KCNKØ: Single, Cloned Potassium Leak Channels Are Multi-Ion Pores

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ABSTRACT KCNKØ was the first clone to show attributes of a leak conductance: voltage-independent potassium currents that develop without delay. Its novel product is predicted to have two nonidentical P domains and four transmembrane segments and to assemble in pairs. Here, the mechanistic basis for leak is examined at the single-channel level. KCNKØ channels open at all voltages in bursts that last for minutes with open probability close to 1. The channels also enter a minutes-long closed state in a tightly regulated fashion. KCNKØ has a common relative permeability series (Eisenman type IV) but conducts only thallium and potassium readily. KCNKØ exhibits concentration-dependent unitary conductance, anomalous mole fraction behavior, and pore occlusion by barium. These observations argue for ion-channel and ion-ion interactions in a multi-ion pore and deny the operation of independence or constant-field current formulations. Despite their unique function and structure, leakage channels are observed to operate like classical potassium channels formed with one-P-domain subunits.

INTRODUCTION

Potassium-selective leak currents appear to be key to excitable membrane function (Goldman, 1943; Hodgkin and Katz, 1949; Hodgkin et al., 1952; Hille, 1975; Adams et al., 1980) but have resisted coherent description. Also called background conductances, their activity across the physiological voltage range explains their broad influence; leak currents mediate resting membrane potential (like inwardly rectifying potassium channels) and alter action potential height and duration (Siegelbaum et al., 1982; Baker et al., 1987; Koyano et al., 1992; Shen et al., 1992; Backx and Marban, 1993; Buckler, 1997). Despite assignment to these important roles in membrane physiology, it remained a matter of controversy whether leak currents were carried by unique molecular entities or simply accumulated from seepage through other transport pathways. The challenge to characterization in native cells was inherent; leak was naturally obscured by voltage and ligand-gated channels or subtracted to advance studies of time-dependent currents. Evaluations of native preparations failed even to reach consensus regarding the ionic basis of leak (Jack, 1976; Baker et al., 1987).

KCNKØ (previously *ORK1*) of *Drosophila melanogaster* nerves and muscles was the first channel clone to display characteristics expected for a leak conductance, behaving like an open, potassium-selective, transmembrane hole (Goldstein et al., 1996). Like leak in native cells, whole-cell KCNKØ currents developed without apparent delay in response to voltage steps and were well described as free electrodiffusion by the constant-field current equation (Goldman, 1943; Hodgkin and Katz, 1949). KCNKØ was thus an open rectifier showing a linear current-voltage relationship in symmetric solutions and predominantly outward currents under physiological conditions (Goldstein et al., 1996). Although electrodiffusion theory assumes ions move independently of interactions with other ions or a conduction pathway, real ion channels fail to obey these predictions (Hodgkin et al., 1952; French and Adelman, 1976). Thus, classical potassium channels demonstrate ion-ion and ion-pore interactions in a permeation pathway holding multiple ions simultaneously (Hille and Schwarz, 1978; Yellen, 1984; Neyton and Miller, 1988a; Heginbotham and MacKinnon, 1993; Lu and MacKinnon, 1994; Doyle et al., 1998).

Potassium channel subunits with a two-P-domain, four-transmembrane-segment (2P/4TM) predicted topology were initially recognized in the genome of a nematode (Ketchum et al., 1995; Wei et al., 1996). The first functional example, *KCNKØ*, now belongs to a large collection of 2P/4TM channel genes (Goldstein et al., 1998) designated the *KCNK* family (and KCNK proteins) by the Human Genome Organization (HUGO). Thus far, all *KCNK* genes that function in experimental cells are potassium-selective leak currents (*KCNK2*, *-3*, *-4*, *-5*, and *-9*) (Fink et al., 1998; Goldstein et al., 1998; Reyes et al., 1998; Kim et al., 2000; Lopes et al., 2000); other *KCNK* genes have yet to display reproducible activity although their message is found in native cells (*KCNK1*, *-6*, *-7* and *-8*) (Goldstein et al., 1998; Chavez et al., 1999; Pountney et al., 1999; Salinas et al., 1999; Bockenhauer et al., 2001).

Because of their unique behavior (leak), novel subunit organization (two P domains), large number (50+ genes), and wide expression in mammalian tissues (apparently all), we sought to characterize the conductance behavior of KCNKØ at the single-channel level. This study shows that despite its apparent bilateral symmetry and variation at sites important to pore formation in other potassium channels

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(Doyle et al., 1998; Roux and MacKinnon, 1999) ion permeation proceeds by similar mechanisms in KCNKØ and classical potassium channels formed by tetrameric assembly of one-P-domain subunits.

MATERIALS AND METHODS

Molecular biology

The 619-amino-acid isolate of *KCNKØ* used in this work and described (Goldstein et al., 1996) is now understood to lack residues 620-1001 of the wild-type protein expressed in native fly tissues (Goldstein et al., 1999). As considered in another report (Zilberberg et al., 2000), the open-channel attributes of the truncated variant are indistinguishable from those of the full-length channel; conversely, truncation alters the frequency and duration of the long-lasting closed state and diminishes sensitivity to regulation, and this was the advantage of using the truncated variant in this study on the open state. cRNAs were transcribed using T7 RNA polymerase and the mMessage mMachine kit (Ambion, Austin, TX). Transcripts were quantified using a spectrophotometer and by comparison with control samples separated by agarose gel electrophoresis and stained with ethidium bromide.

Electrophysiology

Xenopus laevis oocytes were isolated and injected with 46 nl of solution containing 0.2 or 2 ng of cRNA (for whole-cell or patch studies, respectively) as described (Goldstein et al., 1996). To extend the life of oocytes after cRNA injection incubation buffers contained (in mM): 83 KCl, 10 NaCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES, pH 7.5 with NaOH with penicillin/streptomycin (100 U/ml) and gentamycin (10 μg/ml). Whole-cell currents were measured 1–4 days after cRNA injection (on-cell patches 2–4 days later). For two electrode recordings the amplifier was an Oocyte Clamp from Warner Instruments Corp. (Hamden, CT), and data were filtered at 1 kHz and sampled at 4 kHz. For patch-clamp, the amplifier was an EPC-9 amplifier (HEKA Elektronik, Lambrecht, Germany), and data were stored on videocassettes. For off-line analysis, patch records were sampled at 50 kHz with ACQUIRE software (Bruxton Corp., Seattle, WA) and digitally filtered at 1 kHz or as indicated. Kinetic analyses were performed on patches judged to contain only one channel on the basis of the single-current level. Closed- and open-time durations were determined using half-amplitude threshold detection (Colquhoun and Sigworth, 1995) and TAC single-channel analysis software (Bruxton Corp.). Dwell-time distributions were plotted on a logarithmic time axis and a square-root vertical axis to best discern event populations (Sigworth and Sine, 1987). Dwell-time histograms were fitted with TacFit software (Bruxton Corp.) using a sums-of-exponential-probability density function and maximum likelihood method.

For whole-cell experiments, the pipette contained 3 M KCl and the bath solution contained (in mM): 140 KCl, 1 $MgCl_2$, 0.3 $CaCl_2$, 5 HEPES, pH 7.5 with KOH. For on-cell patch-clamp experiments, pipette and bath solutions contained (in mM): 140 KCl, 2 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES, pH 7.4 with KOH. For inside-out, patch-clamp experiments, bath solutions contained (in mM): 140 KCl, 2 $MgCl_2$, 5 EGTA, 5 HEPES, pH 7.2 with KOH. Solutions with pH 5 and 9 were made with 10 mM 2-[*N*-morpholino]ethanesulfonic acid and 2-[*N*-cyclohexylamino]ethanesulfonic acid, respectively. Puratronic grade potassium chloride was purchased from Alfa Aesar (Ward Hill, MA). All ionic changes were made by isotonic substitution with a chloride salt except for work with thallous ions where nitrate salts and a ground bridge were employed. All experiments were conducted at room temperature.

RESULTS

Whole-cell KCNKØ currents openly rectify

Channel behaviors to be studied at the single-channel level were first assessed in whole-cell mode. As noted previously (Goldstein et al., 1996), macroscopic KCNKØ currents appear to rise instantaneously in response to changes in membrane voltage without evidence for an activation threshold (Fig. 1 *A*); currents are steady with maintained voltage without signs of inactivation after steps to positive (60 mV) or negative (−150 mV) potentials. Current-voltage relationships are linear when potassium levels are equal on both sides of the membrane and nonlinear under asymmetric conditions (Fig. 1 *A*); as external potassium is varied, currents are well approximated by the Goldman-Hodgkin-Katz current equation for free electrodiffusion through an open, ion-selective partition (Goldman, 1943; Hodgkin and Katz, 1949). This response to asymmetric solutions is referred to as Goldman, or open, rectification to differentiate it from current asymmetries (inward or outward) that are maintained even with symmetric ionic conditions (Goldstein et al., 1996, 1998).

Whole-cell KCNKØ currents exhibit anomalous mole-fraction behavior

KCNKØ channels are selective for potassium compared with sodium (Goldstein et al., 1996) and bi-ionic, whole-cell reversal potential measurements reveal an Eisenman type IV relative permeability series ($Tl^+ > K^+ > Rb^+ > NH_4^+ > Cs^+ \gg Na^+, Li^+$; Table 1) as observed for many cloned voltage-gated and inwardly rectifying potassium channels. Like other potassium channels, KCNKØ also shows strict control over the conductance of monovalent cations: only thallous and potassium ions pass readily through the channel (Fig. 1 *B* and Table 1). Despite its high relative permeability, rubidium is at least sevenfold less conductive than potassium based on whole-cell (Table 1) and single-channel (shown below) determinations.

In mixed bath solutions of thallium and potassium, whole-cell KCNKØ currents are smaller than in pure solutions of either ion (Fig. 1 *B*). Observing a conductance minimum with a solution containing two permeant ions (compared with either pure solution) is called an anomalous mole-fraction effect. In a pore that holds a single ion at a time, conductance is expected to increase (or decrease) monotonically with an increase in the mole fraction of a permeant ion, even in a mixed solution. The anomalous mole-fraction effect has been argued to indicate a pore that can simultaneously accommodate multiple ions with differing affinities. This suggests that KCNKØ, like other cloned potassium channels, employs ion-binding sites and a multi-ion pore.

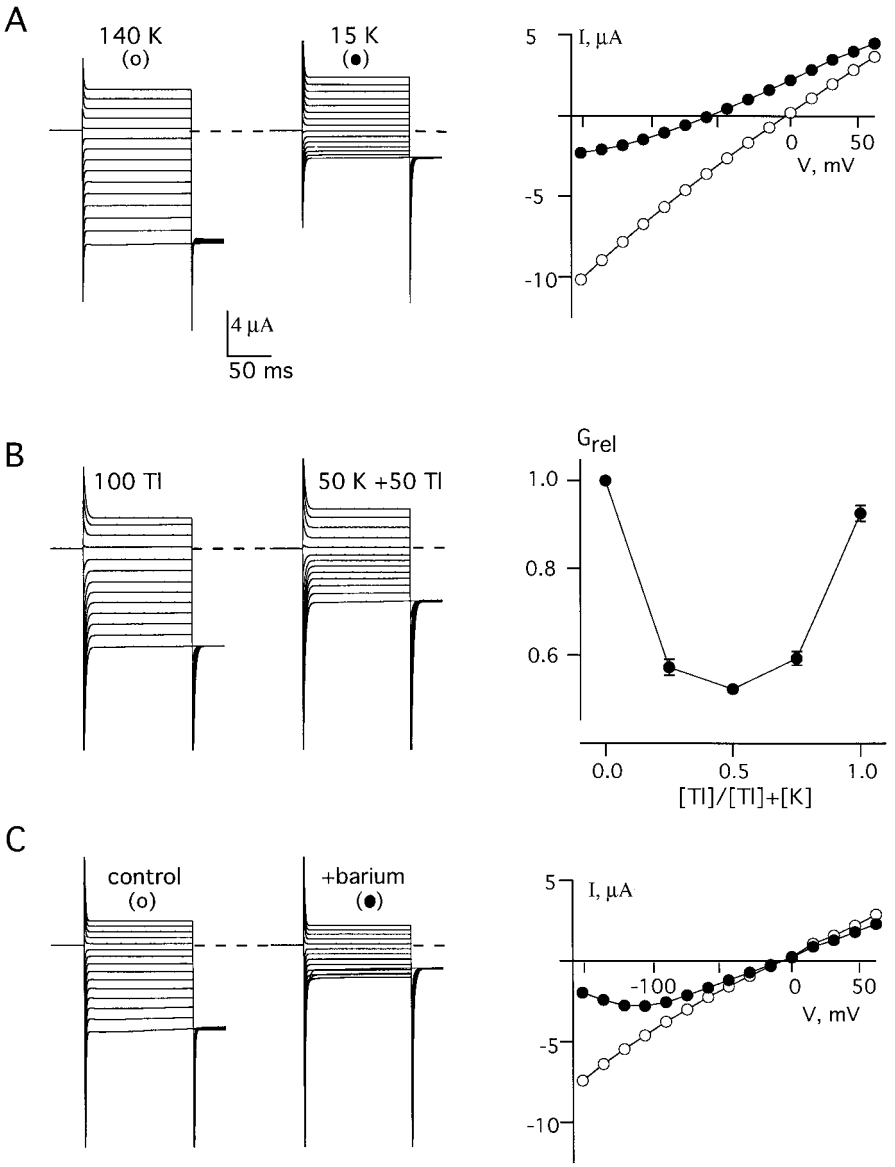

FIGURE 1 Whole-cell KCNKØ currents: open rectification, anomalous mole-fraction behavior, and barium blockade. Channels were expressed in *Xenopus* oocytes and studied by two-electrode voltage clamp (see Materials and Methods) with 125-ms steps to −150 to 60 mV in 15-mV increments from the zero current holding potential followed by a 125-ms step to −150 mV with an interpulse interval of 1 s. (*A*) Representative currents measured with constant perfusion of 140 mM KCl (○) and then 15 mM KCl (●) bath solution. The plot is the current-voltage relationship for this cell. (*B*) Representative currents in 100 mM $TlNO_3$ and then 50 mM $TlNO_3$ with 50 mM KNO_3. The plot is the macroscopic conductance at −120 mV relative to 100 mM KNO_3 at various mole fraction ratios with thallium (mean ± SEM; n = 5 cells). (*C*) Representative currents in control solution (140 mM KCl; ○) and then with 5 mM added barium (●). The plot is the steady-state current-voltage relationship for these cells.

Whole-cell KCNKØ currents are inhibited by barium

KCNKØ shows voltage-dependent blockade by external barium ions like other cloned potassium channels (Fig. 1 *C*). Barium, similar in size to potassium but with two charges, has been shown to bind in potassium channel pores thereby physically occluding the ion conduction pathway (Miller et al., 1987; Neyton and Miller, 1988a; Doyle et al., 1998; Jiang and MacKinnon, 2000). If the same blocking mechanism operates in KCNKØ channels, another discrepancy with the operation of independence is manifest: interaction of a potassium homolog (Latorre and Miller, 1983) and the pore. As macroscopic measurements are at best mechanistically suggestive, we next characterized KCNKØ at the single-channel level.

Single-KCNKØ-channel open bursts last for minutes

Single KCNKØ channels move between minutes-long open bursts and closures (Fig. 2 *A*). Long closures are tightly regulated and the subject of another report (Zilberberg et al., 2000). In this work, open channels are studied exploiting a KCNKØ variant that displays wild-type burst attributes and diminished occupancy of the inter-burst closed state (Materials and Methods). KCNKØ channels in on-cell mode with approximately symmetric 140 mM potassium open in bursts with open probability (P_o) close to 1 across a broad voltage range (Fig. 2 *B*). Open bursts appear more flickery and open probability decreases slightly at depolarized potentials (Fig. 2 *A*). To assess the basis for voltage-dependent flicker, open and closed dwell-time histograms were as-

TABLE 1 Relative permeability and conductance for macroscopic KCNKØ currents

Test ion (X)	V_{rev}, mV (mean ± SEM)	Relative permeability, P_X/P_K (mean ± SEM)	Relative conductance, G_X/G_K (mean ± SEM)
T1	12 ± 1	1.60 ± 0.03	0.93 ± 0.02
K	−2.0 ± 0.5	0.92 ± 0.02	1
Rb	−20 ± 1	0.45 ± 0.02	0.14 ± 0.02
NH_4	−46 ± 2	0.17 ± 0.01	0.24 ± 0.02
Cs	−57 ± 2	0.108 ± 0.007	0.10 ± 0.01
Na	−101 ± 3	0.019 ± 0.002	0.09 ± 0.01
Li	−103 ± 4	0.018 ± 0.002	0.09 ± 0.02

Macroscopic reversal potentials (V_{rev}) were assessed by two-electrode voltage clamp under nearly bi-ionic conditions with 100 mM of the indicated cation in the external solution. Values represent the mean ± SEM for six oocytes. Relative permeability was calculated according to $P_X/P_K = 1/\exp(-FV_{rev}/RT)$ assuming internal potassium was 100 mM. Conductance was calculated according to $G_X = I_X/(E - E_{rev})$ at −120 mV.

sessed at −90 mV ($P_o = 0.99$; Fig. 2, *B* and *C*) and 60 mV ($P_o = 0.93$, Fig. 2, *B* and *D*).

During the open burst, KCNKØ channels visit one open state and three closed states (mean duration ~0.2, 2, and 100 ms). It is the frequency of the briefest closure that increases at positive voltages (Fig. 2, *C* and *D*, *middle panel*); this decreases mean open time 60-fold, from 120 ms at −90 mV to 2 ms at +60 mV (Figs. 2, *C* and *D*, *left panel*). Neither the relative frequency nor mean duration of the two longer open burst closures are found to be altered by voltage (Fig. 2, *C* and *D*, *right panel*; 100 Hz). Voltage dependence of briefest open burst closures appears to be inherent to the channel protein rather than the effect of membrane potential on a charged blocker; when KCNKØ is studied in off-cell patches, channel behavior is not changed by use of ultra-pure potassium (to reduce metal contaminants), adjustment of the pH from 7.0 to 5.0 or 9.0, or alteration in magnesium level (from 2.0 to 10 mM or nominally zero) or calcium level (from 2.0 mM to no added calcium with 5 mM EGTA) on either side of the membrane (not shown).

Instantaneous currents are due to open single channels

Macroscopic KCNKØ currents appear to develop with no delay in response to voltage changes (Fig. 1). This is expected for channels already open at the holding voltage before the voltage step. However, a capacitance transient associated with charging a whole cell might obscure the activation of an extremely rapid, voltage-gated channel and conceal time-dependent channel activation. To confirm that KCNKØ currents develop instantaneously, the activation of single channels is studied at −120 and 60 mV in on-cell mode (with approximately symmetric 140 mM potassium) and ensemble traces are constructed (Fig. 3). Visits of the channel to the inter-burst, long-lasting closed state provide null traces to achieve subtraction of capacitance currents. An instantaneous rise in unitary (Fig. 3 *A*) and ensemble (Fig. 3 *B*) currents with steps to negative and positive membrane potentials reveals that KCNKØ channels are open before changes in voltage. Conversely, no openings are seen with pulses between −120 and 60 mV delivered during the long-lasting closed state ($n = 70$; four patches).

Single KCNKØ channels openly rectify

Open rectification of macroscopic currents (Fig. 1 *A*) is inherent to single KCNKØ channels and not an occult effect of gating (Fig. 4). Thus, single channels studied in on-cell mode with approximately symmetric 140 mM potassium demonstrate a linear single-channel current voltage relationship with a unitary slope conductance of 60 pS. Under asymmetric conditions (15/~140 mM potassium achieved by isotonic substitution with sodium), the single-channel current-voltage relationship rectifies (and conforms approximately to the current equation).

Single KCNKØ channels show concentration-dependent conductance

According to independence, a channel would exhibit a linear relationship between conductance and permeant ion concentration under symmetric conditions. As for classical potassium channels, and consistent with ion-channel interaction, KCNKØ single-channel conductance shows saturation as potassium is increased symmetrically across inside-out off-cell patches (Fig. 5). A fit to the Michaelis-Menten equation gives values for K_m and g_{max} of 45 ± 7 mM and 79 ± 3.8 pS, respectively. This K_m is similar to that of an inward rectifier potassium channel (Lu and MacKinnon, 1994) and approximately sevenfold less than that observed with *Shaker* channels (Heginbotham and MacKinnon, 1993). At still higher levels of symmetric potassium (not achieved here) a conductance decrease would argue for operation of a multi-ion pore (Hille and Schwarz, 1978; Lu and MacKinnon, 1994).

KCNKØ single channels pass rubidium and ammonium poorly

Macroscopic measurements suggest a classical relative permeability series for KCNKØ channels but low rubidium and ammonium conductance (Table 1); this was confirmed in single-channel studies. In on-cell configuration with 140 mM rubidium or ammonium in the pipette (a pseudo bi-ionic arrangement), outward potassium currents are observed but no inward currents (Fig. 6). Based on extrapolated reversal potentials, both ions move through KCNKØ channels with a unitary conductance that is too small to measure; rubidium conductance is at least 20-fold less than that for potassium based on estimated minimal detectable

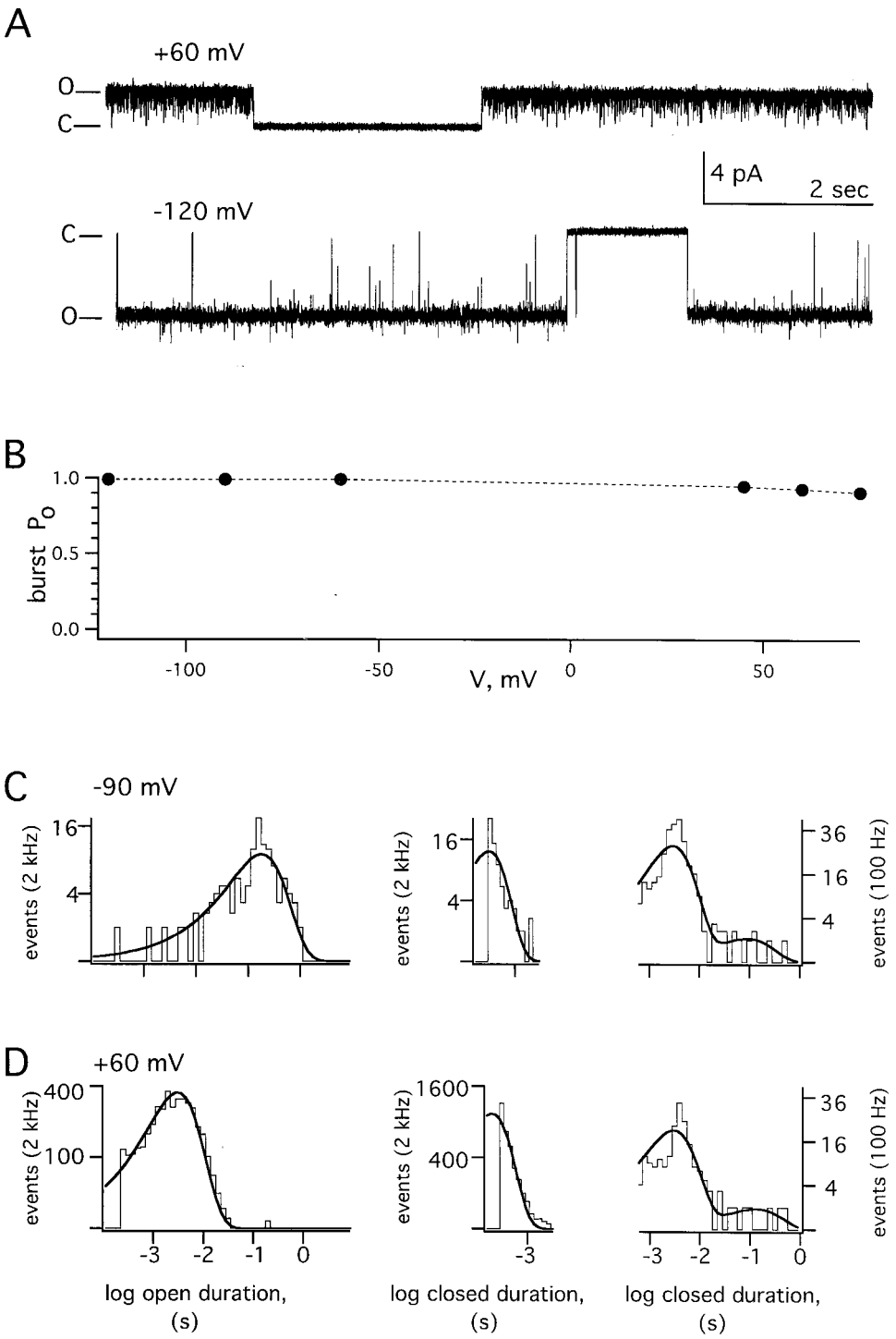

FIGURE 2 Single KCNKØ channel open probability. Single channels in *Xenopus* oocytes were studied in on-cell mode (Materials and Methods) with 140 mM KCl in the pipette and bath solution at the indicated voltages. (*A*) Representative single-channel record at 60 and −90 mV, filtered at 500 Hz. Open (O) and closed (C) state levels are indicated. (*B*) The plot is open burst open probability versus membrane potentials. Each data point represents an average P_o obtained from six to nine on-cell single channel patches, filtered at 2 kHz and analyzed using half-amplitude threshold for event detection. Error bars are smaller than the symbols. Total recording time analyzed at each voltage was 1–3 min. (*C, left panel*) Open-time histogram from 21 s at −90 mV, filtered at 3 kHz; τ = 120 ms. (*C, middle panel*) Closed-time histogram from 21 s at −90 mV filtered at 3 kHz; τ = 0.15 ms. (*C, right panel*) Closed-time histogram from 11 min at −90 mV filtered at 100 Hz (relative frequency); τ_1 = 2 ms (0.97); τ_2 = 90 ms (0.03). (*D, left panel*) Open-time histogram from 14.4 s at 60 mV, filtered at 3 kHz; τ = 2 ms. (*D, middle panel*) Closed-time histogram from 14.4 s at 60 mV filtered at 3 kHz; τ = 0.12 ms. (*D, right panel*) Closed-time histogram from 7 min at 60 mV filtered at 100 Hz (relative frequency); τ_1 = 2.4 ms (0.97); τ_2 = 120 ms (0.03).

events (Fig. 6, plot). Inward unitary currents are also not observed with 140 mM cesium, sodium, or lithium in the pipette (not shown).

Single KCNKØ channels show anomalous mole-fraction behavior

The relative permeability of thallium in KCNKØ channels is greater than that for potassium, and both ions are measurably conductive in whole-cell configuration (Table 1). At the single-channel level, conductance of thallium is similar to potassium (Fig. 7); however, thallium currents show a lower open probability and more flicker (0.82 ± 0.01 at −120 mV; n = 3). Single-channel observations reveal that the anomalous mole-fraction effect seen at the macroscopic level (Fig. 1 *B*) is due to a decrease in unitary conductance rather than an effect on channel gating (Fig. 7 *B*). KCNKØ

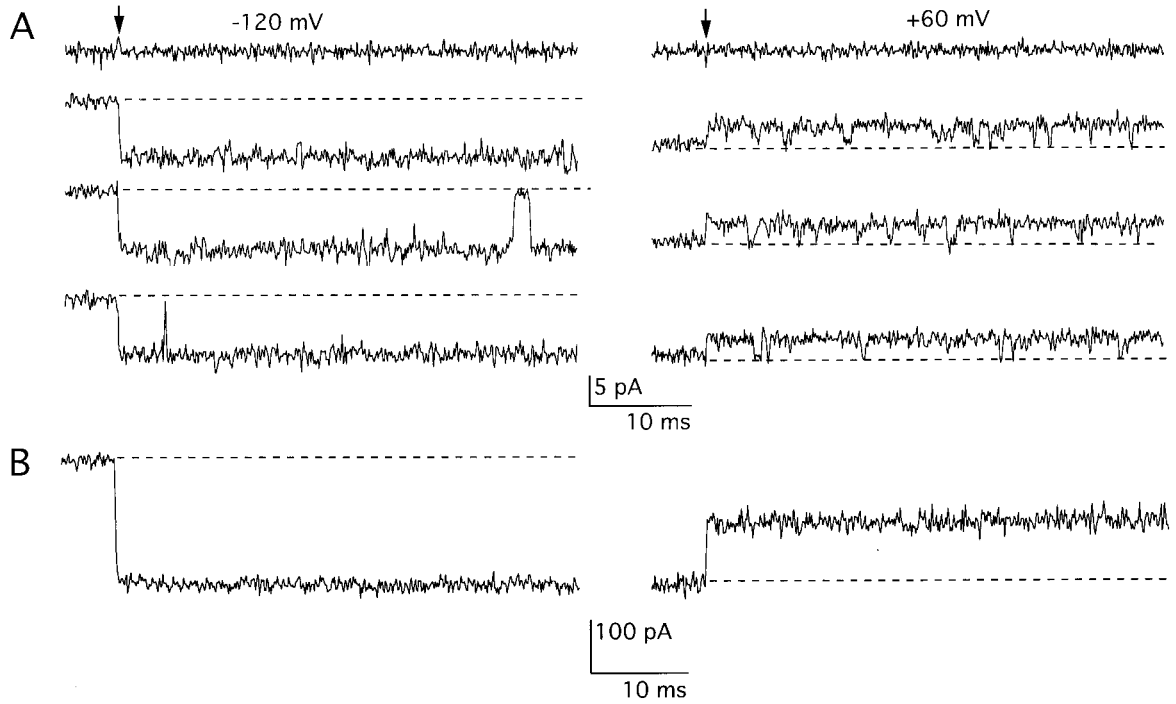

FIGURE 3 Single KCNKØ channels are open at rest. Unitary currents were recorded in on-cell configuration with 140 mM KCl solution in the pipette and the bath at the indicated membrane potentials, filtered at 2 kHz. - - -, 0-mV current level. (*A*) Single-channel currents develop instantaneously in response to voltage steps from 0 to −120 or 60 mV. Three representative traces with an active single channel and one null trace during a long channel closure are shown at each voltage. (*B*) Ensemble of 40 single-channel traces at each voltage from the patches in *A*.

unitary conductance in mixed solutions does not change monotonically with mole fraction but passes through a minimum. This suggests that thallium and potassium ions interact within a multi-ion KCNKØ pore.

Barium inhibition of single KCNKØ channels is via pore occlusion

Inhibition of single KCNKØ channels by barium meets expectations for binding of a blocker in the ion channel pore: first, inhibition displays voltage dependence; second, open time is altered without a change in single-channel conductance; third, block kinetics are consistent with a bimolecular process; finally, block is sensitive to permeant ions on the opposite side of the membrane. Thus, hyperpolarization of the membrane from −60 to −120 mV in the presence of external barium increases channel flicker due to an increase in the time single KCNKØ channels spend in the blocked state (Fig. 8 *A*); this is not associated with a significant effect on unitary current magnitude. The equilibrium dissociation constant (K_i) for external barium at −120 mV is 4.4 ± 0.4 mM and a dose-inhibition relationship is well fit by an isotherm employing a coefficient of 1; this suggests that inhibition results from a moderate affinity interaction between a single barium ion and the channel protein (Fig. 8 *B*).

A bimolecular model is adequate to describe the kinetics of barium block in classical potassium channels (Armstrong and Taylor, 1980; Armstrong et al., 1982; Vergara and Latorre, 1983; Neyton and Miller, 1988a). In those cases, the forward rate constant changes in a linear fashion with the concentration of barium whereas the barium off-rate is independent of blocker level. When the kinetics of block of single KCNKØ channels was determined using open- and closed-time dwell histograms, the leak channel was found to act like classical potassium channels (Fig. 9). Thus, at −120 mV, KCNKØ open probability is ~0.98 and the rate for transition to any closed state is small; under these conditions, the association rate constant for barium is calculated from the reciprocal of the mean open time (Fig. 9 *A*, *left panel*). Consistent with a bimolecular reaction, the barium on-rate is linearly dependent on barium concentration (Fig. 9 *B*). Closed-time histograms show a new closed time with added barium (Fig. 9 *A*, *right panel*). Thus, mean block time can be determined and barium off-rate can be estimated; as predicted, off-rate is independent of barium concentration (Fig. 9 *B*). At −120 mV, these rates (k_{on} = 4.8 × 10^4 s^{-1} M^{-1}; k_{off} = 2.3 × 10^2 s^{-1}) indicate a K_i = 4.8 mM, consistent with values determined from single-channel open probability (Fig. 8 *B*) and two-electrode voltage clamp (4.5 ± 0.4 mM; n = 6; not shown). This slow barium on-rate is like that observed for internal barium entry into

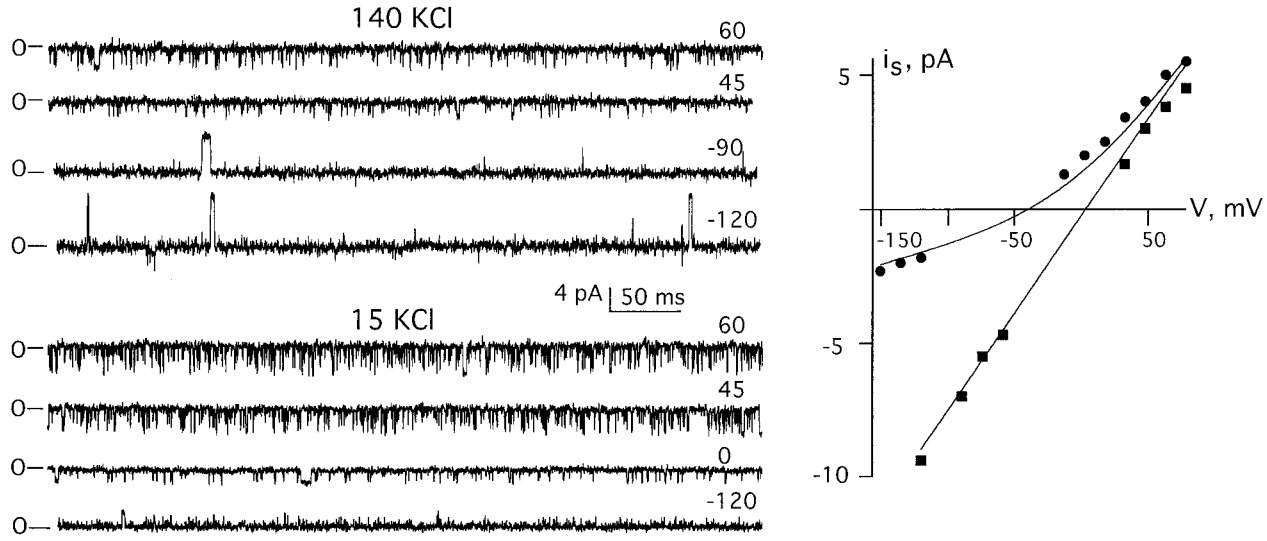

FIGURE 4 Single KCNKØ channels openly-rectify. Unitary currents in 140 mM (■) and 15 mM (●) KCl solution in the pipette and bath recorded as in Fig. 3. The open state is indicated (O). The plot shows single-channel current at a variety of voltages. The lines are fits of the data to the current equation, as previously shown (Goldstein et al., 1996). A linear regression fit of the data with 140 mM KCl gives a single-channel conductance of 60 pS.

calcium-activated potassium channels (Miller et al., 1987) and approximately five orders of magnitude slower than the second-order entry rate for potassium ions, which approach diffusion limitation (Latorre and Miller, 1983; Yellen, 1984).

To characterize the voltage dependence of barium blockade, association and dissociation rate constants are determined at different voltages. As expected for a charged blocker that binds in the pore, the electric field influences binding affinity; the effective electrical distance (δ) suggests barium binds ~60% down the potential drop across the membrane (Fig. 9 *C*, *left panel*).

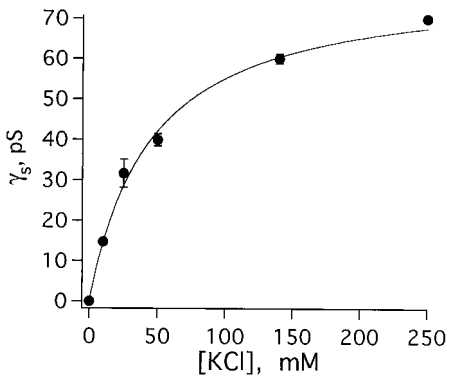

FIGURE 5 KCNKØ single channel conductance saturates with increasing symmetrical potassium. Unitary currents recorded in off-cell configuration at indicated levels of symmetric KCl in the pipette and bath. Points represent the average of three or four inside-out patches. The curve is a fit to the Michaelis-Menten equation $g_s = ([K]g_{max}/[K] + K_m)$ and gives values for K_m and g_{max} of 45 ± 7 mM and 79 ± 3.8 pS, respectively.

Barium block of KCNKØ is also sensitive to the concentration of potassium on the opposite (trans) side of the membrane (Fig. 9 *D*, *right panel*). When potassium bathing inside-out patches is raised from 17 to 70 mM (by isotonic substitution for sodium) with 140 mM potassium and 10 mM barium in the pipette, mean open time is unaffected (2.2 ± 0.4 and 1.8 ± 0.3) whereas open probability increases from 0.180 ± 0.008 to 0.47 ± 0.05 due to a decrease in mean blocked time from 8.4 ± 0.7 to 1.18 ± 0.12 ms; this indicates an approximately sevenfold increase in blocker off-rate with raised trans-potassium. Referred to as a knock-off effect, this suggests that permeant ions traverse the pore from the opposite side of the membrane to destabilize barium on its pore site and supports the idea that multi-ion occupancy (that is, both barium and potassium ions in the conduction pathway) underlies KCNKØ channel function.

DISCUSSION

In this report, we consider the conductance behavior of the two-P-domain, potassium-selective leak channel KCNKØ. Single-channel KCNKØ currents are seen to develop with no apparent delay and to conform to predictions of the current equation, that is, to openly rectify. Single KCNKØ channels exhibit saturation of unitary conductance with increasing symmetric potassium levels, anomalous mole-fraction behavior, and findings consistent with physical occlusion of the pore by barium. These attributes argue that KCNKØ leak channels employ permeation mechanisms like those found in classical potassium channels formed of one-

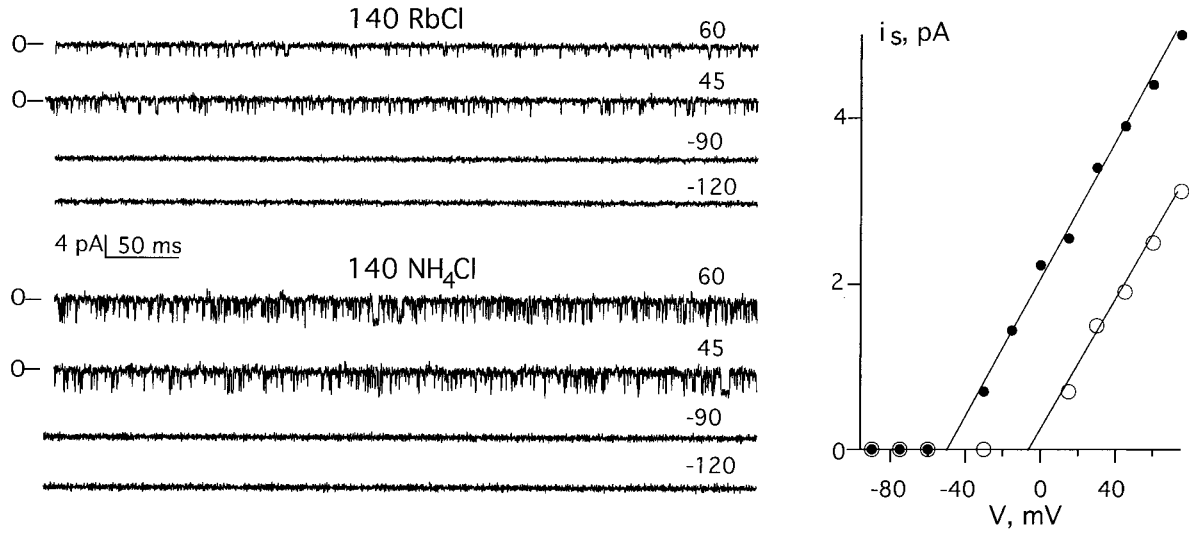

FIGURE 6 KCNKØ unitary currents with rubidium and ammonium are small. Representative unitary currents recorded in on-cell mode as in Fig. 4 in 140 mM RbCl or NH_4Cl solution in the pipette and bath at the indicated potentials. The open state is indicated (O). The plot is the single-channel current at various voltages for the channels shown. Lines are linear fits to the data and indicate single-channel conductances of 38 and 40 pS and reversal potentials of −10 and −50 mV for rubidium (○) and ammonium (●), respectively.

P-domain subunits: ion-channel and ion-ion interactions in a multi-ion pore.

Currents without delay indicate that voltage plays only a minor role

KCNKØ channels open in long-lived bursts (Fig. 2 *A*) and visit an equally long-lasting closed state in a tightly regulated (non-voltage-dependent) fashion (Zilberberg et al., 2000). Single-channel recordings confirm that currents rise immediately with voltage steps because channels are often open (Fig. 2 *B*) before a step to a new potential (Fig. 3). Open bursts maintain an open probability of ~1 across the physiological voltage range despite brief closures with mean duration ~0.2, 2, and 100 ms (Fig. 2). Although voltage does alter open probability to a limited degree (by changing the frequency of visits to the shortest intra-burst closed state, ~0.2 ms), neither the duration of open bursts nor entry into the burst state from the long inter-burst closed state are affected by potential.

Ion-channel and ion-ion interactions

KCNKØ has a unitary slope conductance of ~60 pS in approximately symmetric 140 mM potassium, and its unitary current-voltage relationship rectifies in reasonable accord with the constant-field current equation when potassium levels are unequal (Fig. 4). That equation considers a theoretical current-voltage relationship based on a Nernst-Planck continuum model for electrodiffusion in which the membrane is treated as a homogeneous slab of uniform thickness (Goldman, 1943; Hodgkin and Huxley, 1952; Hodgkin et al., 1952). The model does not include the notion of ion-binding sites or ion-ion interactions (Hodgkin and Keynes, 1955). However, four lines of evidence argue that ions traversing the KCNKØ pore interact with the channel protein and each other.

First, independence predicts a linear relationship between unitary conductance and ion concentration under symmetrical conditions; conversely, KCNKØ demonstrates saturation of its single-channel conductance-voltage relationship (Fig. 5) consistent with ion-channel interactions (Hille, 1992). Second, independence predicts a monotonic rise in unitary conductance as the concentration of a permeant ion in a mixture is increased; conversely, KCNKØ exhibits a unitary conductance minimum when thallous and potassium ions are mixed at constant ionic strength (Fig. 7). Although anomalous mole-fraction behavior is consistent with ion-ion interaction in a multiple ion pore (Neher, 1975; Hagiwara et al., 1977; Sandblom et al., 1977; Hille and Schwarz, 1978; Almers and McCleskey, 1984; Hess and Tsien, 1984; Eisenman et al., 1986; Heginbotham and MacKinnon, 1993; Sesti et al., 1995; Dang and McCleskey, 1998; Kiss and Korn, 1998), the phenomenon can be modeled with a one-site pore (Armstrong and Neyton, 1992).

Third, KCNKØ demonstrates attributes expected for barium inhibition by a simple pore-occlusion mechanism (supporting both ion-channel and ion-ion interaction): voltage dependence, a new nonconducting blocked state without a change in single-channel conductance, bimolecular kinetics, and enhanced barium dissociation rate with increased potassium level on the opposite side of the membrane (knock-off; Figs. 8 and 9). Finally, KCNKØ demonstrates relative permeability and conductivity like classical potassium chan-

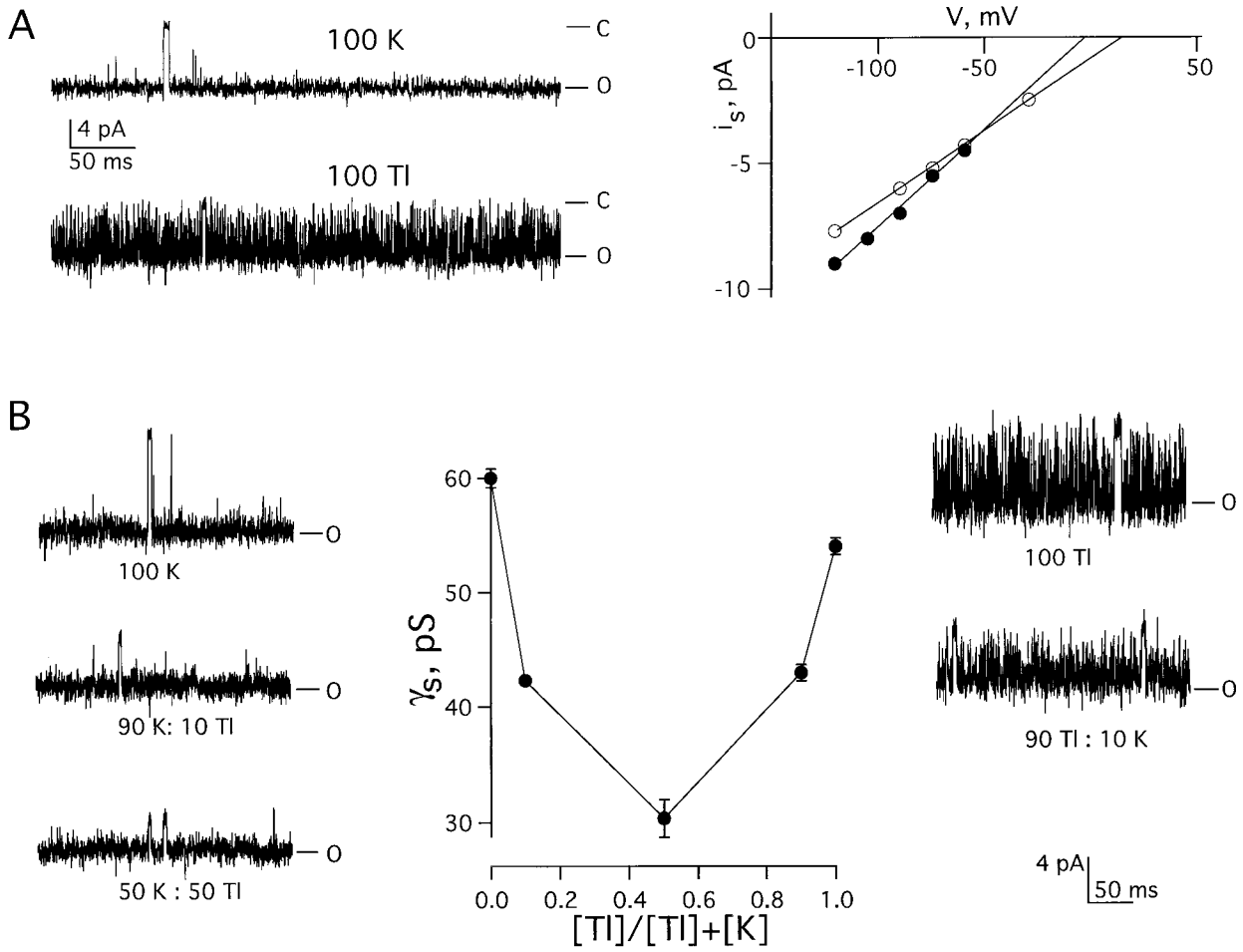

FIGURE 7 Single KCNKØ channels pass thallium and show anomalous mole fraction behavior. Unitary currents recorded in on-cell mode as in Fig. 4. The open state is indicated (O). (*A*) Representative currents recorded with 100 mM KNO_3 or 100 mM $TlNO_3$ in the pipette at −120 mV. The plot is the single-channel current-voltage relationship in 100 mM KNO_3 (●) and 100 mM $TlNO_3$ (○). (*B*). Single-channel conductance at various mole-fractions of thallium and potassium. Representative raw current traces for 100 mM KNO_3, 90 mM KNO_3 + 10 mM $TlNO_3$ and 50 mM KNO_3 + 50 mM $TlNO_3$, 10 mM KNO_3 + 90 mM $TlNO_3$ and 100 mM $TlNO_3$ at −120 mV, filtered at 2 kHz. The plot is unitary conductance at −120 mV at various mole ratios (mean ± SEM; n = 3–5 patches).

nels with multi-ion pores (Fig. 6 and Table 1). A robust effect of rubidium on reversal potential despite a small absolute conductance is observed in other potassium channels (Blatz and Magleby, 1984; Yellen, 1984; Eisenman et al., 1986; Ashcroft et al., 1989; Heginbotham and MacKinnon, 1993; Silver et al., 1994; Matsuda, 1996) where it has been interpreted as evidence that ions do not move independently of one another inside the conduction pathway (Hille, 1975).

The KCNK superfamily of leak channels

Members of the two-P-domain potassium channel superfamily have increased rapidly since the cloning of TOK1 (Ketchum et al., 1995) from *Saccharomyces cerevisiae* (with a 2P/8TM predicted topology) and *KCNKØ* from *Drosophila melanogaster* (Goldstein et al., 1996). The clan now includes over 50 isolates from nematodes (Ketchum et al., 1995; Wei et al., 1996), plants (Czempinski et al., 1997), and mammals (see below) that, like KCNKØ, have a 2P/4TM predicted topology. Although the channels vary widely in their regulation (Zilberberg et al., 2000), thus far, all are potassium-selective background conductances, that is, leak channels that pass current across the physiological voltage range.

Among the two-P-domain channels are now examples of outwardly, openly, and inwardly rectifying conductances. TOK1 was the first clone to demonstrate outward rectification, that is, a channel that passes outward potassium currents in a fashion coupled to the reversal potential for potassium (Ketchum et al., 1995; Vergani et al., 1997); a plant two-P-domain channel is also outwardly rectifying (Czempinski et al., 1997). Subsequent to cloning of *KCNKØ* (Goldstein et al., 1996), two genes for mammalian

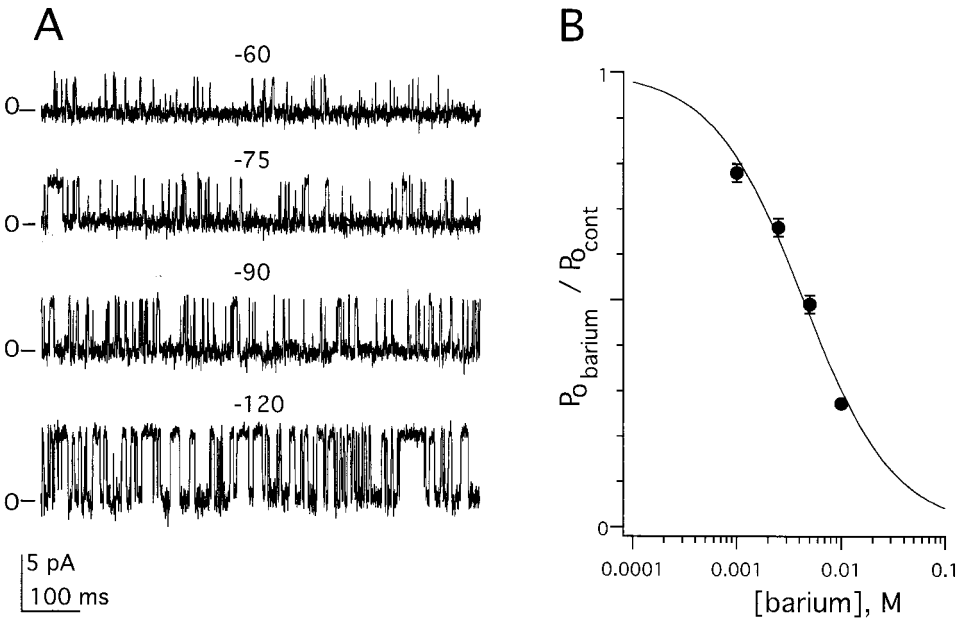

FIGURE 8 Barium blocks single KCNKØ channels. Single-channel records in on-cell configuration as in Fig. 4, sampled at 50 kHz, filtered at 1 kHz. (*A*) Representative single-channel records with 140 mM KCl and 5 mM barium in the pipette at the indicated holding potentials. The open state is indicated (O). (*B*) Dose response for external barium block at −120 mV. Data fit to $1 - (1/1 + ([Ba]/K_i)^h)$ gave $h = 1.0 \pm 0.1$, the concentration required to achieve half block; $K_i = 4.4 \pm 0.4$ mM.

open rectifiers were isolated, *KCNK3* (Duprat et al., 1997; Leonoudakis et al., 1998; Manjunath et al., 1999; Lopes et al., 2000) and *KCNK4* (Fink et al., 1998), and two genes for channels with mixed open-outward rectification behavior, *KCNK2* (Fink et al., 1996; Goldstein et al., 1998) and *KCNK5* (Reyes et al., 1998). *KCNK9* encodes a weak inward rectifier (Kim et al., 2000). Other *KCNK* genes yield messenger RNA in native cells but do not show reproducible function in experimental cells; these may lack obligatory accessory subunits or regulatory influences, or they may function inside the cell rather than at the plasma membrane. Genes of this type include *KCNK6* (Chavez et al., 1999; Pountney et al., 1999). *KCNK7/KCNK8* (Salinas et al., 1999; Bockenhauer et al., 2001), and *KCNK1* (Goldstein et al., 1998; Pountney et al., 1999), an isolate originally suggested to encode an inwardly rectifying channel and dubbed TWIK (Lesage et al., 1996a).

A shared attribute of KCNK leak channels is regulated activity. Thus, we show elsewhere that KCNKØ channels open and close in a tightly controlled, non-voltage-dependent fashion (Zilberberg et al., 2000). KCNKØ is found to have a 300-residue pore-forming domain (containing the two P loops and four predicted transmembrane segments) and a 700-residue carboxyl-terminal regulatory domain that serves to integrate signals from multiple second messenger pathways employing protein kinase A, C, and G. Activation increases open probability of single KCNKØ channels to ~1, inhibition reduces it to ~0.05, and truncation to remove the 700-residue carboxy terminus yields unregulated, but otherwise wild-type, leak channels. In a similar (if less robust) manner, KCNK2 is moderately inhibited by protein kinase C and A (Fink et al., 1996) and activated by arachidonic acid, mechanical stretch, or lowered intracellular pH (Patel et al., 1998, 1999; Maingret et al., 1999); KCNK3 is suppressed by lowered external pH (Duprat et al., 1997; Kim et al., 1998; Leonoudakis et al., 1998; Lopes et al., 1998; Manjunath et al., 1999) in a potassium-dependent fashion (Lopes et al., 2000) and down-regulated in motoneurons by neurotransmitters (Talley et al., 2000); KCNK4 is moderately increased by unsaturated fatty acids and membrane stretch (Fink et al., 1998; Maingret et al., 1999), and KCNK5 is inhibited by external acidification (Reyes et al., 1998).

Ion conduction is similar in one- and two-P-domain potassium channels despite structural differences

Although it remains unproven, it seems likely that both P domains in 2P/4TM subunits contribute to pore formation and that channel complexes are dimeric. (A report supporting this stoichiometry for the *KCNK1* product TWIK/hOHO demonstrated oxidative formation of subunit dimers but remains controversial as effects on function were also reported (Lesage et al., 1996b), and others find this gene to be nonfunctional in oocytes (Goldstein et al., 1998; Pountney et al., 1999)). Assuming that two KCNKØ subunits form each channel, its pore is predicted to have twofold symmetry rather than the fourfold arrangement of classical channels formed with one-P-domain subunits. This is notable when KCNKØ is considered in light of the only potassium channel with a determined high-resolution structure, KcsA (Doyle et al., 1998). The potassium selectivity filter in KcsA is supported by an extended network of aromatic residues stabilized by hydrogen bonds in tetrameric fashion (indeed,

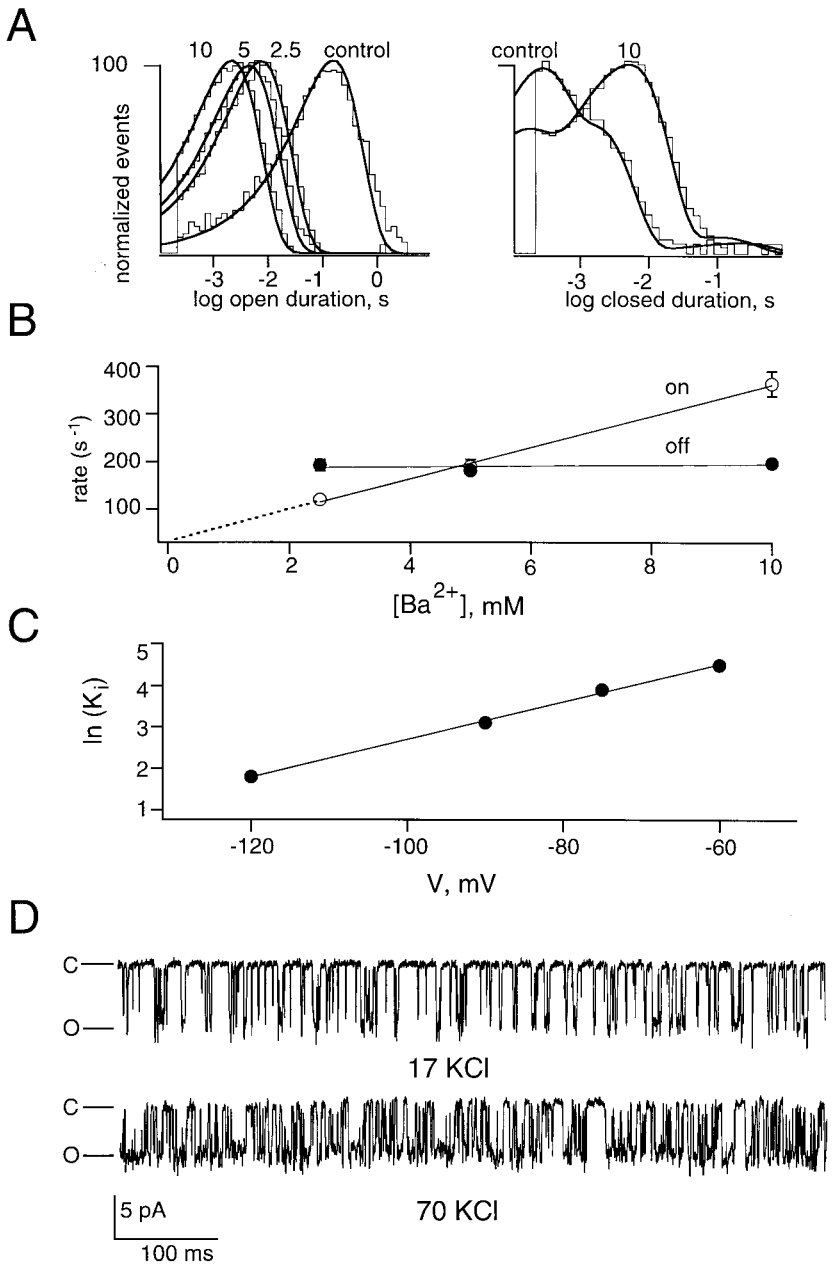

FIGURE 9 Pore block of single KCNKØ channels by barium. Single-channel records in on-cell and off-cell configurations are as in Figs. 4 and 5. (*A*) Barium decreases open time and produces a new closed state. Open-time (*left panel*) and closed-time (*right panel*) histograms analyzed from single KCNKØ channels at −120 mV, filtered at 1 kHz, under control conditions (140 mM KCl) and with indicated concentrations of external barium. Recording time for analyses were 9.6 min for control and 4.2, 4.5, and 2.2 min with 2.5, 5, and 10 mM barium, respectively. Normalized event counts are presented. Mean open time in the absence of barium was 156 ms; open times in the presence of external barium (level in mM) were 8. 3(2.5), 4.9 (5), and 2.5 (10) ms, respectively. Mean closed times (relative frequency) in the absence of barium were $\tau_1 = 0.25$ (0.64), $\tau_2 = 1.67$ (0.36), and $\tau_3 = 200$ ms (0.003); and with 10 mM external barium were $\tau_1 = 0.15$ (0.18), $\tau_2 = 1.35$ (0.13), $\tau_{block} = 5.72$ ms (0.7), and $\tau_3 = 100$ ms (0.006) . (*B*) Changing barium level alters on-rate but not off-rate. Each data point represents three to six on-cell patches studied at −120 mV with 140 mM KCl solution in the pipette and the indicated barium concentration. Pseudo-first-order association rate constant (K_{on}[barium], ○) and dissociation rate constant (K_{off}, ●) were estimated from the inverse of mean open and blocked times, respectively (Moczydlowski; 1986; Neyton and Miller, 1988b). (*C*) Block by barium is voltage dependent. Barium inhibition constants (K_i) were calculated at the indicated voltages from the estimated off- and on-rate (k_{off}/k_{on}, $n = 3$–5; error bars are smaller than symbols). The electric distance was calculated according to $K_i(V) = K_o\exp(-z\delta FV/RT)$, where $K(0)$ 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 binding site (Woodhull, 1973). A $z\delta$ of 1.14 ± 0.04 suggests barium experiences ~60% of the field at its site in the pore. (*D*) Barium block is sensitive to potassium on the opposite side of the membrane. Inside-out patches were formed with 140 mM KCl and 10 mM barium in the pipette and studied with 70 or 17 mM KCl in the bath at −90 mV (three patches each condition). Mean closed time increases and open probability decreases with lower potassium on the opposite side of the membrane; values are reported in the text. Open (O) and closed (C) states are indicated.

refinement assumed this symmetry). Bilateral symmetry and differences at several key residues that contribute to the KcsA network suggest that KCNKØ may form its selectivity filter by a modified strategy (key network residues that vary between the P domains are bold: KcsA, AL**WW**S-VETATTVG**Y**G**D**LYP; KCNKØ P_1, AF**FF**AFTVCSTVG-**Y**G**N**ISP; and KCNKØ P_2, SL**YY**SYVTTTTIG**F**G**D**YVP).

Thus, KCNKØ channels display permeation attributes analogous to those observed in classical multi-ion potassium channels formed with one-P-domain subunits: discrimination among monovalent cations, saturating unitary conductance, anomalous mole-fraction behavior, and pore blockade by barium. These characteristics dispute the operation of independence in leak channels, indicate the inadequacy of the Goldman-Hodgkin-Katz current equation to rationalize open rectification, and show that novel mechanisms are not required to describe the function of leak channels.

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