not only by a relief from block but also from an interaction of TEA with the gating mechanisms controlling the deactivation of the channel.

Effects of internal TEA on DRK1 deletion mutants. Three deletion mutants of DRK1 were used to investigate the possible involvement of the putative intracellular amino- and carboxyl-terminal domains in the ability of internal TEA to inhibit this channel (Fig. 6A). K⁺ channels expressed in oocytes upon injection of cRNA encoding a deletion mutant lacking 318 amino acids at the carboxyl terminus (Δ C318) (26), which lacks most of the carboxyl-terminal tail of DRK1, or of cRNA encoding a DRK1 channel molecule, which lacks a major part of both its amino and carboxyl termini (Δ N139 Δ C318), were inhibited by internal TEA with the same affinity as wild-type DRK1 (Fig. 6B). Furthermore, the same effectiveness of internal TEA was found in a deletion mutant lacking the carboxyl terminus almost completely (Δ C416), which differs from the other deletion mutants because it is missing a region close to

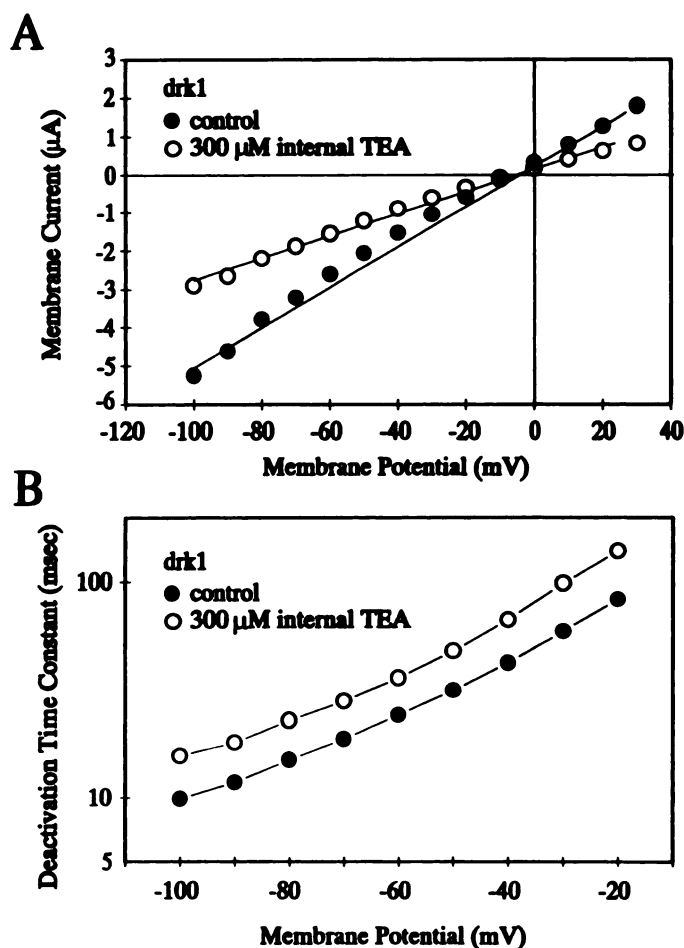


Fig. 5. Effect of TEA on DRK1 current-voltage relationship in high K⁺ solution. Oocytes expressing DRK1 were bathed in a solution containing 100 mM K⁺ ions in order to shift the K⁺ reversal potential toward more positive values and to obtain information on the effects of internal TEA on inward and outward K⁺ currents. Tail currents were studied by a protocol consisting of a standard 300-msec depolarizing pulse to +30 mV, followed by a 500-msec hyperpolarizing test pulse to different potentials (from +30 to -100). A, Instantaneous I/V of DRK1 channels obtained in the absence and in the presence of 300 μ M internal TEA. The values were obtained by extrapolating the time point at the beginning of the tails, by fitting the tail current at different potentials to a single exponential function. B, Time constants of this exponential function. Data from a single oocyte are reported (the experiment was repeated in three more oocytes, with similar results).

the sixth putative transmembrane domain (S6) that is particularly rich in negative charges in the parent channel DRK1 (9). The IC₅₀ values (in μ M) for the internal TEA-induced inhibition of the current expressed by the DRK1 deletion mutants were 181 ± 18 ($n = 4$), 192 ± 22 ($n = 6$), and 111 ± 47 ($n = 4$) for Δ C318, Δ N139 Δ C318, and Δ C416, respectively. These values were not significantly different from the IC₅₀ of the wild-type channel DRK1 (165 ± 43 ; $n = 7$) (Table 1). More extensive deletions at the amino terminus (Δ N174) or at the carboxyl terminus (Δ C444) failed to produce functional DR K⁺ channels.

Time-dependent block of DRK1 by TPpA. Fig. 7 shows the effects of superfusion of oocytes expressing DRK1 channels with a solution containing the lipid-soluble TEA derivative TPpA. In the control current trace, DRK1 did not inactivate during the 350-msec test pulse, nor did internal or external TEA block produce any changes in inactivation (see Fig. 1),

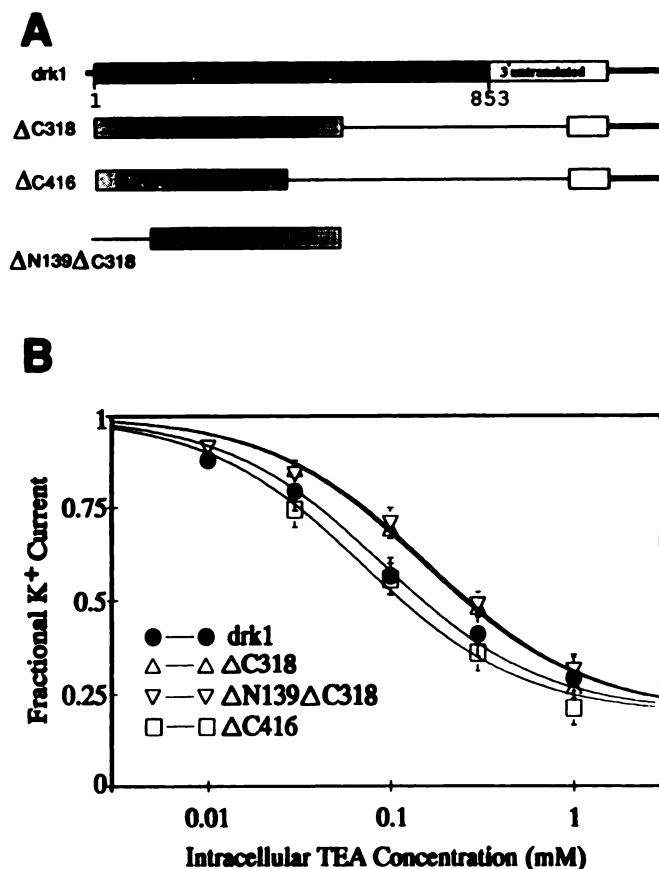


Fig. 6. DRK1 and deletion mutants show the same sensitivity to internal TEA. **A**, Coding and untranslated region of DRK1 and of the deletion variants studied. The coding region is shaded and the six (S1–S6) putative transmembrane segments are shown in black. The DRK1 mutants have the deletion indicated by a thin line. **B**, Fractional K^+ current expressed in oocytes injected with runoff transcripts of DRK1 and its deletion mutants, plotted as a function of internal TEA concentration. Solid line, best fit of the experimental points for a one-to-one drug-to-receptor binding scheme. Each value is the mean \pm standard error of four to 10 determinations, obtained in oocytes of at least two different batches.

although in the presence of high concentrations of internal TEA activation and deactivation were slowed (see Fig. 5B). When superfused in the presence of TPeA, the current, after a few tens of milliseconds, gradually declined during maintained depolarization (Fig. 7A), showing an apparent inactivation. This effect resembles the observations made by Armstrong (24) and by French and Shoukimas (27) on the interaction of quaternary ammonium ion derivatives of various chain lengths with the DR of the squid giant axon. However, the data of Armstrong (24) and of French and Shoukimas (27) were obtained by internal perfusion of the axon with these compounds, whereas smaller effects were detected when long-chain TEA derivatives were superfused externally (24). The observation that, in the squid axon, TPeA does not act on an internal site when applied externally is not surprising, because it is known that Schwann cells surrounding the squid axon act as a diffusion barrier. However, it should be noted that, also in the experiments of Armstrong (24), prolonged exposure to lipid-soluble TEA derivatives applied externally caused a detectable inactivation of the K^+ current. Therefore, it is likely that TPeA can diffuse across the oocyte plasma membrane and act, with high affinity, on the internal site of the DR channels. This

view is supported by the fact that, in DRK1, the effect of TPeA on the steady state current at the end of the depolarizing pulse shows a marked voltage dependence (Fig. 6B), which resembles the effect of internal injection of TEA in the same DR channel, in the sense that the IC_{50} showed a decrease with membrane depolarization. Because the IC_{50} for TPeA showed an e -fold change for 79 ± 9 mV, the fraction of membrane electric field experienced by TPeA, δ , corresponded to 0.3 ± 0.02 ($n = 4$). The onset of the effect of TPeA was much slower (Fig. 7, C and D) than that of external TEA (Fig. 7E). This, together with the observation that no recovery of the blocked current occurred upon washout of the drug, is consistent with an internal site of action. The slow onset of the blocking effects of TPeA, together with a progressively increasing block during the experiment, presumably due to drug diffusion and accumulation into the oocyte, prevented accurate determination of the IC_{50} . Although it is clear that TPeA was more potent than TEA, it is likely that the reported values of the IC_{50} for TPeA are probably overestimated. We were unable to reproduce this effect by internal injection of TPeA into the oocytes, probably due to an accumulation of the lipophilic drug into the abundant cytoplasmic granules.

Clearance of TPeA from its internal binding site by external K^+ . The results shown in Fig. 5 suggest that inward K^+ currents can relieve the internal block by TEA. In the case of TPeA, we obtained much better evidence for this hypothesis by comparing the rates of recovery from TPeA-induced inactivation when the principal extracellular cation was K^+ or the less permeable Na^+ . The experiments were performed with a DRK1 deletion mutant ($\Delta C416$) because this K^+ channel clone, in contrast to the parent DRK1 channel (26), lacks voltage-dependent inactivation, thus simplifying the study of drug-induced inactivation. Fig. 8 shows that the rate of recovery from TPeA-induced inactivation was very slow in external Na^+ at -70 mV and was very much faster in external K^+ . With a 4-sec period between conditioning and test pulses, there was almost no recovery of the test pulse currents in Na^+ but complete recovery in external K^+ . After a period of 15 sec between pairs of pulses used in these experiments, there was incomplete recovery of the conditioning pulse in Na^+ , whereas recovery was complete in 120 mM K^+ solution. In 120 mM Na^+ solution, the block was relieved faster when the membrane potential during recovery was increased from -70 to -140 mV. As shown in Fig. 8C, the time course for the recovery from block was monophasic. Mean values for the recovery time constants in 120 mM Na^+ solution were 7.1 ± 1.3 sec ($n = 4$) and 2.6 ± 0.4 sec ($n = 3$), at recovery potentials of -70 and -140 mV, respectively. The time constant in 120 mM K^+ solution at -70 mV averaged 0.9 ± 0.2 sec ($n = 3$).

Discussion

The results presented here suggest that DR-type K^+ channels from mammalian brain have two different sites for TEA binding, one that is located at the external surface of the channel protein and another that is only accessible from the cytoplasmic side of the membrane.

The internal and external sites show marked differences in their affinities for TEA. Because the TEA molecule has approximately the same size as a hydrated K^+ ion, it is generally believed that TEA blocks the channel by binding to a site in the mouth of the channel (24). Because TEA is too large to

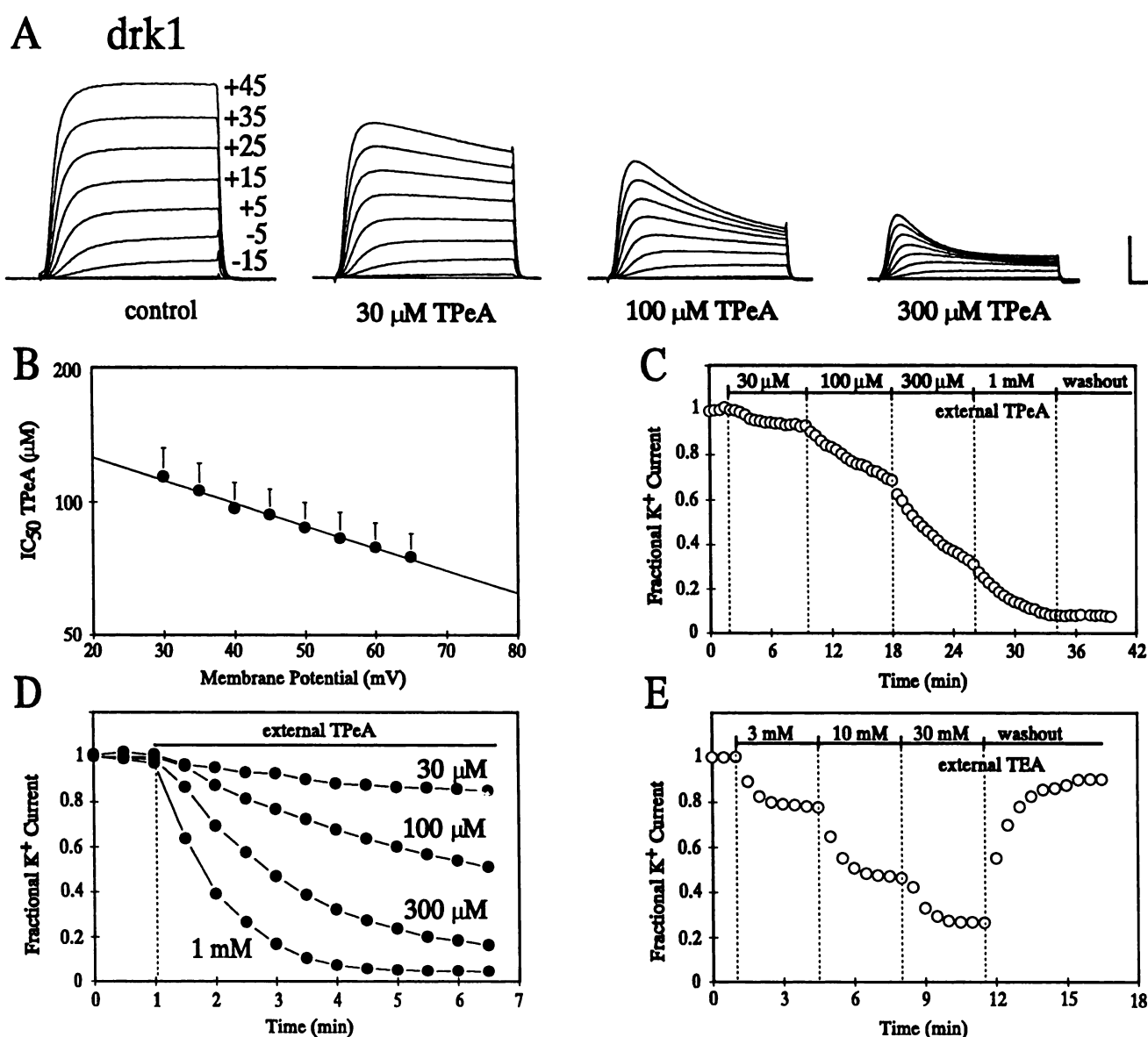


Fig. 7. Effect of the quaternary ammonium ion derivative TPcA on DRK1. TPcA was added to the medium superfusing oocytes injected with DRK1 cRNA. **A**, Current traces 3 min after exposure to the indicated concentrations of TPcA. Each current trace was produced in response to membrane depolarization to potentials indicated to the right of the control curve. Scale bar, 1 μ A and 100 msec. **B**, IC₅₀ values for inhibition of the current at the different potentials (as described in Results and in the legend to Fig. 3). **D**, Effects of superfusion of a DRK1-expressing oocyte with the indicated concentrations of TPcA on the steady state current at the end of a depolarizing pulse to +20 mV repeated each 30 sec. **C**, Same experiment repeated, but in this case the same oocyte was exposed to increasing concentrations of TPcA. **E**, For comparison, the effect of external TEA on DRK1, using the same experimental protocol.

move into deeper regions of the channel structure, it prevents K⁺ ion flux along the conduction pathway. The location of the TEA binding site in the membrane electric field can be inferred from the voltage dependence of block. Although almost no voltage dependence was found for the external TEA block, a clear voltage dependence is associated with the internal TEA binding site. The existence of differences in the voltage dependence of the external TEA site among the different channels is not surprising. External TEA causes a voltage-independent block of DR in frog node of Ranvier (28, 29), of ATP-sensitive K⁺ channels of frog skeletal muscle (22), and of other cloned DR K⁺ channels expressed in *Xenopus* oocytes (30). However, DR channels from frog skeletal muscle (31), as well as Ca²⁺-dependent K⁺ channels from pituitary GH₃ cells (32), were

blocked by TEA in a voltage-dependent manner, although in both cases the TEA binding site sensed <20% of the membrane electric field. In contrast, the internal TEA binding site has a strong voltage dependence in the DR of the squid giant axon (27) (this channel has no external site), in frog node of Ranvier (28), and in the ATP-sensitive K⁺ channel (22). Furthermore, the internal TEA binding site in DRK1 shows some similarities to the site in squid axon, with respect to the existence of a hydrophobic region in the vicinity of the TEA binding site. This hydrophobic region is thought to be responsible for the increase in potency shown by lipophilic derivatives of TEA, e.g., TPcA. A decrease in the dissociation rate constant may explain the apparent inactivation of the current elicited by the depolarizing step observed in the presence of this compound.¹

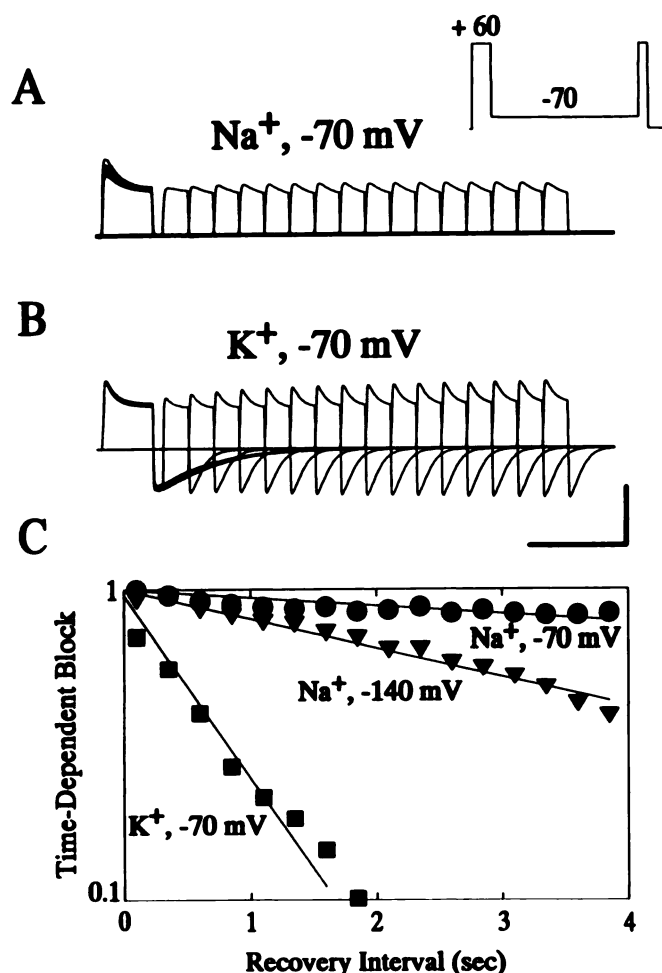


Fig. 8. Time course of recovery from TPeA block. Oocytes injected with cRNAs encoding for the DRK1 deletion mutant $\Delta C416$ were exposed, under voltage clamp, to 0.5 mM TPeA for 8 min to allow TPeA to accumulate intracellularly and were then superfused continuously with drug-free solution containing either 120 mM Na^+ or 120 mM K^+ . The time course of recovery from TPeA block was monitored using the double-pulse protocol shown in the *inset*, consisting of a 500-msec conditioning pulse to +60 mV and a variable recovery period at a more negative potential, terminated by a 250-msec test pulse to +60 mV. The holding potential between trials was -90 mV. The protocol was repeated with different recovery periods, at intervals of 15 sec. The envelope of the superimposed peak test currents traces the recovery from the time-dependent block developed during the conditioning pulse, such that the briefest recovery interval gave the smallest test pulse current. **A**, Set of currents evoked by paired conditioning and test pulses during exposure to 120 mM Na^+ solution, with recovery potential at -70 mV. **B**, Same experiment performed in 120 mM K^+ solution. Vertical and horizontal calibration bars, $1 \mu\text{A}$ and 1 sec, respectively. **C**, Time course of recovery from block, plotted semilogarithmically as a function of the length of the recovery interval between conditioning and test pulses. Time-dependent block = $1 - (I_p - I_r)/(I_{\text{max}} - I_r)$, where I_p = peak test pulse current, I_r = residual current at the end of the conditioning pulse, and I_{max} = maximum conditioning pulse current. Solid lines, linear regression fits to the data.

In our experiments, inward K^+ currents appear to relieve internal TEA and TPeA block. The results are, therefore, consistent with the traditional interpretation of open-channel block. Furthermore, single-channel studies on these cloned K^+ channels are also consistent with TEA blockade of open channels.¹

Among the mammalian DR channels studied, the affinities for internal and external TEA may differ by 40-fold. This suggests the existence of a different molecular architecture on

or around these sites. Alignment of the amino acid sequences of these K^+ channels shows that the core region, including the six putative transmembrane domains (S1–S6), is well conserved, in contrast to the putative cytoplasmic amino and carboxyl termini, which show a far more pronounced divergence. In particular, the putative intracellular carboxyl terminus region flanking the sixth transmembrane domain (S6) displays a high density of negative charges in DRK1, RCK1, and RCK2. Most of these charges are missing in the sequence of r-NGK2. However, the fact that in DRK1 the removal of the amino and of the carboxyl termini did not modify the sensitivity of the channel to internal TEA supports the idea that the internal TEA binding site is located within the core region of DRK1, extending from amino acid 139 to amino acid 437 and including all six putative transmembrane domains (about one third of the complete sequence of 853 amino acids).

Additional support for a core location of the TEA binding sites came from experiments in which a short segment of 21 amino acids in the region corresponding to the S5–S6 linker (33) was transferred from the donor r-NGK2 clone to host DRK1 clone (17). The resulting chimeric DRK/NGK clone showed the single-channel conductance and profile for internal and external TEA block of the donor r-NGK2. The corresponding region in the related SHA K^+ channel clone has been altered by point mutations, which have defined residues influencing external TEA binding (D431 and T449) (16) and produced large changes in ion selectivity (F433 and T441) (34). Further insights on the location of the TEA binding sites may come from a comparison of the primary sequences of DRK1, r-NGK2, and RCK2 (Fig. 9). The K at 382 of DRK1, close to position T449 in SHA, which is known to influence external TEA binding (16), could repulse TEA, and this might explain the relative low affinity of the external TEA block in this clone. The M aligned to this position in RCK2 may be too bulky, but, being uncharged, might contribute to the intermediate affinity for external TEA block observed in this channel. The Q of r-NGK2 or the V of RCK1 at this position may allow high affinity TEA binding. As far as the internal TEA site is concerned, the presence of an L in r-NGK2 at position 400, aligned with V at positions 374 in DRK1, 373 in RCK1, and 424 in RCK2, could

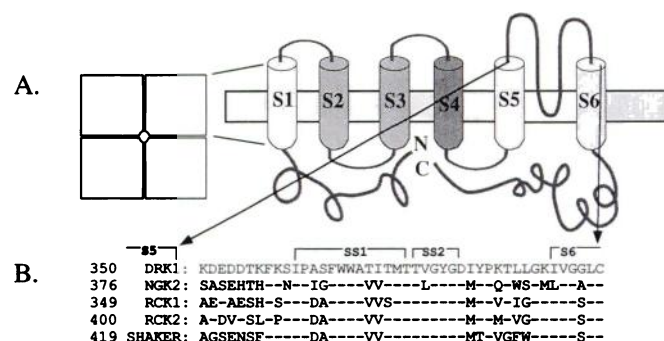


Fig. 9. Schematic drawing of the putative structure of K^+ channels and alignment of the amino acid sequences in the S5–S6 linker of DRK1, r-NGK2, RCK1, RCK2, and SHA. **A**, Model of the topography of a voltage-gated K^+ channel. Left, four identical repeats arranged about a central pore; right, a single repeat with its six putative transmembrane segments. The linker between S5 and S6 is thought to make a hairpin within the membrane (33). **B**, Comparison among DRK1, r-NGK2, RCK1, RCK2, and SHA channels of the amino acid sequences in the S5–S6 linker. Dashes, residues identical to DRK1. Numbers on the left, first residue in each of the aligned sequences.

be responsible for the relative low affinity of the internal TEA site in this clone. It should be emphasized that recent experiments performed in SHA have shown that a single point mutation (T to S at position 441) causes a 10-fold reduction in the potency of internal TEA in blocking the channel (35). However, this position is strictly conserved among the four clones utilized in the present investigation and, therefore, cannot be solely responsible for the differences in internal TEA block observed between r-NGK2 and the other channels. Further mutagenesis experiments are, therefore, required to identify the exact amino acid residues that might contribute to the formation of the internal and external TEA binding sites in these channels.

In conclusion, the results of the present study show that, 1) not only do different DR K⁺ channels exhibit different TEA sensitivities, but the profile for the sensitivity to external application (r-NGK2 \approx RCK1 > RCK2 > DRK1) is different from that for internal application (DRK1 = RCK1 = RCK2 \gg r-NGK2); 2) internal TEA is highly effective in blocking single-channel currents in DRK1 and RCK2, and far less effective in r-NGK2; 3) block at the internal site is clearly voltage dependent, whereas block at the external site is virtually voltage independent; 4) the internal TEA binding site is located within the K⁺ conduction pathway, because the block by TEA and the TEA derivative TPcA appears to be relieved by inward K⁺ current; and, 5) in DRK1, deletion of 318 amino acids at the carboxyl terminus, alone or in combination with a 139-amino acid deletion at the amino terminus, did not cause any significant alteration in the ability of internally applied TEA to inhibit the channel. No effect on DRK1 block by internal TEA was observed when a more extensive deletion (416 amino acids) at the carboxyl terminus was tested.

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Send reprint requests to: M. Taglialatela, Department of Molecular Physiology and Biophysics, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030.