Activation-Dependent Subconductance Levels in the drk1 K Channel Suggest a Subunit Basis for Ion Permeation and Gating

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ABSTRACT Ion permeation and channel opening are two fundamental properties of ion channels, the molecular bases of which are poorly understood. Channels can exist in two permeability states, open and closed. The relative amount of time a channel spends in the open conformation depends on the state of activation. In voltage-gated ion channels, activation involves movement of a charged voltage sensor, which is required for channel opening. Single-channel recordings of drk1 K channels expressed in *Xenopus* oocytes suggested that intermediate current levels (sublevels) may be associated with transitions between the closed and open states. Because K channels are formed by four identical subunits, each contributing to the lining of the pore, it was hypothesized that these sublevels resulted from heteromeric pore conformations. A formal model based on this hypothesis predicted that sublevels should be more frequently observed in partially activated channels, in which some but not all subunits have undergone voltage-dependent conformational changes required for channel opening. Experiments using the drk1 K channel, as well as drk1 channels with mutations in the pore and in the voltage sensor, showed that the probability of visiting a sublevel correlated with voltage- and time-dependent changes in activation. A subunit basis is proposed for channel opening and permeation in which these processes are coupled.

INTRODUCTION

Ion channels are a large class of membrane proteins found in all cell types, where they perform a wide variety of physiological functions. All channels cloned so far seem to a share a common architecture of homologous subunits or domains surrounding a central aqueous pore. Whereas ligand-gated channels assemble as pentamers (Unwin, 1993), voltage-gated channels consist of four domains or subunits (Li et al., 1994). In voltage-gated K channels the four subunits are identical and contain six putative transmembrane segments, S1–S6. The highly conserved P region contained in the linker between S5 and S6 is thought to line the narrow part of the pore (Yellen et al., 1991; Yool and Schwarz, 1991; Hartmann et al., 1991). Positive charges in S4 (Liman and Hess, 1991; Papazian et al., 1991; Perozo et al., 1994), together with negative charges in S2 and S3 (Papazian et al., 1995; Seoh et al., 1996), form the voltage-sensing machinery. Membrane depolarization causes a translocation of the charged S4 segment (Yang and Horn, 1995; Larsson et al., 1996; Mannuzzu et al., 1996; Yang et al., 1996), which is thought to induce conformational changes leading to an increased probability that the channel will open (Bezanilla and Stefani, 1994). The structural assignments of the voltage sensor and the pore-forming region have set the stage for investigating the molecular basis of two fundamental channel properties: channel opening and ion permeation.

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The behavior of individual ion channels can be directly observed with the patch-clamp technique (Hamill et al., 1981). Experiments that use this approach show that single ion channels switch stochastically between what seems to be two states: a closed state in which there is no measurable ion permeation and an open state in which the permeation rate is both constant and specific for a particular channel. Although permeation appears to be a binary, all-or-nothing process, channels contain multiple subunits, each of which makes a contribution to the pore lining. In the case of voltage-dependent K channels the four subunits are identical, presumably forming a pore with fourfold rotational symmetry. When such a K channel opens, all four subunits must be able to undergo the same conformational change, irrespective of the physical basis for channel opening and permeation. Therefore, in addition to the homomeric open and closed states, there are heteromeric states in which some of the subunits are "closed" and others are "open" (Fig. 1 *A*). Every time a channel undergoes a transition between the closed and the open states, it has to visit these intermediate states. These heteromeric pore conformations have thus far been ignored by assuming that transitions between the homomeric open and closed states are concerted (i.e., exhibit strong positive cooperativity) and that only the homomeric open state permeates ions. The results shown here for the drk1 K channel (Frech et al., 1989) expressed in *Xenopus* oocytes suggest, however, that 1) this cooperativity may break down in partially activated channels and 2) the heteromeric pore conformations may give rise to intermediate current levels.

Sublevels associated with transitions

Patch-clamp recordings from individual drk1 K channels showed that most of the opening transitions seemed to go

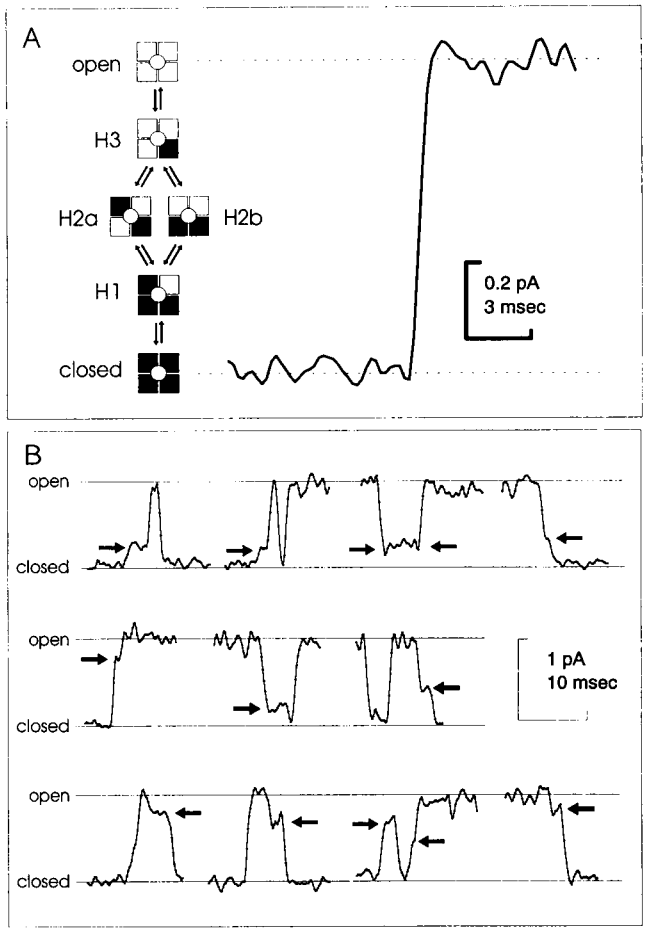

FIGURE 1 Opening transitions: heteromeric conformations and substates. *A*, K channels consist of four identical subunits. When such a channel opens, each subunit must undergo a conformational change from "closed" (*black*) to "open" (*white*), resulting in heteromeric channel conformations (*H1–H3*), which the channel must visit during every transition. A representative single-channel opening transition is shown, in which the channel appears to go directly from the zero-current closed level to the open level. The transition has a finite duration that is due to low-pass filtering (500 Hz) and has a smooth appearance. *B*, Opening and closing transitions of drk1, selected from the same recording used in *A*, illustrating that shoulders (*arrows*) are present in a minority of the transitions. Some shoulders have durations of several milliseconds, exceeding the rise time of the low-pass filter.

directly from the closed to the fully open state (Fig. 1 *A*). In many cases, however, the channel was seen to pause briefly at an intermediate current level, giving the appearance of a shoulder (Fig. 1 *B*). Such shoulders may result from short-lived subconductance levels visited during the transitions (Fig. 2A), or they could be filter artifacts produced by brief periods of unresolved, fast switching between the closed and open states (Fig. 2 *B* and *C*). Occasionally, sublevels seemed to be well resolved, with a duration that exceeded the filter rise time severalfold. It is, therefore, unlikely that the sublevels were produced by the relatively slow interconversion between open and closed conformations illustrated in Fig. 2 *B*. However, when the rate of interconversion is increased sufficiently (Fig. 2 *C*), intermediate current levels result that are difficult, if not impossible, to distinguish from true subconductance levels (see also Moss and Moczydlowski, 1996). As it is not clear a priori what mechanism underlies the intermediate current levels in drk1, these levels will be referred to as sublevels. The term sublevel implies only that current levels are somewhere between the closed and the fully open levels. The interpretation of sublevels either as true subconductance levels or as filter artifacts resulting from fast-switching activity has important implications for the molecular basis of permeation and gating.

Because the short-lived sublevels observed for drk1 were usually associated with transitions between the closed and the open states, it was hypothesized that these sublevels correspond to the heteromeric channel conformations, H1–H3. To test this hypothesis we developed a formal model of channel behavior that explicitly takes into account the conformations of individual subunits. With this model, specific testable predictions were made regarding the voltage and the time dependence of the putative heteromeric pore conformations.

A functional model of the channel based on subunit conformations

A minimal model was developed based on subunit conformations rather than on (hypothetical) channel states. Since Hodgkin and Huxley's (1952) seminal modeling of conductance changes that underlie excitability, it has been assumed that activation (movement of the voltage sensors) is required for channel opening. More recently, it has become clear that activation is necessary, but not sufficient, for channel opening. That activation and channel opening involve two separate conformational changes, in the voltage sensor and in the pore, respectively, is supported by ample experimental evidence (Hoshi et al., 1994; Schoppa et al., 1992; Zagotta et al., 1994a). The minimal model to describe the behavior of a subunit is therefore a linear scheme containing three states: resting, active, and open (Fig. 3 *inset*). Combining four identical subunits in a channel produces a triangular 15-state model (Fig. 3). Because activation probably requires more than one conformational change in each subunit (Perozo et al., 1993; Zagotta et al., 1994b), this model describes only the final step in the activation pathway. Of the fifteen states, only six states have homomeric pore conformations. The remaining nine states correspond to the heteromeric conformations shown in Fig. 1 *A*.

The structure of the model is a direct consequence of 1) the basic assumption that activation is necessary but not sufficient for channel opening and 2) the fact that K channels consist of four identical subunits. If the nine heteromeric states are highly unstable and do not support ion permeation, they can be ignored. Doing so reduces the model to the popular six-state linear ($C \rightleftarrows C \rightleftarrows C \rightleftarrows C \rightleftarrows C \rightleftarrows O$) scheme used to describe activation of voltage-dependent K channels (Koren et al., 1990; Zagotta and Al-

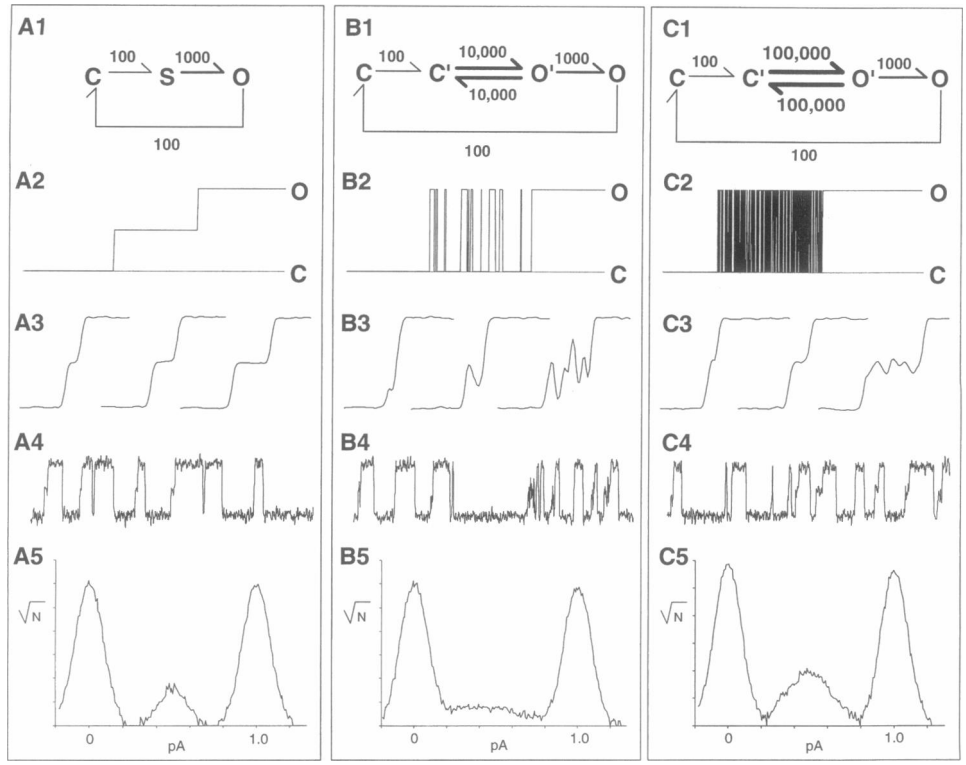

FIGURE 2 Origins of intermediate current levels. Two origins for intermediate current levels are 1) subconductances and 2) fast-switching activity. In the latter case the rate of switching determines the stability of the sublevel, which is illustrated here by use of Markov models to simulate single-channel activity (VanDongen, 1996). *A1*, *B1*, *C1*, Three Markov models were used, each with a stable open (O) and closed (C) state, with an average lifetime of 10 ms. The current through a (fully) open channel was 1 pA. The channel can go from C to O only by passing through either a 50% subconductance level S (*A*) or a combination of an unstable closed and an open state, C′ and O′, respectively, each with an average lifetime of 100 μs (*B*) or 10 μs (*C*). *A2*, *B2*, *C2*, Examples of unfiltered, noise-free C–O transitions. For model *B* the stable closed and open levels are separated by a brief period of fast C′–O′ switching containing on average 10 openings. For model *C* the same period contains on average 100 openings. The duration of the records shown is 4 ms. *A3*, *B3*, *C3*, The effect of low-pass filtering is illustrated. Simulated ideal data were sampled at 140 kHz, a small amount of white noise (0.05 pA rms) was added, and the result was low-pass filtered at 1 kHz and resampled at 10 kHz. Records shown have a duration of 8 ms. For all three models the channel visits intermediate current levels when going from C to O. For model *A* the duration of the shoulders varies (exponentially) but the amplitude is highly reproducible. In *B* the sublevel amplitude is highly variable, because the low-pass filter integrates over only a small number of C′–O′ levels. The fluctuations seen in the third example result from the fast-switching behavior, not from the added noise. In *C3* the sublevels are much more stable, resembling those in *A3*. *A4*, *B4*, *C4*, Same simulations as in row 3, except that more noise (0.5 pA rms) was added before filtering at 1 kHz. Records shown have a duration of 200 ms. *A5*, *B5*, *C5*, Amplitude histograms constructed from 20 records similar to those for row 4, omitting sample points arising from transitions (VanDongen, 1996; Tyerman et al., 1992). The square root of the number of observations is used to enhance small peaks arising from intermediate current levels. Whereas slow switching (10 kHz) results in a broad distribution of intermediate current levels (*B5*), much faster switching (100 kHz) results in a peak that is only slightly broader (*C5*) than the peak caused by a real subconductance level (*A5*).

drich, 1990). In this case, only homomeric pore conformations are used, and the final opening transition involves a concerted movement of all four subunits. Our subunit–sublevel hypothesis introduces only one new aspect: Heteromeric pore conformations may give rise to short-lived sublevels. With this assumption, the model predicts the occurrence of highly unusual single-channel behavior. A partially activated channel that attempts to open can visit only short-lived heteromeric conformations. Because it is hypothesized that these heteromeric conformations produce sublevels, single-channel behavior may be dominated by short-lived sublevels if the channel spent enough time in these partially activated conformations. The model therefore predicts a correlation between the state of activation of the channel and the abundance of sublevels. This prediction has been tested using the drk1 K channel as well as mutants that were constructed to increase the single-channel conductance and alter activation properties.

MATERIALS AND METHODS

Site-directed mutagenesis, in vitro mRNA synthesis, and oocyte preparation and injection were performed essentially as described (Wood et al., 1995). Care and handling of *Xenopus* frogs were in accordance with institutional guidelines. Drk1 K channels (Frech et al., 1989) were expressed in *Xenopus* oocytes as previously described (VanDongen et al., 1990). Single-channel behavior was recorded from cell-attached patches (VanDongen et al., 1988) containing a single drk1 channel using commercial patch-clamp amplifier (Axopatch 200; Axon Instruments, Foster City, CA). K channels were activated by step depolarizations from a holding potential of −100 mV. Data acquisition and voltage control were performed with PCLAMP hardware and software (Axon Instruments). Current records were low-pass filtered (eight-pole Bessel filter) at 1.5 kHz before digitization. The bath solution (in mM) was KCl 120, EGTA 10, $MgCl_2$ 1,

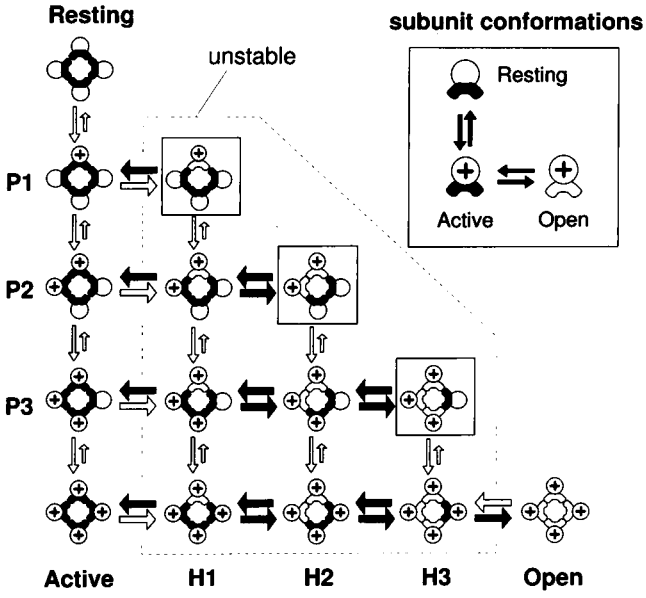

FIGURE 3 Functional model of the channel based on subunit conformations. A minimal model was developed that describes the behavior of a voltage-gated channel containing four identical subunits. Because voltage-dependent activation (movement of the charged voltage sensor) and channel opening (a change in pore conformation) are two separate processes, subunits need to have at least three conformations (*inset*): resting, active, and open. Voltage-dependent activation is modeled as a single step, in which movement of the charged voltage sensor changes the conformation of a subunit from resting to active. A change in the conformation of the subunit's contribution to the pore lining moves the subunits from the active to the "open" state. As activation is required for opening, the open state is accessible only from the resting state. Combining four identical 3-state subunits into a channel results in the 15-state triangular model shown. The three corners of the triangle are homomeric resting, active, and open states. Activation is portrayed as a downward movement; channel opening, as a movement to the right. Heteromeric pore conformations (*H1–H3*) are assumed to be unstable and therefore short lived. Rate constants leaving these nine heteromeric states are represented by filled arrows to indicate that they are much larger than any of the other rate constants. As long as activation is incomplete (*rows P1–P3*) the channel has no access to the fully open state. Heteromeric states at the diagonal are boxed because they are relatively stable owing to lack of forward rate constants.

HEPES 10, pH = 7.20, with KOH. The pipette solution in mM was NaCl 140, KCl 5, $MgCl_2$ 1, HEPES 10, pH = 7.40, with NaOH. For isotonic KCl conditions in Fig. 4 *B* and *D* the same solution was used in the bath and in the pipette, with the following composition (in mM): KCl 240 (Fig. 4 *B*) or KCl 100 (Fig. 4 *D*), EGTA 10, $MgCl_2$ 1, HEPES 10, KOH 60, pH = 7.2, with HCl. Under isotonic KCl conditions channel openings were inward currents but are shown as positive deflections.

We fitted amplitude histograms with a sum of Gaussians by minimizing the sum of squared differences (residuals) between the model and the data, using the variable metric Davidon–Fletcher–Powell method (Rao, 1984). The number of Gaussian components was increased until a minimum in the asymptotic information criterion (AIC) was reached (Akaike, 1981):

$$\mathrm{AIC(RSS)} = N \log(\mathrm{RSS}) + 2P,$$

in which RSS is the sum of squared residuals, N is the number of points, and P is the number of parameters.

Conditional open probabilities are estimated as follows. First, we "remove" the waiting time to the first opening (the "first latency") from each single-channel record by shifting the time base such that the first opening transition occurs at $t = 0$. All records now start with an opening either to a sublevel or to the fully open state. The conditional open probability, $m(t)$, is then calculated as an ensemble average over these time-shifted records (Aldrich et al., 1983; Zagotta et al., 1989). The ensemble average is calculated for sublevels (m_{sub}) and for the fully open state (m_{open}) as follows. First, for each time point t the number of records for which the channel is in a sublevel (S_t), the fully open state (O_t), or the closed state (C_t) is determined. The probabilities are then calculated as follows: $m_{sub,t} = S_t/(S_t + O_t + C_t)$ and $m_{open,t} = O_t/(S_t + O_t + C_t)$. Because the number of records available at each time point can decrease for large t because of the shift in time basis, $(S_t + O_t + C_t)$ rather than the ensemble size N is used for normalization.

RESULTS

Abundant sublevels at threshold

Generally, the occurrence of sublevels is rare in single-channel recordings of drk1 and other K channels (Fig. 4 *A*). This can be explained, in terms of the 15-state model, if activation is much faster than channel opening. Following a depolarization, the channel proceeds directly from the resting state (Fig. 3, *top left*) to the fully activated state (*bottom row*), with a very low probability of opening (*moving to the right*) while in rows P1–P3. Activation kinetics of drk1 are strongly voltage dependent. Whereas the rate of activation is fast at depolarized potentials, drk1 takes several hundred milliseconds to activate completely at threshold (VanDongen et al., 1990). Single-channel behavior was therefore studied near the threshold of activation, where sublevel behavior was predicted to be enhanced. These experiments indeed revealed a greater abundance of sublevels (Fig. 4 *B*). In contrast to the behavior at more depolarized potentials, there were many instances in which openings apparently failed to reach the fully open state.

Because of the small single-channel conductance of drk1, the amplitude resolution of such threshold recordings is marginal, precluding a reliable quantitative analysis. The single-channel conductance was therefore increased threefold by use of a well-documented pore mutation (Hartmann et al., 1991; Kirsch et al., 1992; Taglialatela et al., 1993). As described, this large-conductance channel (drk1-L) activated with normal kinetics and voltage dependence. Single-channel behavior was also normal at depolarized potentials, with the occurrence of sublevels being rare (Fig. 4 *C*). However, step depolarizations to threshold potentials again evoked highly unusual single-channel behavior (Fig. 4 *D*). Most of the early openings were to sublevels, whereas full openings usually appeared only toward the end of the pulse. Once the fully open state was reached, the probability of seeing sublevels decreased substantially.

Enhanced sublevel behavior in a slow-activation mutant

To correlate more rigorously the abundance of sublevels with activation state, the occurrence of partially activated channel conformations was enhanced, using mutagenesis. A mutation in the N-terminal region of the putative voltage sensor, S4, produced a channel (drk1-LS) that displayed very slow and poorly voltage-dependent activation kinetics

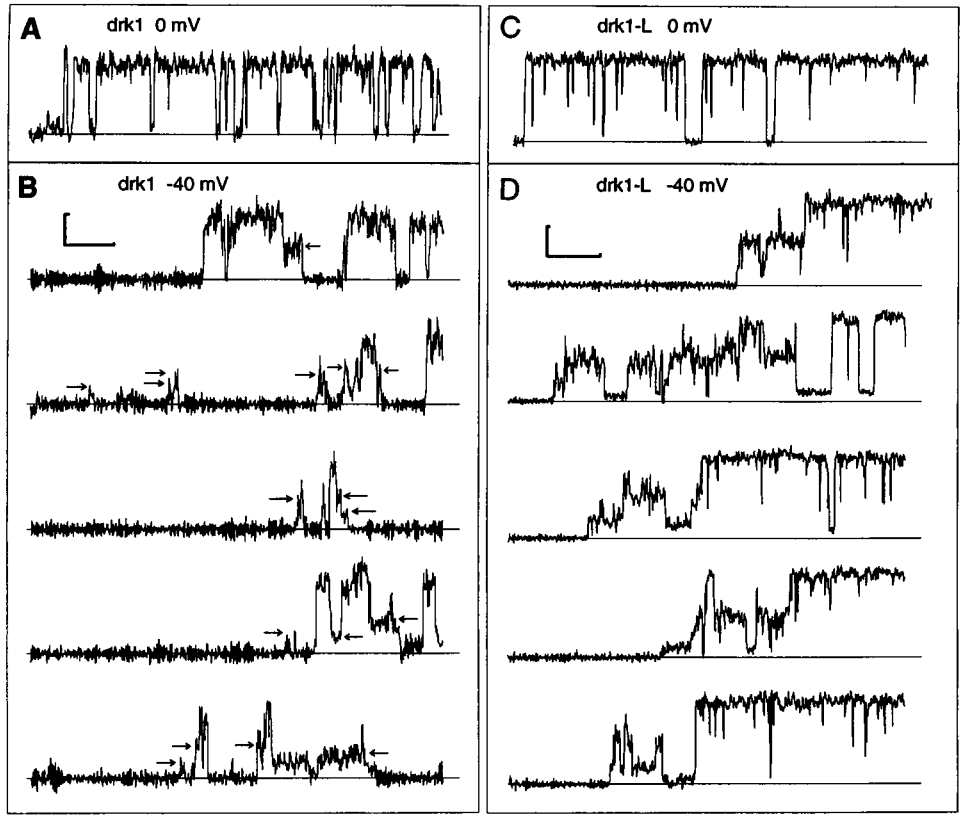

FIGURE 4 Abundance of sublevels at threshold potentials. *A*, Single-channel behavior of drk1 evoked by a step depolarization to 0 mV. Following a short delay, the channel switches stochastically between two current levels, corresponding to the closed and the fully open states. Resolved sublevels are rare. *B*, Single-channel behavior of drk1 evoked by step depolarizations to −40 mV, near the threshold of activation (VanDongen et al., 1990). Many openings, indicated by arrows, were short lived and seemed to be incomplete. *C*, Mutation of residues in the pore region of drk1 to the amino acids found in the ngk2 K channel (*TIT*MTT*V* to *VV*TMTT*L*) increases the single-channel conductance nearly threefold to that of ngk2 (Hartmann et al., 1991). Single-channel behavior of this large-conductance mutant (drk-L) following a depolarization to 0 mV is similar to that of drk1. *D*, Single-channel behavior of drk1-L evoked by step depolarizations to −40 mV. Early openings are usually to sublevels, giving way to full openings toward the end of the pulse. Scale bars are *A*, 0.25 pA, 100 ms; *B*, 0.5 pA, 50 ms; *C*,0.5 pA, 50 ms; *D*, 0.5 pA, 100 ms.

(see below). In contrast to the kinetics of activation, steady-state activation was still quite voltage dependent. This phenotype is consistent with a multistep activation pathway in which the mutation has made one of the steps poorly voltage dependent and slow (Chapman and VanDongen, manuscript in preparation). If the final step of the activation pathway were slowed down, then sublevels would be predicted to be abundant at all potentials. That this in fact occurs was confirmed experimentally, as shown in Fig. 5. Early openings were dominated by sublevels at all membrane potentials. Similar to the behavior of drk1-L at threshold, initial openings were primarily to sublevels, with full openings appearing nearer the end of the pulse.

Estimating the number of sublevels and their amplitudes

Amplitude histograms were constructed from raw single-channel traces, using only sample points with a time derivative that is smaller than 2 standard deviations to prevent transitions from contaminating the histogram (Tyerman et al., 1992; VanDongen, 1996). In addition to peaks that arise from the fully open and closed states, amplitude histograms showed additional peaks corresponding to sublevels (Fig. 6). These histograms were fitted with sums of Gaussians, increasing the number of Gaussian components until the asymptotic information criterion (Akaike, 1981) was optimized. Describing the amplitude distributions typically required six Gaussians. In addition to the two major peaks for the closed and the fully open levels, there were usually four nonequidistant sublevels. The results, summarized in Table 1, show that sublevel amplitudes were 15%, 37%, 58%, and 82% of the fully open amplitude. The probability of being in a sublevel was strongly voltage dependent for drk1-L, decreasing 10-fold between −40 and 0 mV (Table 1). For the slow-activation mutant, drk1-LS, the probability of being in a sublevel was high and relatively voltage independent.

Correlation of sublevel behavior with activation

As previously stated, the model shown in Fig. 3 predicts a greater probability of early openings visiting a sublevel

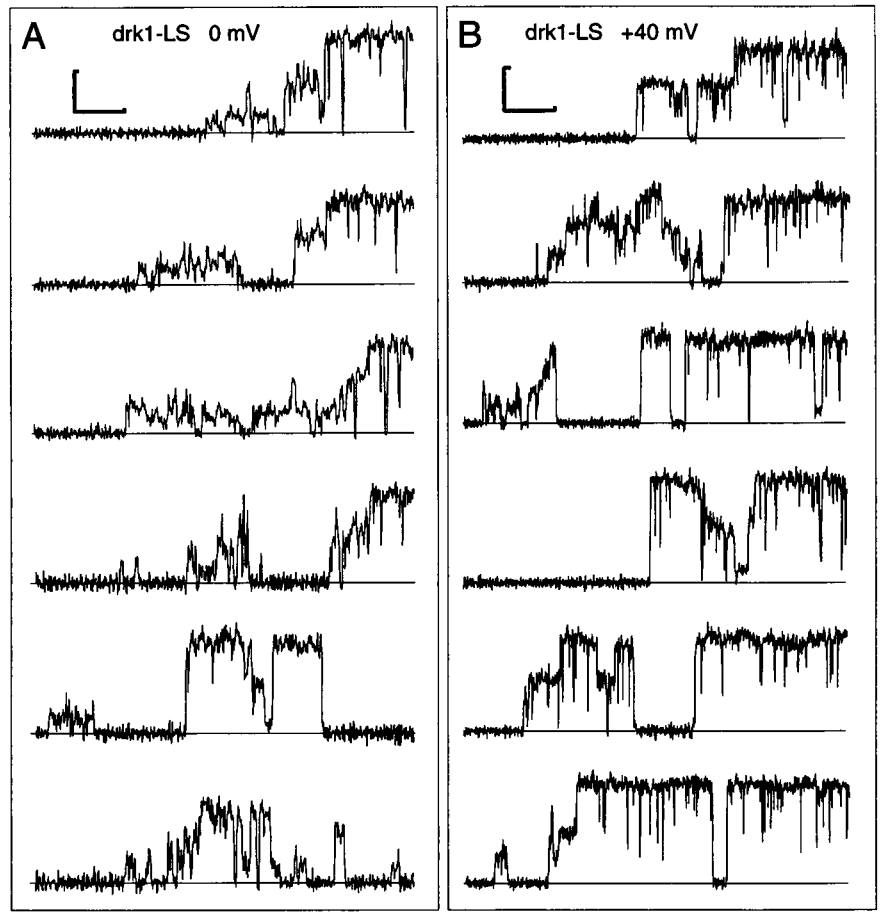

FIGURE 5 Early sublevels at all potentials in a slow-activation mutant. A mutation in the N-terminal segment of S4 of drk1-L slowed the time course of activation and reduced the voltage dependence of the activation kinetics. Three positions were mutated to construct drk1-LS, neutralizing two positive charges. The sequence *RRVV* was changed into *LLVA* at positions 289–292. The time constant of activation varied from 400 to 200 ms between −30 mV and +60 mV in drk1-LS, whereas the time constant for drk1 and drk1-L decreased from >400 ms to <5 ms in this same voltage range. Examples of single-channel behavior of drk1-LS elicited by step depolarizations to 0 and +40 mV are shown in *A* and *B*, respectively. Early openings were often to sublevels, giving way to full level openings toward the end of the pulse. Recording conditions were the same as in Fig. 1. Scale bars are A, 0.5 pA, 100 ms; B, 1.0 pA, 100 ms.

when activation is slow relative to channel opening. Furthermore, as the channel progresses toward the fully activated state, sublevels should give way to full openings. We tested this prediction by estimating $m(t)$, the conditional probability that the channel is open at time t, given that the channel first opened at $t = 0$ (Aldrich et al., 1983; Zagotta et al., 1989). This probability was estimated separately for sublevels and full openings. For this purpose, single-channel records were idealized with the TRANSIT algorithm (VanDongen, 1996). The idealized current levels were then assigned to the nearest conductance state (Fig. 7). Several hundred records were analyzed, and conditional open probabilities were calculated. The results, shown in Fig. 8, confirmed that the conditional probability of being in a sublevel was a function of time and voltage. Under conditions in which activation was slow (drk1-L at −40 mV, drk1-LS at 0 and +40 mV), ~80% of the first openings were to a sublevel. Following the first opening, the probability of being in a sublevel decreased slowly, whereas the probability of being in the fully open state increased. When activation was fast (drk1-L at 0 mV), the initial probability of being in a sublevel was much smaller (28%), and the probability decreased much faster. Fig. 9 illustrates the voltage and time dependence of channel activation for the same data sets. Activation kinetics were strongly voltage dependent for drk1-L, activating slowly at −40 mV and much faster at 0 mV. The S4 mutant, drk1-LS, activated slowly at more-depolarized potentials (0 and +40 mV), and the kinetics were relatively voltage insensitive. Figs. 8 and 9 show that the probability of being in a sublevel has the same time and voltage dependence as channel activation.

DISCUSSION

The observation of short-lived intermediate current levels associated with some transitions between the open and closed states of the drk1 K channel (Fig. 1 *B*) led us to propose the hypothesis that these sublevels originated from heteromeric pore confirmations (Fig. 1 *A*), in which some subunits facilitate ion permeation and others do not. Formalization of this hypothesis led to a structure-function model, which explicitly takes into account the conformation of individual subunits (Fig. 3). This model predicts that sublevel behavior will be correlated with the state of activation of the channel. This prediction was experimentally tested for 1) drk1, 2) a pore mutant channel with a threefold higher single-channel conductance (drk1-L), and 3) an S4 mutant, which activated with slow kinetics that were poorly voltage dependent (drk1-LS). In all cases sublevel behavior was found to correlate with channel activation. The kinetics of sublevel behavior exhibited the same voltage dependence

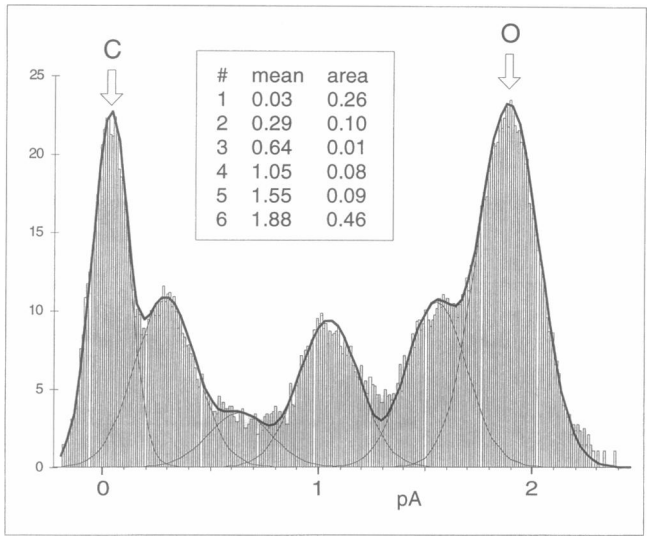

FIGURE 6 Amplitude histograms show four sublevels. Amplitude histograms were constructed from raw current traces, accepting only sample points with a relatively small first derivative (Tyerman et al., 1992), to prevent transitions from contaminating the histogram. An example of such a histogram is shown for drk1-LS at +40 mV (Fig. 5 *B*). The *y* axis is the square root of the number of observations in each bin, which enhances Gaussian components with relatively small areas. The histogram shown here was best described by a sum of six Gaussians. Before histogram construction, current records were filtered at 500 Hz with a finite-impulse-response filter with a Gaussian kernel.

as the activation kinetics (Fig. 9). Furthermore, both the probability of initially opening to a sublevel (Fig. 8) and the overall probability of being in a sublevel (Table 1) depended strongly on voltage in drk1-L but not in drk1-LS. The observation that a mutation of the voltage sensor that affected activation resulted in similar changes in sublevel behavior strongly supports a causal relationship between activation state and sublevel abundance.

Mechanisms of subconductance levels

Subconductance levels have been reported to occur in a large number of channels (Auerbach and Sachs, 1983; Cull-Candy and Usowicz, 1987; Geletyuk and Kazachenko, 1985; Hamill et al., 1983; Jahr and Stevens, 1987; Matsuda, 1988; Meves and Nagy, 1989; Nilius et al., 1989). In Ca-activated K channels a subconductance level was found to be associated with transitions between the closed and the fully open states (Ferguson et al., 1993). Likewise, a period of subconductance behavior was reported to precede full openings in a chloride channel from skeletal muscle (Blatz, 1990). Recently subconductance states were observed in cGMP-gated cation channels in the salamander retina (Taylor and Baylor, 1995). Because as the time spent in sublevels decreased with cGMP concentration, it was suggested that sublevels were associated with partially liganded channels. Similar concentration-dependent subconductance levels have been reported for a chimeric AMPA-kainate receptor (Rosenmund and Stevens, 1996).

One possible mechanistic explanation for subconductance levels is that the channel has a multibarrel structure (Hunter and Giebisch, 1987). However, low-resolution structural images seem to rule out the existence of more than one pore in both ligand- and voltage-gated channels (Unwin, 1993; Li et al., 1994). A second potential mechanism for subconductance levels involves partial block of the channel by divalents, protons, or toxins, either interacting directly with the open pore or interfering allosterically with permeation (Hess et al., 1989; Lucchesi and Moczydlowski, 1990; Pietrobon et al., 1988; Prod'Hom et al., 1987; Schild and Moczydlowski, 1994). Strong support for the existence of such a mechanism has been obtained for a cloned cGMP-gated channel, where protonation of negatively charged residues in the pore produces subconductance levels (Root and Mackinnon, 1994). Because the sublevels described here depend critically on the state of activation of the channel, it seemed unlikely that they would be caused by protonation of pore residues. To exclude this possibility formally, we recorded single-channel behavior of the slow-activation mutant drk1-LS at pH 5 and 9. The behavior of the channel was found not to be affected by proton concentration (data not shown).

A third potential source for subconductance levels is filter artifacts produced by rapid switching between closed (C) and fully open (O) levels (Fig. 3; see also Moss and Moczydlowski, 1996). Based on the data presented here, it is impossible to determine whether the intermediate current levels resulted from true subconductance levels or from fast C–O switching. Implications of both possibilities are therefore considered.

Interpretation of sublevels: subconductance states versus fast switching

Figure 10 illustrates two classes of Markov models capable of producing activation-dependent sublevels. Following a step depolarization, the channel proceeds from a resting state to the open state. During this activation process the channel traverses several partially activated, intermediate conformations in which the activation state of the subunits, as well as the number of activated subunits, increases (Sigworth, 1993; Zagotta et al., 1994b). Some of the partially activated states have access to channel conformations that produce sublevels. In Fig. 10 *A* the sublevels are caused by true subconductance states. In Fig. 10 *B* they result from filter artifacts produced by rapid C–O switching. Because the channel has the ability to open both with and without visiting a resolvable sublevel, states that produce sublevels were modeled as an optional, parallel pathway. Both situations depicted in Fig. 10 challenge the common assumption that complete activation must precede channel opening. Partially activated channels seem to have access to open states, although these states are very short lived. Stable openings apparently still require full activation.

If fast C–O switching produces sublevels, then partially activated channels must have (brief) access to the fully open

TABLE 1 Summary of amplitude distributions

	drk1-L −40 mV		drk1-L 0 mV		drk1-LS 0 mV		drk1-LS +40 mV		
	Amp (%)	Area (%)	Amp (%)	Area (%)	Amp (%)	Area (%)	Amp (%)	Area (%)	All Amp (%)
Closed	0 (3)	29 ± 7 (3)	0 (10)	34 ± 5 (10)	0 (8)	47 ± 4 (8)	0 (8)	32 ± 5 (8)	0 (29)
Sub1	12 ± 2 (3)	7.9 ± 4.0 (3)	15 ± 3 (3)	1.0 ± 0.6 (10)	15 ± 1 (8)	8.2 ± 1.7 (8)	15 ± 1 (8)	7.9 ± 1.1 (8)	15 ± 1 (22)
Sub2	42 ± 4 (2)	1.0 ± 0.6 (3)	(0)	0.0 ± 0.0 (10)	33 ± 3 (4)	1.5 ± 0.8 (8)	38 ± 5 (3)	0.5 ± 0.3 (8)	37 ± 4 (9)
Sub3	53 ± 1 (2)	4.1 ± 4.2 (3)	64 ± 5 (3)	0.4 ± 0.2 (10)	58 ± 1 (8)	4.3 ± 1.1 (8)	58 ± 1 (8)	2.5 ± 0.9 (8)	58 ± 2 (21)
Sub4	82 ± 3 (3)	1.7 ± 0.3 (3)	85 (1)	0.1 ± 0.1 (10)	80 ± 2 (5)	3.0 ± 1.1 (8)	82 ± 2 (7)	1.8 ± 1.1 (8)	82 ± 2 (16)
Open	100 (3)	56 ± 6 (3)	100 (10)	64 ± 5 (10)	100 (8)	36 ± 3 (8)	100 (8)	54 ± 5 (8)	100 (29)
P (sub)		15 ± 2		1.4 ± 0.3		17 ± 2		13 ± 2	

Amplitude histograms were fitted with sums of Gaussians (Fig. 6) for drk1-L at −40 mV (N = 3) and 0 mV (N = 10) as well as for drk1-LS at 0 mV (N = 8) and +40 mV (N = 8). The number of Gaussian components was optimized as described. Sublevel amplitudes are expressed as a percentage of the fully open state; relative areas are also listed as percentages. All values are given in the following format: mean ± SE (N). The probability of being in a substate, P(sub), was calculated by summing the relative areas for the four sublevels. In drk1-L P(sub) decreases from 15% at −40 mV to 1.4% at 0 mV. In drk1-LS P(sub) is relatively high at all potentials: 17% at 0 mV and 13% at +40 mV. The 15%, 58%, and 82% sublevels were observed in 22, 21, and 16 of 29 histograms, respectively. The 37% level was detected in only 9 out 29 histograms, and it usually represented less than 1% of the data points.

state. There are two mutually exclusive implications of this situation. The first possibility is that a conformational change in the pore-forming region of each subunit is not required for complete channel opening. Alternatively, a resting subunit can be coerced to go to the "open" conformation by a neighboring activated subunit. In either case, the channel is capable of opening fully without being completely activated.

Sublevels produced by rapid C–O switching are characterized by broadened amplitude distributions. The lack of broadening observed in the data presented here implies that rate of switching would have to be extremely high ($>10^5$ s^{-1}; Fig. 2) and the lifetimes of the closed and open states would have to be extremely short (<10 μs). Because the resolvable fully open and closed states seen following complete activation are several orders of magnitude more stable, the fast-switching model (Fig. 10 *B*) results in a discontinuity in channel behavior when the last subunit activates.

In the subconductance model (Fig. 10 *A*), partially activated channels can visit partially conducting states. This implies that individual subunits are capable of supporting ion permeation. That is not to say, however, that individual subunits accomplish this by independently contributing a single pore to an overall multibarrel structure. The observation that sublevel amplitudes are nonequidistant, in fact, argues against four parallel, identical, and independent permeation pathways.

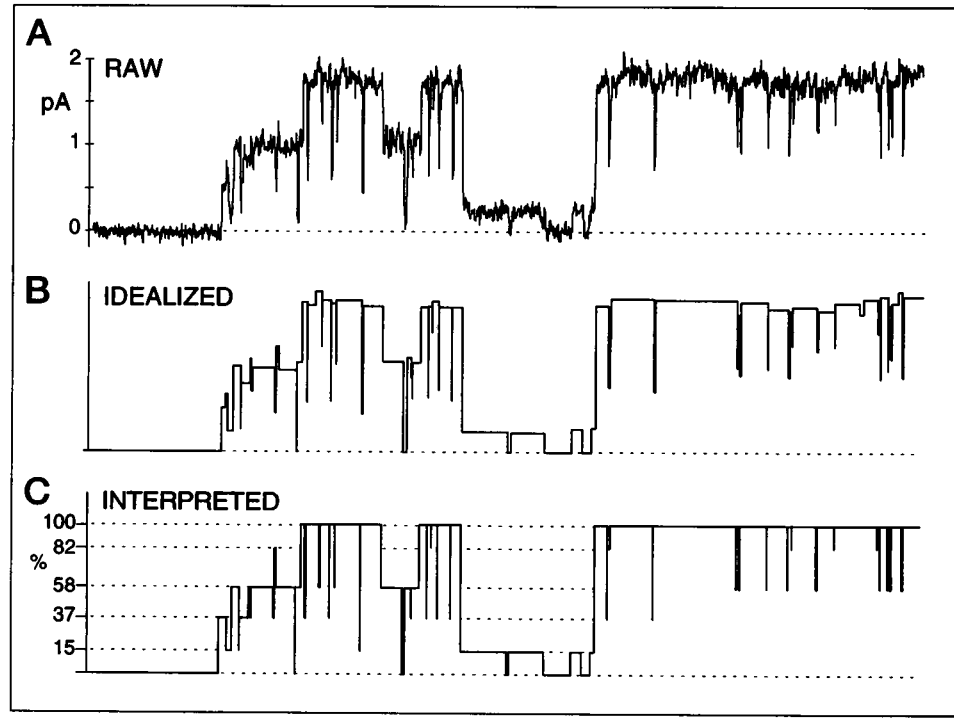

FIGURE 7 Idealization and Interpretation of single-channel data. *A*, Example of a single-channel record of drk1-LS obtained by a 750-ms step depolarization to 0 mV. Recording conditions were the same as for Fig. 1. *B*, Idealization of the record in *A* by the TRANSIT algorithm. *C*, "Interpretation" of the idealization in *B* using the five conductance levels from Table 1. Idealized levels are assigned to the closest conductance state. The result is used for the subsequent statistical analysis.

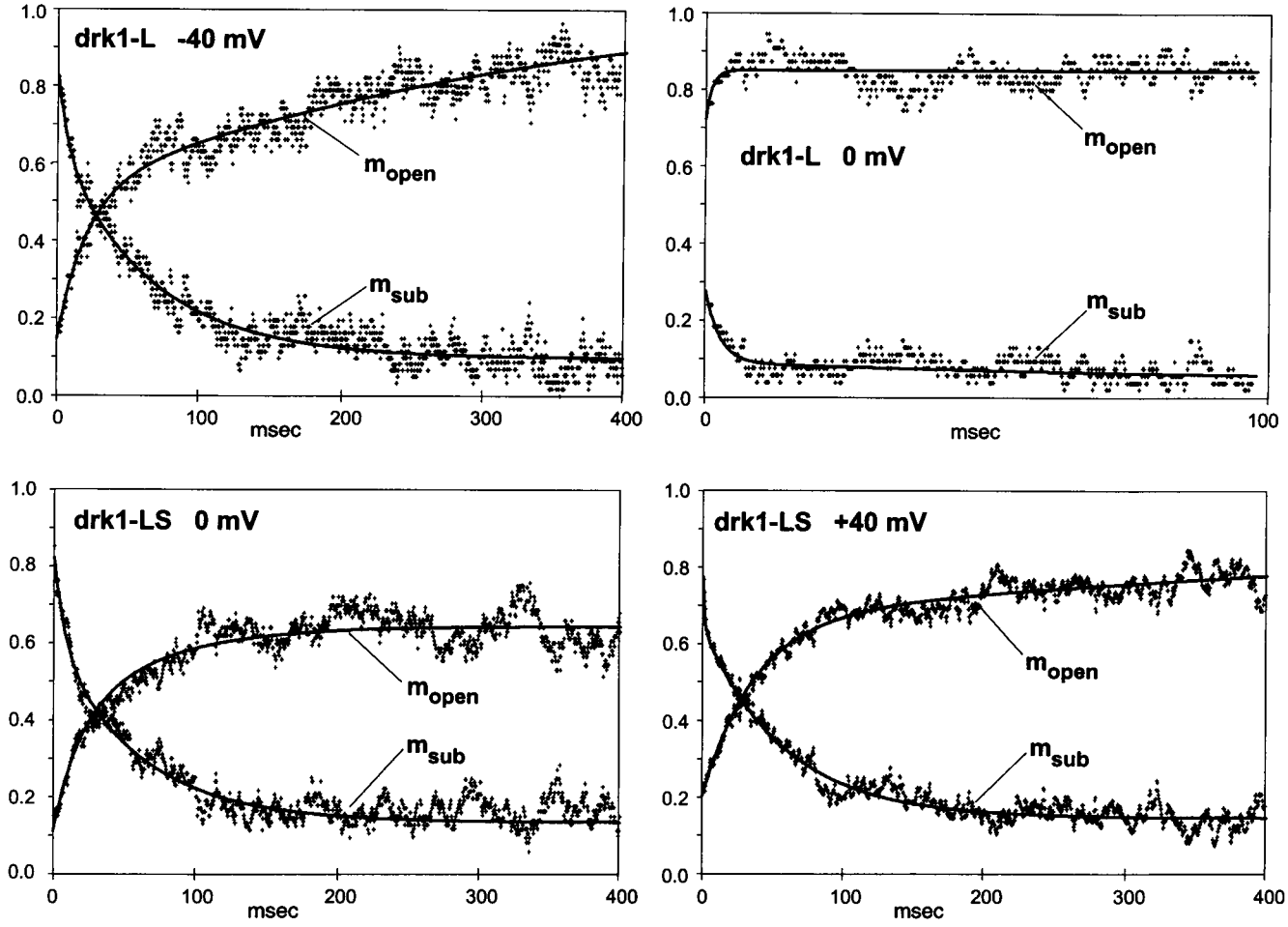

FIGURE 8 Voltage and time dependence of sublevel behavior: conditional open probabilities. The conditional probabilities of being in a sublevel, m_{sub}, or in the fully open state, m_{open}, were estimated from idealized and "interpreted" single-channel data (see Fig. 7) as described in Materials and Methods. The probabilities were well fitted with a sum of two exponentials (*solid lines*). Following the first opening at $t = 0$, m_{sub} decreases over time, whereas m_{open} increases with similar kinetics. For drk1-L at threshold (−40 mV), the probability of being in a sublevel is high initially (~80%) and decreases slowly to ~10%. The probability of being in the fully open state follows an inverse process. At a more depolarized potential (0 mV) the initial value of m_{sub} is lower (28%), and it decreases much faster to below 10% (note the difference in time scale). Again, m_{open} shows the complementary behavior. The conditional probabilities for drk1-LS behave similarly, except that the kinetics are slow and show relatively little voltage dependence.

Theoretical models of permeation do allow for multiple conductance levels arising from a single pore (Dani and Fox, 1991). Additionally, the idea that individual subunits may permit ion permeation is supported by experiments in which subunits containing lethal point mutations in the pore could be rescued by coassembly with functional subunits (Pascual et al., 1995; Lu and Miller, 1995). These observations, in conjunction with the data presented here, strongly support a role for individual subunits in determining the overall functionality and behavior of ion channels.

Sublevels and heteromeric subunit conformations

The correlation of sublevel abundance with the state of activation can be explained by Markov models similar to the general models shown in Fig. 10. Whereas such Markov models may be capable of accurately describing the behavior of the channel, their "states" cannot be unambiguously interpreted as specific channel conformations. A model based on specific subunit conformations is illustrated in Fig. 3. If the heteromeric pore conformations (*columns H1–H3*) are allowed to produce sublevels, then this model is capable of describing the data presented above. The model then offers a straightforward explanation for the subconductance interpretation of sublevels: the permeation rate of the channel increases with the number of subunits in the "open" conformation. To make the model compatible with the interpretation of sublevels as fast-switching artifacts requires that additional states be added, producing a more complex model that requires additional assumptions to be made.

If sublevels result from heteromeric pore conformations, then the four observed sublevels (Fig. 6 and Table 1) may correspond to the four heteromeric channel conformations shown in Fig. 1 *A*. It is tempting to assign the 15% and 82% levels to H1 and H3, respectively, although we have not

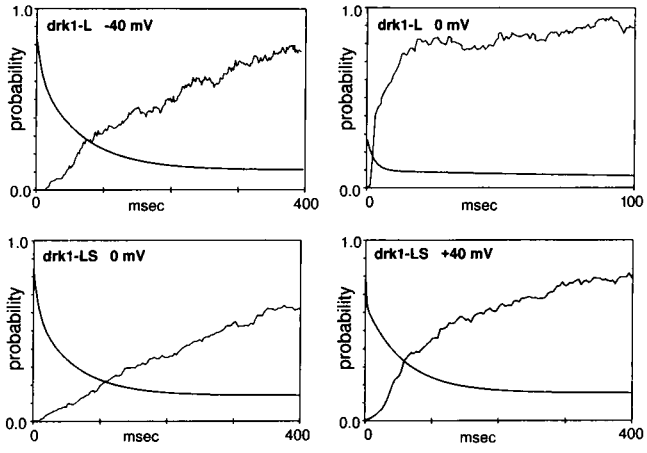

FIGURE 9 Voltage and time dependence of activation. Activation kinetics were characterized for the same four data sets used in Fig. 8. Current ensemble averages were calculated and converted into open probabilities by dividing by the current through a (fully) open channel. Records in which the channel failed to open (nulls) were omitted from the calculation. The activation kinetics for drk1-L are very voltage dependent (*top panels*). Activation of drk1-LS was slow and much less voltage dependent (*bottom panels*).

presented direct evidence that this would be correct. The 37% and 58% levels would then be assigned to H2a and H2b or vice versa. Independently of the assignment, the existence of four nonequidistant subconductance levels suggests that the contribution of an individual subunit to permeation (subconductance model) or gating (fast switching model) is not independent of the conformation of the other subunits. This idea is supported by experiments in which small and large conductance subunits were combined. The conductance of channels containing two small and two large conductance subunits depended critically on the subunit arrangement (Liu et al., 1996).

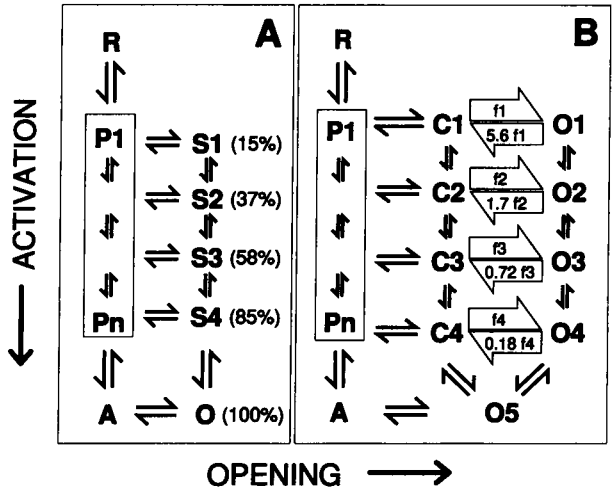

FIGURE 10 Two classes of models describing activation-dependent sublevels. Two classes of models were derived to describe the experimental results. Each model has the same general structure. In each case activation proceeds from the resting state (*R*) through a series of n partially activated states (P1–P*n*) to reach the fully activated state (*A*). The stable open state (O or O5) is accessible only from the fully activated state. *A*, In the first model sublevels arise from true subconductance states (S1–S4), which are accessible from the partially activated states. *B*, The second model is identical to the one in *A*, except that each S state was replaced by a rapidly interconverting C–O pair (Fig. 2). The ratios of forward and reverse rates were constrained to produce apparent subconductance levels of 15%, 38%, 58%, and 82%.

Symmetry, cooperativity, and (in)stability

The relative stability of the homomeric open and closed states implies the existence of stabilizing interactions between neighboring subunits with the same pore conformation. When the first subunit in a homomeric channel moves to a new conformation, it destabilizes the conformation of both of its neighbors, thereby making a conformational change for them more likely. The circular fourfold symmetry of the channel further enhances the instability of heteromeric states and consequently results in a short lifetime for the associated sublevels. Therefore, these levels are usually unresolved, and most transitions between the fully open and the closed states appear smooth, devoid of sublevels. Recordings from large-conductance K channels at extremely low temperatures (−35°C) seem to support the existence of unstable subconductance behavior associated with at least some opening and closing transitions (Miodownik and Nonner, 1995).

In the model shown in Fig. 3, channel openings appear concerted when the channel is fully activated (i.e., is at the bottom row). There are two reasons why sublevels may become more apparent when the channel is partially activated (*rows P1–P3*). First, the stable homomeric open state is not accessible, so all openings are to sublevels. Second, the partially activated, heteromeric open states in which all active subunits contribute to permeation (boxed states in Fig. 3) are relatively stable, because they lack forward rate constants. A channel therefore can get "trapped" in a sublevel that would otherwise be too short lived to be resolved.

Subunit basis for permeation and gating?

Ion permeation and channel opening have historically been treated as properties of the whole channel. The role of the individual subunits in these processes has not been explicitly investigated. Based on the evidence presented above, we propose that whenever a K channel opens, the ion permeation pathway is formed in four discrete steps. In each step an additional subunit moves from a conformation that does not support ion permeation ("closed") to one that does ("open"). There are two ways in which an "open" subunit can support permeation: 1) by allowing the channel to switch rapidly between open and closed conformations and 2) by facilitating the movement of ions through the pore. The difference between an "open" and a "closed" subunit conformation is not known, although the free energy difference between the two is probably small. The channel lining

that a subunit contributes to the pore presents a complex energy surface to a permeating ion (Roux and Karplus, 1994). Ions could be prevented from translocating in the "closed" conformation because of a relatively high energy barrier (e.g., a nonpolar region) or a well that is too deep (e.g., a high-affinity binding site). A conformational change in a subunit that reduces the height of the barrier or the depth of the well would enable the channel to support ion permeation. In this scenario ion permeation and channel opening are coupled: the same structure that controls permeation is also responsible for opening and closing the channel.

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