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Authors

Sesti, Federico
Tai, Kwok-Keung
Goldstein, Steve AN

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MinK Endows the I_{Ks} Potassium Channel Pore with Sensitivity to Internal Tetraethylammonium

Federico Sesti, Kwok-Keung Tai, and Steve A. N. Goldstein

Section of Developmental Biology and Biophysics, Departments of Pediatrics and Cellular and Molecular Physiology, Boyer Center for Molecular Medicine, Yale University School of Medicine, New Haven, Connecticut 06536 USA

ABSTRACT I_{Ks} channels are heteromeric complexes of pore-forming KvLQT1 subunits and pore-associated MinK subunits. Channels formed only of KvLQT1 subunits vary from I_{Ks} channels in their gating kinetics, single-channel conductance, and ion selectivity. Here we show that I_{Ks} channels are more sensitive to blockade by internal tetraethylammonium ion (TEA) than KvLQT1 channels. Inhibition by internal TEA is shown to proceed by a simple bimolecular interaction in the I_{Ks} conduction pathway. Application of a noise-variance strategy suggests that MinK enhances blockade by increasing the dwell time of TEA on its pore site from ~ 70 to $370 \mu\text{s}$. Mutation of consecutive residues across the single transmembrane segment of MinK identifies positions that alter TEA blockade of I_{Ks} channels. MinK is seen to determine the pharmacology of I_{Ks} channels in addition to establishing their biophysical attributes.

INTRODUCTION

Amino acids lining the conduction pathway in ion channels influence their selectivity, unitary conductance, and gating kinetics and serve as receptor sites for clinically important medications, including analgesics, anticonvulsants, and antiarrhythmics (Hille, 1992). Quaternary ammonium ions (like tetraethylammonium, TEA) that block potassium channels have proved useful as probes of potassium channel structure and function (Armstrong, 1971; Miller, 1988; Yellen, 1998). Thus mutations in ion channel proteins that change blocker affinity have established the contribution of residues to pore formation (MacKinnon and Yellen, 1990; Yellen et al., 1991; Slesinger et al., 1993; Lopez et al., 1994; del Camino et al., 2000) and helped to identify sites that move during channel gating transitions (Choi et al., 1991; Holmgren et al., 1997; Yellen, 1998).

I_{Ks} channels are complexes of KvLQT1 and MinK subunits (Barhanin et al., 1996; Sanguinetti et al., 1996). The duration of the cardiac action potential is strongly influenced by I_{Ks} channel function (Sanguinetti and Jurkiewicz, 1990, 1991). Consequently, inherited mutations of KvLQT1 or MinK are associated with long QT syndrome (LQTS), a disorder that predisposes to cardiac arrhythmia and sudden death (Q. Wang et al., 1996; Splawski et al., 1997). Mutations in MinK that produce LQTS have been found to diminish potassium flux by decreasing I_{Ks} single-channel conductance and/or altering I_{Ks} channel gating; this serves to explain the prolongation of cardiac action potential du-

ration in affected individuals (Splawski et al., 1997; Sesti and Goldstein, 1998).

Human KvLQT1 is a voltage-gated potassium channel subunit with ~ 600 residues, six predicted membrane-spanning domains, and one pore-forming P loop. Human MinK has 129 residues and a single transmembrane domain (Takumi et al., 1988; Murai et al., 1989). MinK is the founding member of an emerging superfamily, the MinK-related peptides (MiRPs) (Abbott and Goldstein, 1998; Abbott et al., 1999). MiRPs establish the functional characteristics of mixed channel complexes in native tissues, determining their gating kinetics, unitary conductance, regulation, and pharmacology. Thus channels formed only of KvLQT1 subunits activate rapidly, exhibit current saturation, and have a small single-channel conductance; in contrast, coassembly of KvLQT1 and MinK subunits yields channels that function like native cardiac I_{Ks} channels: they activate slowly, do not achieve current saturation with prolonged depolarizing pulses, have a fourfold greater unitary conductance (Pusch, 1998; Sesti and Goldstein, 1998; Yang and Sigworth, 1998), and exhibit distinctive responses to a variety of activators and inhibitors (Busch et al., 1997).

In this study we compare blockade of KvLQT1 and I_{Ks} channels by TEA. Channels formed with MinK are found to be more sensitive to internal TEA (and less sensitive to external TEA) than channels formed by KvLQT1 subunits alone. Internal TEA is seen to block I_{Ks} channels by a pore occlusion mechanism, a mechanism previously observed for external TEA (Goldstein and Miller, 1991; Tai and Goldstein, 1998). MinK appears to increase the dwell time of TEA on its internal pore site. Twenty-seven consecutive sites in MinK are evaluated by mutation, and three are found to modify inhibition by TEA. Changes at two sites exposed to the extracellular milieu increase block by external TEA, while substitutions at a residue on the cytoplasmic side of the selectivity filter decrease inhibition by internal TEA. Previously, MinK residues were shown to be exposed in the outer portion of the I_{Ks} channel pore (K.-W. Wang et al.,

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Address reprint requests to Dr. Steve A. N. Goldstein, Section of Developmental Biology and Biophysics, Departments of Pediatrics and Cellular and Molecular Physiology, Boyer Center for Molecular Medicine, Yale University School of Medicine, 295 Congress Avenue, New Haven, CT 06536. Tel.: 203-737-2214; Fax: 203-737-2290; E-mail: steve.goldstein@yale.edu.

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1996) and in the deep pore on both sides of the selectivity filter (Tai and Goldstein, 1998). This report indicates that MinK also contributes to the form and function of the cytoplasmic portion of the ion permeation pathway.

EXPERIMENTAL PROCEDURES

Molecular biology

Mutants of rat and human MinK were produced with the QuikChange mutagenesis kit (Stratagene, La Jolla, CA), followed by insertion of altered gene fragments into translationally silent restriction sites, as previously described (K.-W. Wang et al., 1996; Tai et al., 1997; Tai and Goldstein, 1998). All sequences were confirmed by automated DNA sequencing. Human MinK (S38 isoform) and KvLQT1 cDNAs were gifts from R. Swanson (Merck) and M. T. Keating and M. Sanguinetti (University of Utah), respectively. cRNAs were made for rat genes in pSD (Tai and Goldstein, 1998) and for human genes in pBF2 (Sesti and Goldstein, 1998), using the mMessage mMachine kit (Ambion, Austin TX). Transcripts were quantified by spectroscopy and compared with control samples separated by agarose gel electrophoresis stained with ethidium bromide.

Electrophysiology

Oocytes were isolated from *Xenopus laevis* frogs, defolliculated by collagenase treatment, and injected the following day with 46 nl of sterile water containing 5 ng KvLQT1 and 0 or 1 ng MinK cRNA. Mixtures of cRNAs were prepared immediately before injection with a calibrated pipette. All experiments were performed at room temperature. As reported previously (Tai and Goldstein, 1998), at times longer than 30 h after cRNA injection, there is no evidence of contamination of human I_{Ks} channel currents by complexes containing the KvLQT1-like subunit endogenous to oocytes; the two channel types can be differentiated by their sensitivity to methanethiosulfonates. Data are presented as mean \pm SEM, with the number of cells or patches indicated.

Whole oocyte currents were measured 2–4 days after injection by two-electrode voltage clamp (Oocyte Clamp; Warner Instruments, Hamden, CT) with constant perfusion. Data were sampled at 1 kHz and filtered at 0.25 kHz; leak correction was performed off-line. To study the effect of internally applied TEA in whole-cell mode, cells were microinjected with 2.3 nmol TEA (or more as indicated) in unbuffered sterile water, and studies were undertaken 20–30 min thereafter. Assuming an average solute space of 400 nl for an oocyte (K.-W. Wang et al., 1996), injection of 2.3 nmol TEA corresponds to a concentration of \sim 10 mM TEA in the cytosol. Oocytes studied 20–70 min after TEA injection showed no leak currents and stable block parameters. To study external block, TEA chloride was substituted for NaCl in ND-96 (in mM): 96 NaCl, 2 KCl, 1 MgCl₂, 0.3 CaCl₂, 5 HEPES (pH 7.6).

Patch currents were recorded 2–3 days after cRNA injection, using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA), a Quadra 800 computer, and ACQUIRE software (Instrutech, Great Neck, NY) and stored unfiltered on VHS tape. For noise variance analyses data were sampled at 50 or 100 kHz and filtered through a four-pole Bessel filter at 10 or 25 kHz. Data were analyzed with TAC (Instrutech) and IGOR (WaveMetrics, Lake Oswego, OR) software packages. The bath and the pipette solutions contained (in mM) 100 KCl, 10 HEPES, and 2 EGTA (pH 7.5 with KOH). TEA chloride was added at the indicated concentrations in patch studies without isotonic substitution, as differences were not observed between compensated and noncompensated controls. As indicated, NaCl and KCl were replaced by the chloride salt of a test monovalent cation.

Data fitting

Isochronal open probability curves were fit to the Boltzmann equation:

$$\frac{1}{1 + \exp[(V_{1/2} - V)/V_s]} \quad (1)$$

where V is the applied voltage, $V_{1/2}$ is the half-maximum voltage, and V_s is the slope factor. Dose-response curves were fit to the function

$$\frac{1}{1 + ([TEA]/K_{1/2})^n} \quad (2)$$

where $K_{1/2}$ is the concentration of TEA needed to achieve 50% block and n is the Hill coefficient.

The voltage dependence of block was modeled assuming a single receptor site. The corresponding energy profile was composed of two barriers and one well following (Woodhull, 1973), with the external barrier assumed to be infinitely high, so that the blocked current was

$$\frac{I}{I_{\max}} = I_{\min} + I_0 \exp\left[\frac{z(1 - \delta)eV}{kT}\right] \quad (3)$$

where e , k , T , and z represent, respectively, the electronic charge, the Boltzmann constant, absolute temperature, and the valence on the blocker. I_{\min} is the value of the blocked current for $V \rightarrow \infty$, and I_0 is related to the component of the energy profile that is voltage-independent. δ is the apparent electrical distance and represents the fraction of the transmembrane voltage drop experienced by the blocking particle.

Noise variance analysis

We employed nonstationary noise variance (Sigworth and Zhou, 1992) to determine the unitary current and open probability. All-points amplitude histograms were fit to the Gaussian function:

$$\exp\left[-\frac{(I - I_a)^2}{2\sigma^2}\right] \quad (4)$$

where I and I_a are the measured and mean macroscopic current, respectively, and σ^2 is the noise variance. Unitary current, $i_{s.c.}$, and number of channels in the patch, N , were obtained by a fit to

$$\sigma^2 = -\frac{I_a^2}{N} + i_{s.c.}I_a \quad (5)$$

I_{Ks} currents are characterized by slow development and failure to reach saturation. Currents elicited in the first 120 ms of each test pulse showed no time delay and were assumed to be non-channel-dependent; these leak currents and their variances were subtracted.

RESULTS

Internal TEA block of KvLQT1 and I_{Ks} channels

In whole-cell mode, microinjection of TEA produced no inhibition of KvLQT1 channels but blocked I_{Ks} channels readily (Fig. 1 A). To evaluate this difference quantitatively, KvLQT1 and I_{Ks} channels were studied in excised membrane patches in the absence and presence of internally applied TEA. Channels containing MinK were again seen to be more sensitive to TEA applied from the cytosolic face of the membrane (Fig. 1 B). Dose-response curves constructed

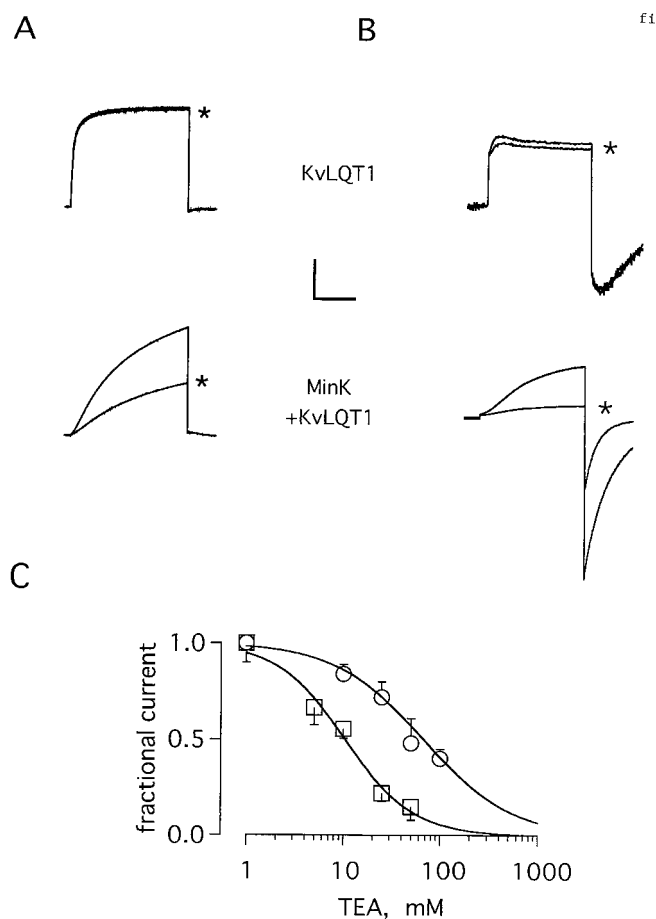


FIGURE 1 MinK endows I_{Ks} with sensitivity to internal TEA blockade. (A) Whole-cell mode. Current traces of an oocyte expressing KvLQT1 subunits alone or MinK and KvLQT1 subunits before and after (*) exposure to internal TEA by microinjection of 2.3 nmol TEA (Materials and Methods). Currents were elicited by 10-s pulses from a holding potential of -80 to 0 mV and are shown after leak subtraction. Scale bars represent 200 nA for KvLQT1 alone, 800 nA for MinK + KvLQT1, and 2 s. (B) Excised patch. Current traces in inside-out macropatches from oocytes expressing KvLQT1 subunits alone or MinK and KvLQT1 subunits before and after (*) exposure to 25 mM internal TEA. Currents were elicited by 6-s pulses from holding a potential of -80 to 40 mV. A classical "hook" is apparent in the KvLQT1 tail current envelope. Scale bars represent 50 pA for KvLQT1 alone, 200 pA for MinK + KvLQT1, and 2 s. (C) Dose response in excised patch mode. Inhibition of current by increasing levels of internal TEA for KvLQT1 channels (\circ) or MinK + KvLQT1 channels (\square). The theoretical lines were constructed according to Eq. 2 from groups of five patches studied at 40 mV. Error bars are SEM; determined values are reported in Table 1.

from groups of four to six patches exposed to various concentrations of TEA revealed equilibrium inhibition constants (K_i) of 11 ± 3 mM for I_{Ks} channels and 58 ± 7 mM at 40 mV for channels formed by KvLQT1 subunits alone (Fig. 1 C and Table 1). In both cases, the relationship of fractional current and TEA concentration fit well to Eq. 2 with a coefficient of ~ 1 , suggesting that a single TEA molecule inhibited one ion channel complex.

Attributes of internal TEA block suggest a pore-directed mechanism

As internal TEA inhibits other potassium channels by direct pore occlusion, we sought to confirm that this mechanism also applied to I_{Ks} channels. Three lines of evidence supported the conclusion. First, inhibition by internal TEA was voltage dependent, with an effective electrical distance ($z\delta$) of 0.88 (Fig. 2 B). This suggested that TEA moved outward through $\sim 12\%$ of the transmembrane electric field to reach its binding site. Second, current reduction by internal TEA was sensitive to the species of monovalent cation in the solution bathing the opposite side of the membrane (Fig. 2 C). Thus external potassium and rubidium ions were very effective in reducing blockade by internal TEA, whereas cesium ions had some effect and impermeant lithium ions did not alter inhibition. Each of the external cations decreased the magnitude of internal TEA block according to its rank order in the relative permeability series for open I_{Ks} channels (Goldstein and Miller, 1991), which is consistent with the idea that the external ions traversed the pore to diminish the affinity of TEA for its internal binding site. Third, internal TEA had only small effects on activation or deactivation kinetics consistent with a model in which the channel opened before the blocker could bind (Fig. 3, A and B). Thus time constants for activation at 40 mV in the absence and presence of internal TEA (at a concentration that achieved 50% blockade) were 4 ± 1 and 3 ± 1 s in the absence and presence of blocker, respectively; time constants for deactivation at -80 mV were 1.0 ± 0.4 and 0.7 ± 0.3 s, respectively. Internal TEA also did not significantly alter the half-maximum voltage for isochronal activation (Fig. 3 C).

MinK decreases the bandwidth required to resolve blocking events by internal TEA

Open-channel blockade can sometimes be observed directly if single channels bind an inhibitor in a stable fashion. However, two factors make it difficult to assess the lifespan of TEA- I_{Ks} channel complexes. First, open I_{Ks} channels "flicker" rapidly between the open and closed states (Sesti and Goldstein, 1998). Second, binding and unbinding of TEA occurs at a rate that is fast relative to customarily employed filter frequencies (not shown). A resolution of both problems was afforded by increasing filter bandwidth. Previously, this allowed determination of unitary current magnitude of I_{Ks} channels by noise variance analysis (Sesti and Goldstein, 1998; Yang and Sigworth, 1998). While single-channel current of I_{Ks} channels was underestimated at 500 Hz (0.17 ± 0.03 pA, Fig. 4, A and B, left), it was seen to be 0.51 ± 0.02 pA at a bandwidth of 10 kHz (Fig. 4, A and B, right) or above (Sesti and Goldstein, 1998).

Increasing bandwidth also allowed assessment of TEA blocking events. At 500 Hz, 10 mM internal TEA decreased

TABLE 1 Functional parameters of KvLQT1 and wild-type and mutant I_{Ks} channels in excised patches

KvLQT1 with	K_i TEA (mM)	n_H	$i_{s.c.}$ (pA)	$V_{1/2}$ (mV)	V_s (mV)	τ_{act} (s)	τ_{deact} (s)
No MinK	58 ± 7	1.0 ± 0.1	0.1 ± 0.0	-6 ± 0	9 ± 2	0.05 ± 0.01	0.5 ± 0.2
Wild-type MinK	11 ± 3	1.2 ± 0.1	0.5 ± 0.2	18 ± 3	17 ± 3	4.1 ± 0.6	1.2 ± 0.2
S68C-MinK	67 ± 11	1.0 ± 0.1	0.5 ± 0.2	54 ± 3	13 ± 3	2.9 ± 0.3	0.2 ± 0.0
S68T-MinK	23 ± 3	1.2 ± 0.2	0.6 ± 0.2	54 ± 1	13 ± 2	4.9 ± 1.2	0.6 ± 0.1
S68Y-MinK	58 ± 7	1.1 ± 0.1	0.5 ± 0.2	43 ± 4	16 ± 4	3.2 ± 1.1	0.6 ± 0.2

Equilibrium inhibition by internal TEA (K_i) and the Hill coefficient (n_H) were determined by fitting to Eq. 2 for data collected at 40 mV; $z\delta$ for internal TEA was 0.88 (Fig. 2 B). Unitary current ($i_{s.c.}$) was determined by nonstationary noise analysis, using Eqs. 4 and 5 for data at a test voltage of 40 mV, sampled at 50 kHz and filtered at 10 kHz. Half-maximum activation voltage ($V_{1/2}$) and slope factor (V_s) were estimated by fitting normalized peak currents at -80 mV to Eq. 1. Activation and deactivation kinetics (τ_{act} , τ_{deact}) were estimated at 40 mV and -80 mV, respectively, with data fit to the function $I_o + I_i \exp(-t/\tau)$. Determinations were made for four to six patches.

both the apparent current magnitude visible by eye (Fig. 4 A, left) and unitary current estimated by Eq. 5 from ~ 0.17 to 0.08 ± 0.02 pA (Fig. 4 B, left). Conversely, data evaluated at 10 kHz suggested no decrease in maximum current magnitude (Fig. 4 A, right) and offered an estimated unitary current with TEA of 0.51 ± 0.02 pA (Fig. 4 B, right),

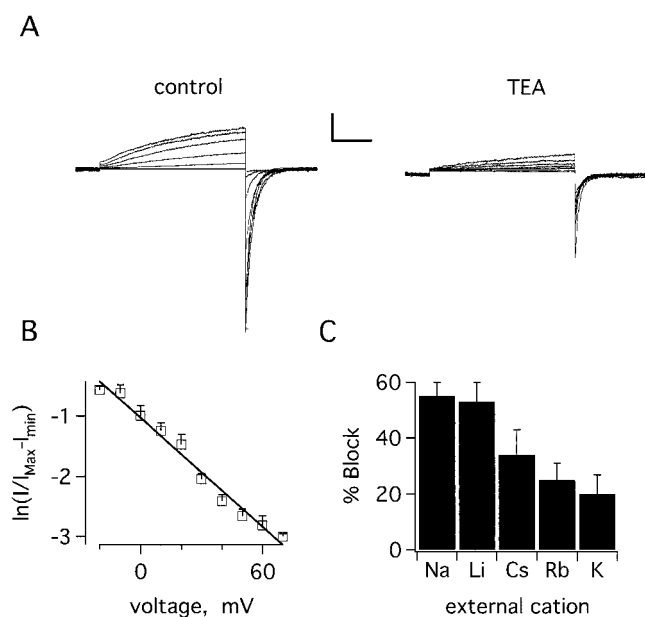


FIGURE 2 Internal TEA blockade of I_{Ks} channels is dependent on the voltage and permeability of monovalent cations on the opposite side of the membrane. (A) Currents in excised inside-out patches in the absence (control) and presence of 10 mM internal TEA. Protocol: 6-s pulses from -80 mV to -20 up to 80 mV in 10-mV steps. Scale bars represent 0.4 nA and 2 s. (B) Voltage dependence of blockade in excised inside-out patches. Current was measured at the end of 6-s pulses to the indicated voltage as in A in the absence (control) and presence of 10 mM TEA. The line is according to Eq. 3 and gives an estimate for $z\delta$ of 0.88 ± 0.05 . (C) Trans-ion effects in whole-cell mode. Current was measured at the end of pulses as in Fig. 1 A in the absence and presence of ~ 10 mM internal TEA, with the indicated monovalent cations substituted for sodium and potassium in the bath for oocytes expressing wild-type rat MinK and human KvLQT1; the percentage of blocked current \pm SEM is plotted for groups of 8–12 cells.

identical to the value without blocker. Indeed, fractional unitary current with TEA (defined as proportional to the fractional variance, σ_{TEA}^2/σ^2 ; see Eq. 5) is expected to approach one with increasing bandwidth. (This follows naturally from description of ion channel transitions between states as power spectra composed of $k - 1$ Lorentzian components, where k is the number of states the channel can visit (Colquhoun and Hawkes, 1977); a single blocked state adds another component and allows a general description of fractional variance:

$$\frac{\sum_{j=1}^{n+1} b_j \arctan(2\pi f/\lambda_j)}{\sum_{j=1}^n b_j^* \arctan(2\pi f/\lambda_j^*)}$$

where * indicates the absence of the blocker, λ_j are corner frequencies, and b_j are coefficients. In the two limiting cases of low ($f \rightarrow 0$) or wide ($f \rightarrow \infty$) bandwidth (offering poor or ideal resolution, respectively) this relationship approaches a constant, as observed experimentally (Fig. 4 C.) Thus, fractional unitary current approached unity for I_{Ks} channels above 2.7 kHz and above 14 kHz for channels without MinK (Fig. 4 C). At low bandwidth fractional unitary current approached ~ 0.5 because the concentration of TEA was chosen to achieve block of half the current at equilibrium (Fig. 4 C). A similar approach has been applied by others to evaluate the dwell time of TEA homologs in Shaker channels (Baukowitz and Yellen, 1996).

Point mutations identify MinK sites that influence TEA affinity

Screening for sites that alter blockade by TEA was performed in whole-cell mode, using point mutants of rat MinK in which individual sites were altered to cysteine. Fig. 5 A shows that mutations at two positions increased the affinity of external TEA without altering block by internal TEA and that mutation at a third site decreased sensitivity to internally applied TEA without altering external TEA blockade. Similar findings were found for channels formed with human MinK subunits mutated at equivalent positions (Fig. 5 B).

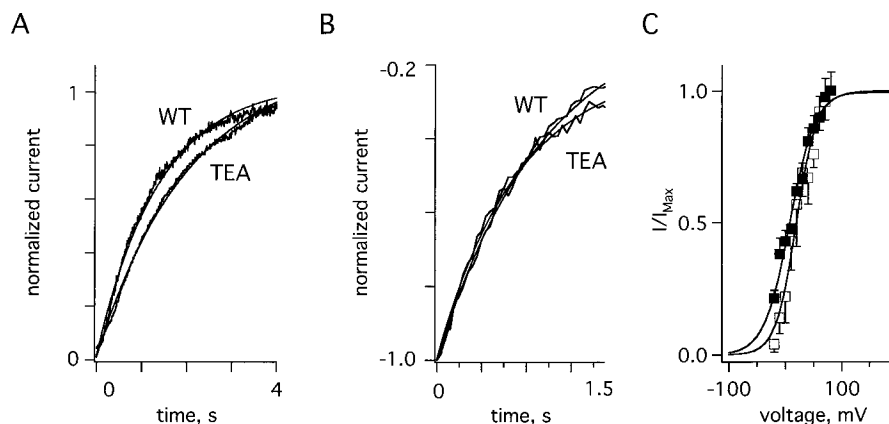


FIGURE 3 Internal TEA has only small effects on activation and deactivation kinetics of I_{Ks} channels in excised outside-out patches. (A) I_{Ks} currents activate similarly in an excised inside-out patch in the absence and presence of 10 mM internal TEA. The time constants for groups of four patches fit with single-exponential functions were 4.0 ± 0.6 and 3 ± 0.6 s at 40 mV in the absence and presence of TEA, respectively. (B) I_{Ks} currents deactivate similarly in an excised inside-out patch in the absence and presence of 10 mM internal TEA. The time constants for groups of four patches fit with single-exponential functions were 1.0 ± 0.4 and 0.7 ± 0.3 s at -80 mV in the absence and presence of TEA, respectively. (C) I_{Ks} current isochronal open probabilities in excised inside-out patches in the absence and presence of 10 mM internal TEA showed a $V_{1/2}$ of 19 ± 3 and 16 ± 3 , respectively.

Channels formed with mutants of human MinK were studied in detail, first in whole-cell mode (Table 2). While external TEA was a weak inhibitor of both KvLQT1 and wild-type I_{Ks} channels, channels formed with F54C-MinK and G55C-MinK were over five times more sensitive to external TEA; these mutations did not alter sensitivity to internal TEA. Conversely, channels formed with S68C-MinK were more than five times less sensitive to internal TEA compared to wild type but showed no change in sensitivity to external TEA (Fig. 5 B and Table 2).

MinK residue S68 influences internal TEA block and channel gating but not unitary current

Channels formed with wild-type MinK or three mutants of MinK altered at position 68 were studied in excised patches (Fig. 6 and Table 1). Compared with channels formed with KvLQT1 subunits alone, MinK increased the unitary channel current by approximately fourfold when evaluated by noise variance (Table 1), as previously reported (Sesti and Goldstein, 1998; Yang and Sigworth, 1998). Assembly of KvLQT1 subunits with S68T, S68C, or S68Y-MinK produced the same increase in single-channel current as wild-type MinK (Table 1). In contrast, all three mutants diminished inhibition by internal TEA without a change in the Hill coefficient. The mutations also altered the half-maximum voltage for activation without significantly changing the slope factor or activation kinetics and speeded deactivation. Fig. 7 A displays the fractional unitary current as a function of bandwidth for channels formed with S68Y-MinK in the presence of 50 mM TEA (these channels are approximately five times less sensitive to TEA than is wild type; Table 1); the fractional current-variance relationship

approached unity above 17 kHz for mutant channels, which is approximately six times greater than the frequency for a current-variance relationship of unity for wild type.

Residue S68 appears to alter internal TEA affinity indirectly

Strategies we used previously to argue for exposure of other MinK residues in the I_{Ks} channel pore do not support a pore location for position 68. Thus channels formed with S68C-MinK did not show altered affinity for internally applied cadmium or zinc (Tai and Goldstein, 1998), nor did they appear to react with water-soluble thiol-reactive methanethiosulfonates similar in size to TEA ($\sim 6\text{--}8$ Å in atomic diameter) (K.-W. Wang et al., 1996; Tai et al., 1997). Although MTS-ES and MTS-ET (the first with a negative charge at neutral pH, the latter with a positive charge) inhibited I_{Ks} channels containing S68C-MinK (Fig. 7 B) as well as those formed with wild-type MinK (not shown), inhibition was rapidly reversible on washing and had no effect on subsequent sensitivity to internal TEA (not shown), which is inconsistent with the formation of a covalent bond. These findings suggest that MinK S68 is not exposed in the pore and that mutations that alter blockade by internal TEA do so indirectly.

DISCUSSION

MinK and the MinK-related peptides (MiRPs) are integral membrane peptides with a single transmembrane span that are active only when coassembled with pore-forming potassium channel subunits. In the resultant complex, the pep-

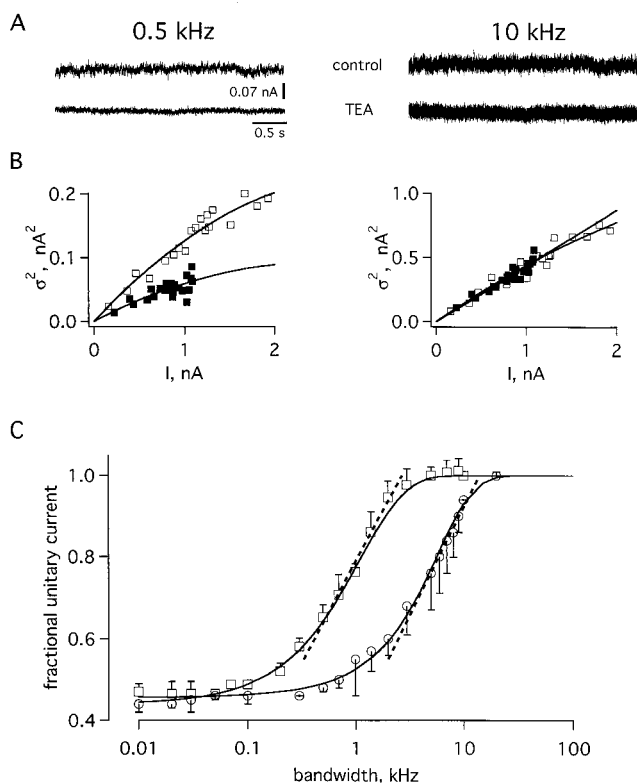


FIGURE 4 MinK increases the residence of TEA on its internal blocking site. (A) Raw current traces at 40 mV with inside-out patches containing I_{Ks} channels in the absence (control) and presence of 10 mM TEA. Data were sampled at 50 kHz and filtered at either 500 Hz (left) or 10 kHz (right), as indicated. The effect of TEA is appreciated qualitatively as a decrease in variation in the current trace at low filter bandwidth (right). (B) Plots display unitary current assessed by noise variance in the absence (□) or the presence (■) of 10 mM TEA at 500 Hz (left) or 10 kHz (right), as indicated. The estimated single-channel current in the patch shown in A was 0.15 and 0.08 pA at 500 Hz (left) and 0.53 and 0.52 pA at 10 kHz (right) in the absence and presence of TEA, respectively. (C) Fractional unitary current versus bandwidth. The fractional unitary current approaches unity as the bandwidth increases. The patches ($n = 3-4$) were exposed to 10 or 50 mM TEA for I_{Ks} channels (□) and KvLQT1 channels (○), respectively, to achieve $\sim 50\%$ inhibition; solid lines are fits to Eq. 1; the dashed lines are extrapolations of the linear portion of the variance-bandwidth relationships and reach 1 at 2.7 kHz for I_{Ks} channels and at 14 kHz for KvLQT1 channels.

tides establish key functional attributes: gating kinetics, single-channel conductance, ion selectivity, regulation, and pharmacology. Heteromeric assembly is required to reconstitute channel behaviors like those observed in native cells. Thus mixed complexes of MinK/KvLQT1 and MiRP1/HERG reproduce the cardiac currents called I_{Ks} and I_{Kr} , respectively, and inherited mutations in all four subunits have been associated with cardiac rhythm disturbances (Abbott and Goldstein, 1998). Studies of MinK have revealed its role in determining I_{Ks} channel ion selectivity (Goldstein and Miller, 1991; Tai and Goldstein, 1998), unitary conductance (Pusch, 1998; Sesti and Goldstein, 1998; Yang and

Sigworth, 1998), gating (Takumi et al., 1991; Splawski et al., 1997; Pusch et al., 1998; Sesti and Goldstein, 1998), modulation by protein kinase C (Busch et al., 1992), regulation by small molecules (Busch et al., 1997), and open-channel blockade by external TEA (Goldstein and Miller, 1991; K.-W. Wang et al., 1996). In this report we demonstrate that MinK also contributes to the structure and function of the internal portion of the I_{Ks} pore, establishing its pharmacology.

MinK endows I_{Ks} channels with increased sensitivity to internal TEA (Fig. 1). Inhibition appears to be via occlusion of the I_{Ks} pore by a single TEA molecule, as in other potassium channels; this conclusion is based on the voltage dependence of internal TEA blockade and the observation that permeant ions on the outside of the membrane diminish inhibition in direct relationship to their position in the relative permeability series (Fig. 2). MinK residue S68 is found to influence block by internal TEA (Fig. 5). However, a cysteine at the site does not react with cadmium, zinc, or agents that covalently modify thiols; this suggests that S68 is not exposed in the aqueous channel pore and that it influences the internal TEA binding site indirectly (Fig. 7).

MinK increases channel sensitivity to internal TEA, apparently by increasing the residence time of the blocker on its internal pore site. Thus fractional unitary current in the presence of TEA increases as filter bandwidth increases (Fig. 4 C), which is as expected if resolution in those periods when the channel is unblocked and open increases with bandwidth (Baukowitz and Yellen, 1996). When TEA is applied at a concentration near K_i , the resolving bandwidth provides an estimate of dwell time at equilibrium, $\sim 370 \mu\text{s}$ for I_{Ks} channels versus $\sim 70 \mu\text{s}$ for channels without MinK.

TEA block proceeds by a bimolecular interaction (Fig. 1) in the open pore (Fig. 2) without changes in channel gating (Fig. 3). In the simplest model for blockade,



where α is the apparent second-order rate constant for TEA binding and β is the first-order rate constant for TEA unbinding, and α is inversely proportional to unblocked time and equal to the product of the true second-order rate constant for binding and the concentration of added TEA. Using dwell-time estimates at K_i based on the resolving bandwidth for fractional unitary current (Fig. 4 C) and Eq. 6 gives the same upper limit for the true second-order rate constants for TEA in I_{Ks} and KvLQT1 channels, ~ 3.7 and $3.6 \mu\text{M}^{-1} \text{s}^{-1}$, respectively. This suggests that the approximately fivefold increase in equilibrium blockade with MinK (Table 1) is secondary to the increased stability of TEA on its pore site. By this same rationale, channels formed with S68Y-MinK show an off-rate for TEA approximately sixfold greater than that for wild-type channels,

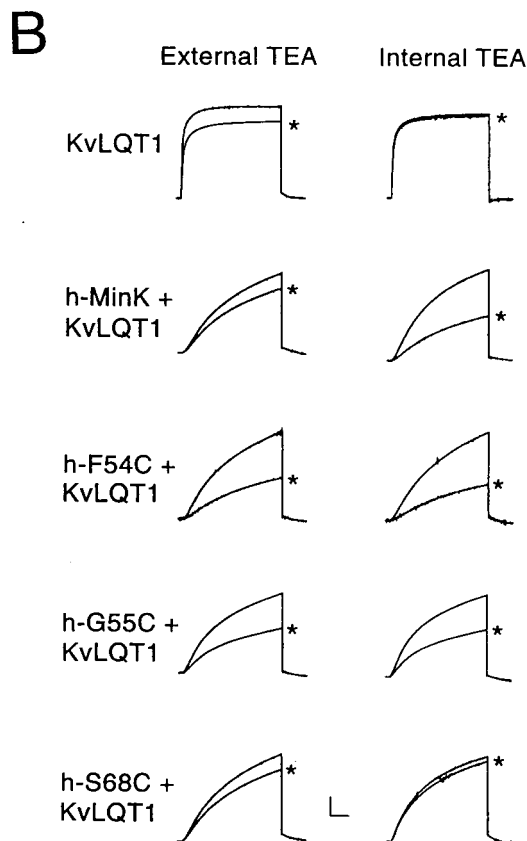
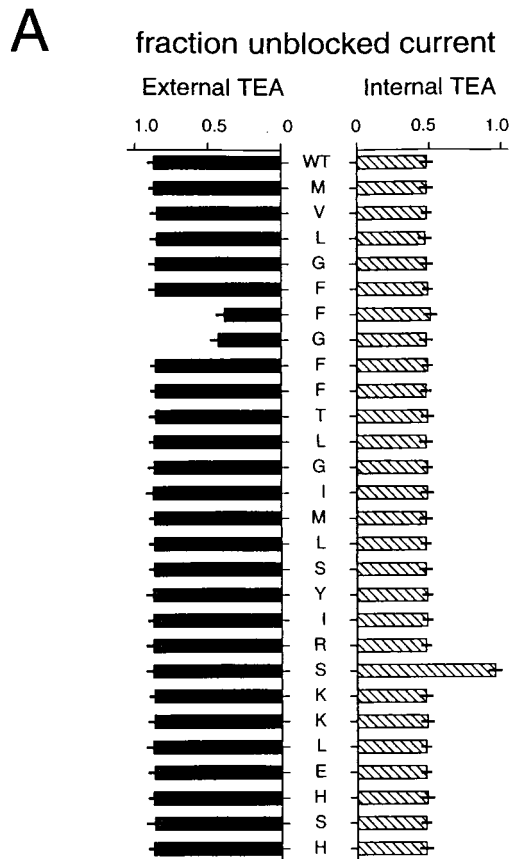


TABLE 2 Blockade of whole-cell current by internal and external TEA

KvLQT1 with	K_i (mM) for internal TEA	K_i (mM) for external TEA
No MinK	$>40 \pm 4$	91 ± 1
Wild-type MinK	5.5 ± 0.6	122 ± 8
F54C-MinK	$>40 \pm 4$	20 ± 5
G55C-MinK	$>40 \pm 4$	23 ± 7
S68C-MinK	$>40 \pm 4$	123 ± 11

The equilibrium inhibition constant (K_i) for internal TEA block of wild-type MinK was estimated from groups of 6–20 oocytes studied at 0 mV by two-electrode voltage clamp before and after microinjection with 0.08, 0.25, 0.7, 2.3, 7, and 20 nmol TEA and fit to Eq. 2. The other channels were studied in groups of 6–8 cells by injection of 20 nmol TEA, and K_i was determined with Eq. 2 to be greater than 40 mM. External TEA was added at 50 or 100 mM by isotonic substitution for NaCl as described in Materials and Methods, and K_i was determined with Eq. 2 to be greater than 40 mM. Values are mean \pm SEM.

with little change in on-rate (Fig. 7 A). Mutations that alter ligand off-rate but not on-rate frequently indicate steric interactions (Goldstein et al., 1994). Thus MinK is implicated in the formation of the internal TEA receptor site. Residue 68 appears to influence the binding site indirectly, as its exposure in the pore could not be demonstrated (Fig. 7 B). These estimates of TEA on- and off-rates are similar to those found in other potassium channels (Stanfield, 1983; Yellen, 1984).

Channels formed by coassembly of MinK and KvLQT1 in oocytes are similar to native cardiac potassium channels in their insensitivity to external TEA and inhibition by low millimolar levels of internal TEA. Thus native I_{Ks} currents that show little change with external TEA but prolongation of action potential duration with cytoplasmic TEA include those in guinea pig myocytes (Ochi and Nishiye, 1974), canine cardiac Purkinje fibers (Ito and Surawicz, 1981), and chick myocytes (Maruyama et al., 1980). A role for MinK residues in the inner portion of I_{Ks} channels is also supported by its influence on the activity of antiarrhythmic agents that act from the cytosol (Busch et al., 1996; Suessbrich et al., 1996).

FIGURE 5 Screening reveals three MinK sites that alter blockade of I_{Ks} channels by TEA. (A) External and internal TEA blockade of channels formed with rat MinK variants (mutated individually from residue M50 to H76) and wild-type KvLQT1 subunits. Currents were elicited in whole-cell mode by 10-s pulses from a holding potential of -80 to 0 mV. The bars represent the mean \pm SEM for the fraction of unblocked current in groups of 5–20 oocytes exposed to 75 mM external TEA or microinjected with 2.3 nmol TEA chloride. The three sites showing significant changes, F55, G56, and S69, are equivalent to F54, G55, and S68 in human MinK. (B) External and internal TEA blockade of channels formed with three human MinK mutants, F54C, G55C, and S68C, and KvLQT1. Traces of sample cells before and after (*) exposure to 75 mM external TEA or microinjection with 2.3 nmol TEA. The vertical scale bar represents 600 nA for F54C and G55C and 100 nA for channels formed with S68C MinK; the horizontal bar represents 2 s.

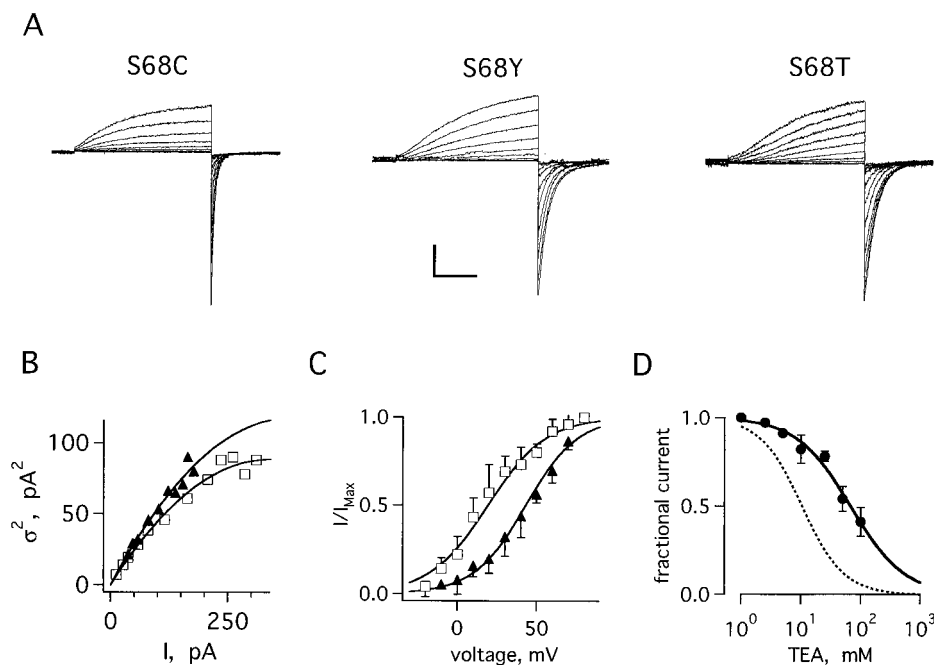


FIGURE 6 Attributes of I_{Ks} channels formed with MinK mutants at S68 in excised patches. (A) Currents in excised inside-out patches during 6-s pulses from -80 mV to -20 up to 80 mV in 10 -mV steps for I_{Ks} channels containing S68C, S68Y, or S68T MinK. (B) Unitary current by noise variance for wild-type and S68Y MinK containing I_{Ks} channels. In these patches estimated single-channel currents at 40 mV were 0.57 pA for wild type (\square) and 0.65 pA for the mutant (\blacktriangle) at 10 kHz. Values for groups of patches are reported in Table 1. (C) I_{Ks} current isochronal open probabilities in excised inside-out patches for wild-type MinK (\square) and S68Y MinK (\blacktriangle); values for groups of patches are reported in Table 1. (D) Dose response in excised patch mode for S68C MinK-containing channels (\bullet). Current was inhibited by increasing levels of internal TEA. The protocol and theoretical fit are as in Fig. 1 C at 40 mV. The dotted line is the fitted curve for wild-type I_{Ks} channels from Fig. 1 C. Values for groups of patches are reported in Table 1.

A rough model for the geometry of the I_{Ks} conduction pathway can be hypothesized based on a compilation of those MinK residues that are accessible in the pore or influence pore function (Abbott and Goldstein, 1998). Seven MinK positions interact with thiol-reactive agents in the I_{Ks} conduction pathway when altered to cysteine (K.-W. Wang et al., 1996; Tai and Goldstein, 1998). Three of these sites (positions 43, 44, and 46 in human MinK) form covalent bonds with externally applied MTS-ES and MTS-ET; mutating these residues also alters the affinity of external TEA, which binds at an overlapping site in the pore (K.-W. Wang et al., 1996). This suggests that the pore is at least ~ 6 – 8 Å wide at this point. The conduction pathway appears to narrow between positions 46 and 54. Thus four MinK sites that do not react with MTS compounds do coordinate smaller cadmium (~ 1.41 Å) and zinc ions: two sites are reached only from the external solution (54, 55) and two only from the inside (56, 57) (Tai and Goldstein, 1998). The two adjacent residues, 55 and 56, behave as if they are separated by the “selectivity filter,” as transmembrane movement of sodium, cadmium, and zinc ions is restricted at these residues. Consistent with the idea that G55 is in proximity to the ion selectivity filter, channels with a cysteine at this site show altered selectivity for cesium and ammonium ions (Goldstein and Miller, 1991)

and are sufficiently altered so that the flux of sodium ions can be measured (Tai and Goldstein, 1998). Here we demonstrate that TEA enters the internal portion of the I_{Ks} conduction pathway, suggesting the pore is also at least ~ 6 – 8 Å wide at this point. This evolving image of the I_{Ks} channel is superficially consistent with the topology of a bacterial potassium channel pore determined by x-ray crystallography (Doyle et al., 1998) and the functionally determined form of a voltage-gated potassium channel (del Camino et al., 2000). Finally, we have argued that four KvLQT1 and two MinK subunits combine to form I_{Ks} channels (Wang and Goldstein, 1995; Sesti and Goldstein, 1998); however, this remains a matter of controversy (Wang et al., 1998).

While functional potassium-selective channels can form by the aggregation of four single P domain subunits around a central pathway (MacKinnon, 1991; Shen et al., 1994; Glowatzki et al., 1995; Doyle et al., 1998), it is obligatory to form heteromeric complexes incorporating MinK and MiRP1 subunits to produce channels that behave like native I_{Ks} and I_{Kr} , respectively (Abbott and Goldstein, 1998; Abbott et al., 1999). Recent cloning of the genes encoding MiRP2, MiRP3 (Abbott et al., 1999), and MiRP4 (Piccini et al., 1999) suggests that the attributes of other ion channels in native cells will reflect their assembly as mixed com-

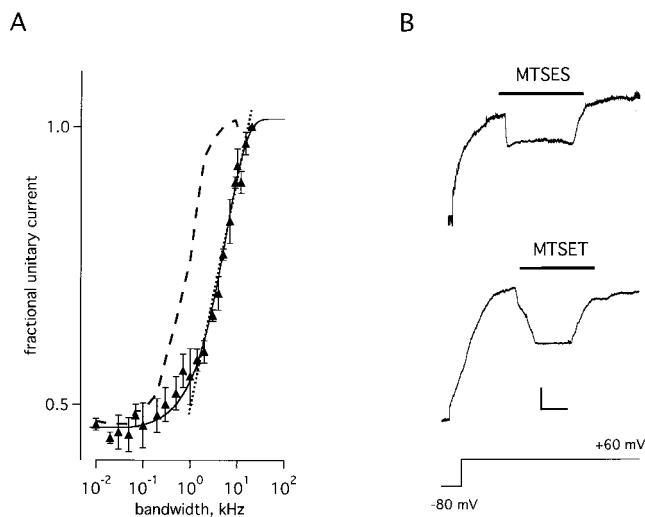


FIGURE 7 MinK mutants at S68 alters the dwell time of internal TEA but is not exposed in the pore. (A) Fractional unitary current for S68Y I_{Ks} channels versus bandwidth. Patches were exposed to 50 mM TEA (\blacktriangle) to achieve $\sim 50\%$ inhibition ($n = 2$); the heavy dashed line is the relationship for wild-type channels from Fig. 4 C. Solid line is fit to Eq. 1; dotted line is an extrapolation of the relationship to unity at 17 kHz for mutant I_{Ks} channels. (B) Blockade of S68C I_{Ks} channels by MTSES and MTSET is reversed by wash-out. Currents are in inside-out patches containing I_{Ks} channels formed with S68C MinK during sustained pulses from -80 to 60 mV exposed to 10 mM MTSES or 5 mM MTSET (bars); scale bars represent 10 s and 100 pA for the patch exposed to MTSES and 500 pA for MTSET.

plexes containing pore-forming subunits and MinK-related peptides.

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