Single *Streptomyces lividans* K\(^+\) Channels

**Functional Asymmetries and Sidedness of Proton Activation**

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**abstract** Basic electrophysiological properties of the KcsA K\(^+\) channel were examined in planar lipid bilayer membranes. The channel displays open-state rectification and weakly voltage-dependent gating. Tetraethylammonium blocking affinity depends on the side of the bilayer to which the blocker is added. Addition of Na\(^+\) to the trans chamber causes block of open-channel current, while addition to the cis side has no effect. Most striking is the activation of KcsA by protons; channel activity is observed only when the trans bilayer chamber is at low pH. To ascertain which side of the channel faces which chamber, residues with structurally known locations were mapped to defined sides of the bilayer. Mutation of Y82, an external residue, results in changes in tetraethylammonium affinity exclusively from the cis side. Channels with cysteine residues substituted at externally exposed Y82 or internally exposed Q119 are functionally modified by methanethiosulfonate reagents from the cis or trans chambers, respectively. Block by charybdotoxin, known to bind to the channel’s external mouth, is observed only when the toxin is added to the cis side of channels mutated to be toxin sensitive. These results demonstrate unambiguously that the protonation sites linked to gating are on the intracellular portion of the KcsA protein.

**key words:** potassium channel • permeation • gating • block

**INTRODUCTION**

The high-resolution structure of the KcsA K\(^+\) channel has invigorated current approaches to the molecular foundations of cellular electrical excitability (Doyle et al., 1998). KcsA is a prokaryotic channel with little sequence similarity to eukaryotic K\(^+\) channels except in the pore-forming region. However, its structure provides compelling explanations for ion permeation and gating phenomena observed over many years in a multitude of K\(^+\) channels. Ironically, functional properties of KcsA have been described only in outline. Single-channel recording, flux measurements, and ligand-binding assays have shown KcsA to be a high-conductance, tetrameric, K\(^+\)-selective channel with an externally located receptor site for charybdotoxin-family peptides (Schrepf et al., 1995; Cortes and Perozo, 1997; Heginbotham et al., 1997, 1998; MacKinnon et al., 1998). While its structure is largely in harmony with models of familiar K\(^+\) channels, an unexpected characteristic of KcsA is its gating by protons (Cuello et al., 1998; Perozo et al., 1999).

As a prelude to a full ion selectivity study of KcsA, we sought to establish a planar lipid bilayer system in which single purified KcsA channels may be recorded accurately and to survey several basic pore properties of the channel. Single KcsA channels can be observed at 5 kHz bandwidth in a low-noise planar bilayer system. We document functionally asymmetric characteristics of KcsA and use several of these to show that protons gate this channel from the cytoplasmic side of the membrane, not the external, side of the membrane.

**MATERIALS AND METHODS**

**Materials**

General chemicals were of reagent grade or higher. High-purity (>99.997%) KCl was obtained from Alfa Inorganics. [2-(trimethylammonium)ethyl]methanethiosulfonate (MTSET, Br salt)\(^1\) and 2-(sulfonatoethyl) methanethiosulfonate (MTSES, Na salt) were

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\(^{1}\)Abbreviations used in this paper: CTX, charybdotoxin; I–V, current–voltage; MTSES, 2-(sulfonatoethyl)methanethiosulfonate; MTSET, [2-(trimethylammonium)ethyl]methanethiosulfonate; POPE, 1-palmitoyl-2-oleoyl phosphatidylethanolamine; POPS, 1-palmitoyl-2-oleoyl phosphatidylglycerol; TEOA, tetraethylammonium.
obtained from Anatrace. Dodecylmaltoside was from Calbio-
chem Corp. and CHAPS from Pierce Chemical Co. Lipids
(Avanti Polar Lipids) were 1-palmitoyl-2-oleoyl phosphatidy-
ethanolamine (POPE) and phosphatidylglycerol (POPG), stored in
sealed ampules at −80°C. Charybdotoxin (CTX) was expressed
in Escherichia coli and purified as described (Stampe et al.,
1994).

Two slightly different constructs of KcsA were used. Most ex-
periments employed a synthetic gene coding for the natural
KcsA polypeptide sequence with a hexahistidine tag added to the
NH₂ terminus. This was derived by truncating the previously de-
scribed “SIK” construct (Heginbotham et al., 1997) after residue
R₁₆₀, the natural COOH terminus. For charybdoxin blocking
experiments, we used a wild-type KcsA construct kindly provided
by Dr. R. MacKinnon (The Rockefeller University, New York) and
“KcsA-TX,” a triple-mutant of this (Q₅₅₈A/T₆₁₅S/R₆₄₆D) that
binds CTX (MacKinnon et al., 1998).

Solutions used for planar bilayer recording are coded accord-
ing to the convention: nKm, where n and m are numbers denot-
ing the concentration (mM) of K⁺ ion, and the pH, respectively.
The solutions also contained an appropriate anionic buffer. Thus,
solution 200K₇ consists of 195 mM KCl/5 mM KOH/10 mM
HEPES, adjusted to pH 7.0 with HCl, and 20K₄ consists of 15 mM
KCl/5 mM KOH/10 mM succinic acid, adjusted to pH 4.0
with HCl.

Purification and reconstitution of KcsA. KcsA was expressed in E.
coli and purified on Ni²⁺-affinity columns as described (He-
ginbotham et al., 1997; MacKinnon et al., 1998). The purified chan-
nel was eluted in 400 mM imidazole at 1–5 mg/ml protein con-
centration quantified by the extinction coefficient at 280 nm
(Heginbotham et al., 1998). Immediately after purification, KcsA
was reconstituted into liposomes at room temperature as follows.
A micellar solution of phospholipids (7.5 mg/ml POPE, 2.5 mg/
ml POPG) and 34 mM CHAPS in reconstitution buffer (450 mM
KCl/10 mM HEPES/4 mM N-methylglucamine, pH 7.0) was pre-
pared as described (Heginbotham et al., 1998), and KcsA protein
was added to final concentrations of 2.5–10 μg/ml, according to
the number of channels per liposome desired. After 20–30 min
incubation, 400 μl of the mixture was passed down a 20-ml
Sephadex G-50 (fine) column equilibrated with reconstitution
buffer. Liposomes eluted in the void volume with a dilution of ap-
proximately threefold and were stored in 75-μl aliquots at −80°C
for up to 3 mo.

Single-channel recording in planar lipid bilayers. Single-channel re-
cordings of KcsA were performed in a horizontal planar lipid bi-
layer, with the following improvements over the system’s previous
description (Chen and Miller, 1996). Partitions used to hold the
bilayer were cut from 80-μm diameter) were
handcrafted by the melt-and-shave method (Wonderlin et al.,
1990). Electrodes were connected to the cis and trans chambers
by salt bridges (2% agar, 200 mM KCl, 5 mM EGTA). Micro-
phonic noise was greatly reduced by enclosing the bilayer cham-
ber in a metal box soundproofed on all surfaces with “dB-Bloc”
plastic noise was greatly reduced by enclosing the bilayer cham-
ber in a metal box soundproofed on all surfaces with “dB-Bloc”
and resistances were in the 1 TΩ range.

For channel insertion, a day’s supply of reconstituted vesicles
was prepared by thawing an aliquot, transferring the suspension
to a glass test tube, and sonicating in a cylindrical bath sonicator
for ~10 s. Vesicles were kept at room temperature throughout the
day’s use, and then discarded. A newly formed bilayer was
ruptured by physical violence, ~1 μl of reconstituted vesicles
were added to the cis solution directly above the open hole and a
new bilayer was immediately spread. Current was monitored at
100–200-mV holding voltages, and if channels failed to appear
within 5 min, the bilayer was ruptured and the procedure was re-
peated. Typically, channels were observed in ~50% of such at-
ttempts. After channel insertion, recording conditions were estab-
lished by perfusion with desired solutions or by dilution of stock
solutions into the bilayer chamber with mixing. In all experi-
ments reported here, 100 mM K⁺ was present on both sides of
the membrane. All data reported and all standard errors dis-
played are based on three to seven independent experiments.

RESULTS

An essential requirement for structure–function analy-
is of any ion channel is a firm knowledge of its orienta-
tion in the membrane under study. Most ion channels
are studied in their native cellular membranes, where
orientation is obvious. However, the KcsA channel is
best investigated as a purified protein reconstituted in
biochemically defined membranes. In such a system,
transmembrane orientation of the channel is not as-
ured and must be empirically established. This study
proceeds towards a single goal: to assign specific func-
tions to defined sides of the KcsA protein. We approach
this goal in two steps. First, we show that several of
the channel’s fundamental properties of gating, perme-
ation, and blockage are asymmetric with respect to the
cis and trans sides of the reconstituted membrane.
Then, the channel’s absolute orientation is established
by assigning specific residues in the KcsA structure to the
corresponding sides of the bilayer.

The planar bilayer system consists of two experimen-
tally accessible aqueous phases: the cis solution to
which KcsA-reconstituted liposomes are added, and the
opposing trans solution. According to the electrical po-
larity convention used here, the cis chamber is the
zero-voltage reference. Single KcsA channels were in-
serted into planar lipid bilayers under asymmetric salt
conditions, and both sides of the bilayer were then
flushed with the desired recording solutions.

Asymmetry of Gating by Voltage and pH

Like other two-transmembrane helix K⁺ channels, KcsA
lacks an S4 voltage sensor. However, its gating shows a
definite, though weak, voltage dependence. Fig. 1 compares
KcsA channel activity at opposite volt-
age polarities in a multiple-channel membrane. At high
positive voltage (175 mV), channel activity is marked by
frequent openings throughout the duration of the
record, a pattern that changes dramatically when volt-
age is reversed in polarity. At high negative voltage
(--175 mV), the channel open probability is much lower. Most channels (>80%) insert into the planar bilayer with this orientation; a minority show reversed orientation, with frequent openings at negative voltages. Thus, KcsA channels preferentially orient in the reconstituted membrane, but the results do not even hint at their absolute orientation; i.e., which side of the bilayer is equivalent to the cytoplasmic or external face of the channel protein.

KcsA is a proton-activated channel. As described by Cuello et al. (1998), the channel opens significantly in planar bilayers only at pH values below ~5. Fig. 2 shows, as in the original observations, that proton binding is linked to gating in a strictly sided manner. KcsA activity is responsive only to the pH of the trans solution; cis pH has no effect. These pH-dependent changes in gating are immediate upon perfusion of the trans side of the bilayer and are fully reversible.

The majority of channels incorporate into the membrane with sensitivity to trans pH, only a minority appearing with reversed sensitivity. (The channels sensitive to cis pH also show reversed voltage sensitivity.) We exploited this asymmetric pH sensitivity to ensure that all channels observed have a single orientation; all subsequent experiments were performed with trans pH 4 and cis pH 7, a maneuver that enforces a perfectly oriented set of active channels by silencing any channel inserting in the "minority" direction.

Asymmetry of Ion Permeation

In symmetric 100-mM K⁺ solutions, KcsA shows open-channel rectification, as seen in the raw recordings and the open-channel current-voltage (I–V) curve of Fig. 3. Channels are well defined in amplitude and do not display the substate behavior reported previously (Schrempf et al., 1995; Cuello et al., 1998). Under these conditions, chord conductances are 56 and 31 pS at 200 and −200 mV, respectively, and zero-voltage slope conductance is 83 pS. The open channel is substantially noisier at negative potentials than at positive. At all potentials, the open state displays noise in excess of the inherent instrumental noise seen during closed intervals; this observation suggests that the open KcsA channel undergoes rapid, unresolved transitions. These transitions do not arise from endogenous blockers since the only cation present in this chemically defined system, aside from K⁺, is H⁺ and since the excess noise is not noticeably altered by changes of cis pH (in the range 4–7), by cis addition of 1 mM EDTA, or by the use of "purissimum grade" KCl in the recording solutions (data not shown).

Asymmetry of Tetraethylammonium Block

Many eukaryotic K⁺ channels are reversibly blocked by tetraethylammonium (TEA), which can bind to two distinct sites located near the two ends of the narrow selectivity filter (MacKinnon and Yellen, 1990; Yellen et al., 1991; Newland et al., 1992). KcsA is also sensitive to TEA applied to either side of the membrane (Fig. 4). TEA reduces the open-channel amplitude in a concentration-dependent fashion, an effect expected for a reversible, low-affinity blocker with kinetics too rapid to be resolved by the recording electronics (Coronado and Miller, 1979). At 200 mV, inhibition constants of 3 and 23 mM are seen for TEA added to the cis and trans solutions, respectively. The difference in affinity from the two sides must arise from intrinsic chemical differ-
ences of the binding sites involved, since the voltage polarity promotes blocker binding from the trans side and hinders it from the cis.

Na⁺ Block: A Hint of Absolute Orientation

A biological imperative of K⁺ channels is to prohibit permeation by Na⁺. However, far from being inert to K⁺ channels, Na⁺ is known to interact with them in a sided fashion, blocking K⁺ currents in nerve and muscle membranes exclusively from the intracellular side (Bezanilla and Armstrong, 1972; French and Wells, 1977; Yellen, 1984). This Na⁺ blocking asymmetry is satisfactorily rationalized in terms of the KcsA structure, which shows a widening of the pore in the center of the membrane (Doyle et al., 1998). Small cations should be able to gain ready admittance to this region from the internal solution, but would be unable to pass further through the narrow K⁺ selectivity filter. A Na⁺ ion in this region would thus block outward, but not inward, K⁺ current. Being rigorously excluded from the externally disposed selectivity filter, Na⁺ would be a functionally inert cation from the extracellular side.

This Na⁺ blocking behavior seen in eukaryotic K⁺ channels is echoed in KcsA (Fig. 5). Addition of 30 mM Na⁺ to the cis side has no effect on the open-channel I–V relation in symmetrical K⁺. In contrast, 10 mM Na⁺ added to the trans side reduces the channel amplitude with voltage dependence strong enough to produce a negative conductance, as seen originally in squid axon K⁺ channels (Bezanilla and Armstrong, 1972). The sidedness of Na⁺ action intimates, by precedent from excitable membrane channels, that the trans-facing side of KcsA is intracellular, and the cis side extracellular.

The Absolute Orientation of KcsA

This conclusion is uncertain, however, because of its reliance on analogy to only a few carefully studied K⁺ channels. We therefore scrutinized the absolute sidedness of KcsA by combining measurements of the functional influences of specific residues with the channel’s known structure (Fig. 6).

Figure 3. Currents through single KcsA channels. Recordings of single KcsA channels were made under conditions of Fig. 1. (A) Representative channel openings at the indicated voltages. (B) Open-channel current-voltage relation. Standard errors are smaller than the width of the points. Solid curve has no theoretical meaning.

Figure 4. TEA blockade of KcsA. (Top) KcsA channels were recorded at 200 mV with indicated TEA concentrations added to the 100-mM K⁺ solutions; these data were filtered at 1 kHz. (Bottom) Open-channel current, i, normalized to the value without TEA, i₀, is shown with single-site inhibition curves, with Kᵢ = 3.2 and 22 mM for cis and trans, respectively.

Figure 6. Location of the external TEA binding site. It is well established in eukaryotic K⁺ channels that the affinity of external TEA block is enhanced by an aromatic residue at the position equivalent to Y82 in KcsA (MacKinnon and Yellen, 1990; Heginbotham and MacKinnon, 1992; Kavanaugh et al., 1992). Accordingly, we examined the effects of substitutions at Y82 on TEA block (Fig. 7).
The affinity of TEA blockade from the cis side is responsive to substitution here, with tyrosine producing the strongest block ($K_i = 3.2$ mM) and the nonaromatic substitutions showing weaker blocking affinities ($Y82C, K_i = 23$ mM; $Y82T, K_i = 143$ mM). In contrast, TEA inhibition from the trans side is insensitive to these replacements. These results argue that position 82, known from the structure to be externally exposed, faces the cis aqueous solution.

Covalent modification of Y82C. The introduction of cysteine at position 82 offers an independent means of assessing the channel’s orientation. Devoid of cysteine, KcsA is an ideal target for analysis by site-specific modification. In eukaryotic $K^+$ channels, ion permeation is affected by substitutions at this position (MacKinnon and Yellen, 1990), as may be easily understood from the KcsA structure, which places Y82 close to the external opening of the narrow pore (Fig. 6). We therefore sought functional evidence of chemical modification of Y82C by MTSET, a cationic, water-soluble sulfhydryl-modifying reagent.

The Y82C substitution preserves the basic KcsA properties of trans pH sensitivity and voltage-dependent gating, but it alters the shape of the open-channel I–V curve, nearly eliminating the rectification normally observed (Fig. 8). The I–V curve was unchanged immediately following addition of 70 $\mu$M MTSET to the cis compartment, but $\sim$3 min of exposure to the reagent led to a distinct asymmetry. This rectification resulted from a decrease in cisto-trans current while leaving current in the reverse direction unchanged, as expected from an electrostatic influence of a positive charge near the channel’s cis entryway. This effect persisted after removal of the MTSET by perfusion, and it was not reversed by several minutes of exposure to 5 mM dithiothreitol. Since MTSET is membrane impermeant (Holmgren et al., 1996), and since the acidic pH of the trans side greatly favors formation of the thiolate nucleophile, the reagent-induced alteration in I–V curve further supports a cis-facing location of residue 82. We have not attempted to determine the number of subunits modified by MTSET under these conditions. However, prolonged application of the reagent leads to the disappearance of the channel, and so we suspect that channels like the one shown in Fig. 8 have not
been modified on all four subunits. In unsurprising control experiments without the cysteine substitution at Y82, MTSET had no effect (data not shown).

The modification of Y82C provoked a supplementary experiment. We have already seen that mutations at Y82 affect external TEA block, and we anticipate from experiments on the equivalent position in Shaker channels (MacKinnon and Yellen, 1990) that modification of this residue by MTSET would greatly reduce TEA affinity. Single Y82C channels were held at 200 mV (K⁺ current towards the cis side), and upon cis addition of 10 mM TEA, open-channel current was reduced by ~35% (Fig. 9). MTSET was then added to the cis side. Initially, there was no effect of the reagent, but after ~1 min, an increase of open-channel current occurred (concurrently with a shortening of open times). This increase reflects the expected decrease of TEA affinity arising from the introduction of a trimethylammonium group near the blocking site. This result corroborates the cis exposure of position 82.

Charybdotoxin sensitivity of KcsA. Scorpion venom peptides of the charybdotoxin family block eukaryotic K⁺ channels by binding to a receptor site in the outer vestibule, thereby occluding the conduction pathway (Miller, 1995). Wild-type KcsA fails to bind CTX, but a triple mutant in the CTX-receptor region, KcsA-Tx, reveals a binding site for radiolabeled CTX (MacKinnon et al., 1998), albeit with rather low affinity (0.1–1 μM).

Fig. 10 demonstrates block of single KcsA-Tx channels by CTX added to the cis side. As expected (MacKinnon et al., 1998), CTX is without effect on the wild-type channel and is profoundly inhibitory on KcsA-Tx. The toxin acts by shortening open times and by modestly lengthening closed times, as expected for a bimolecular process slow enough to resolve individual toxin-block events. CTX action is fully reversed upon perfusion with toxin-free solutions, as illustrated by the open-time histograms of Fig. 10 B. It is not our purpose to carry out a detailed analysis of toxin block, which would minimally require dissecting a four-state model (Anderson et al., 1988). Qualitatively, however, the toxin dwell-time appears to be close to the normal closed times in the absence of toxin (10–100 ms). Furthermore, analysis of the shortening of open times
leads to an estimated bimolecular association rate constant of $\sim 4 \times 10^8 \text{M}^{-1}\text{s}^{-1}$, 60-fold higher than that seen for Shaker channels at a similar ionic strength (Goldstein and Miller, 1993). In additional experiments, CTX-K27H, a toxin variant with greatly reduced affinity for Shaker $K^+$ channels (Goldstein et al., 1994), had a minimal effect on KcsA-Tx (data not shown). Thus, CTX block of KcsA mirrors its action in eukaryotic systems and buttresses the argument (MacKinnon et al., 1998) for fundamentally similar toxin receptor sites in eukaryotic and prokaryotic $K^+$ channels. The results dictate a cis-facing location for this externally exposed receptor site in KcsA. We did not perform experiments with trans addition of CTX, since the acid conditions on the trans side would make the expected negative results meaningless; the conclusion of a cis-facing CTX receptor stands without these controls.

**DISCUSSION**

These experiments exploit a fully oriented reconstituted system in which single KcsA channels, purified af-

**Figure 9.** Effect of MTSET on TEA sensitivity of KcsA-Y82C. Time line (top) indicates the sequence of addition of TEA (10 mM) and MTSET (350 $\mu$M). Currents were recorded from Y82C at 200 mV. All-points amplitude histograms were constructed from 30–40 s of continuous data. The data shown are from a single experiment; the experiment was repeated two more times with similar results.
ter high-level expression in E. coli, can be studied electrophysiologically. We have taken pains to document functional asymmetries in KcsA to assign absolute sidedness to the system. Eight separate asymmetries were examined: voltage-dependent gating, proton activation, open-channel rectification, block by Na\textsuperscript{+}, TEA, and CTX, and covalent modification of channels with cysteines substituted on either side of the membrane. Three independent lines of evidence establish the channel’s orientation in the bilayer. The cis solution bathes the extracellular face of the channel protein containing the CTX receptor and the aromatic TEA blocking site. The trans solution bathes the intracellular face containing Q119.

These assignments unambiguously demonstrate that the protonation sites linked to KcsA gating face the intracellular solution. This orientation makes sense from a statistical standpoint: of the 56 carboxylate groups in KcsA (16 asp, 36 glu, 4 COOH termini), 44 are located at intracellular positions; all 44 histidine residues in the His-tagged protein are also intracellular. Previously, Cuello et al. (1998), arguing from protection of liposome-reconstituted KcsA against proteolysis, had concluded the reverse: that extracellular protons gate the channel. Our own results, in contrast, show robust proteolysis of KcsA under similar conditions (data not shown).

Our results are perplexing from a biological perspective. Indeed, they suggest strongly that this channel is not gated by low pH in its native membrane, since Streptomyces, like most bacteria, tightly regulates cytoplasmic pH near neutrality. If the physiological role of KcsA is in fact to gate the K\textsuperscript{+} conductance of the bacterial

Figure 10. CTX block of KcsA. KcsA wild-type or KcsA-Tx triple mutant channels were held at 150 mV in 100 mM K\textsuperscript{+} solutions; bovine serum albumin (50 \(\mu\)g/ml) was also included in the cis solution to suppress nonspecific binding of CTX to the chamber walls. (A) Records are displayed before (control) or after (+CTX) addition of 1 \(\mu\)M CTX to the cis chamber. (Top) Wild-type KcsA; (bottom) KcsA-Tx. (B) Representative open-time histograms of KcsA-Tx collected from 30 s of continuous data for each condition indicated. Time constants were determined from single-exponential fits to the histograms.

Figure 11. MTSES modification of KcsA-Q119C. Open-channel I–V curves were determined on the Q119C mutant before and after addition to MTSES in the trans chamber. After collecting control records, the trans chamber was perfused with 100K7 solution containing 200 \(\mu\)M MTSES, and the reaction was allowed to proceed for 2 min. Fresh 100K4 solution without MTSES was then reintroduced, and the I–V curve was recorded. Data shown in the figure are from a single experiment; similar results were obtained in three separate bilayers.
membrane, then it is likely that some factor other than pH, perhaps an as yet unrecognized partner protein, provides control of gating. Questions of this kind must remain unresolved until the physiological purposes of prokaryotic K⁺ channels are clarified.

In the course of assessing KcsA orientation, we have also shown that pore characteristics of this prokaryotic channel are remarkably similar to those of many well-studied eukaryotic K⁺ channels. The functional familiarity of KcsA is not surprising, given how closely the structural features proposed for eukaryotic K⁺ channels match those actually observed with KcsA (Doyle et al., 1998; Yellen, 1998). But it is nevertheless important to confirm this functional similarity by experiment, since KcsA provides the first opportunity for a direct structure–function assault on an ion-selective channel.

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