Activation of recombinant human SK4 channels by metal cations

Ying-Jun Cao, Khaled M. Houamed*

Department of Anesthesia and Critical Care, University of Chicago, S. Maryland Ave., Box 4028, Chicago, IL 60637, USA

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Abstract The effects of metal cations on the activation of recombinant human SK4 (also known as hIK1 or hKCa4) channels, expressed in HEK 293 cells, were tested using patch clamp recording. Of the nine metals tested, cobalt, iron, magnesium, and zinc did not activate the SK4 channels when applied, at concentrations up to 100 μM, to the inside of SK4 channel-expressing membrane patches. Barium, cadmium, calcium, lead, and strontium activated SK4 channels in a concentration-dependent manner. The rank order of potency was at $Ca^{2+} > Pb^{2+} > Cd^{2+} > Sr^{2+} > Ba^{2+}$.

Key words: Potassium channel; Calcium; Divalent metal; Calmodulin

1. Introduction

Small-conductance calcium-activated potassium channels (SK channels) are expressed by neurons, smooth muscle, neuroendocrine cells, and hematopoietic cells [1–4]. SK channels regulate neuronal electrical excitability and hormone secretion from endocrine cells, and are involved in lymphocyte activation [3,5–7]. Recent cloning and functional expression of SK channel genes has revealed that these channels belong to the superfamily of ion channels which includes voltage-activated K^+ channels (typified by *Shaker*), calcium-activated large conductance K^+ channels (BK channels), and cyclic nucleotide gated channels [3,8–12]. Moreover, SK channels have been implicated in disorders such as schizophrenia, bipolar affective disorder [12,13], diamond blackfan anemia [14], and myotonic muscular dystrophy [15].

The cloning and expression of recombinant SK channels has shown that, although these channels are activated by sub-micromolar concentrations of intracellular calcium, no recognizable Ca^{2+} binding motifs can be found in the channel sequences [8,10]. Therefore, in the absence of amino acid sequence landmarks, understanding the underlying mechanisms of SK channel activation must be preceded by structure-function studies. There is considerable literature on the interaction of metal cations with calcium binding proteins including calmodulin, troponin, and the SK and BK channels. In previous studies with native channels, the molecular identity of the channels under study was uncertain. In this study, recombinant human SK4 channels were used to explore the identity and potency of activation by metal cations.

2. Materials and methods

2.1. Cells

The experiments were performed on a cell line derived from human embryonic kidney fibroblasts (HEK 293). These cells permanently express recombinant hSK4 channels under the control of a cytomegalovirus promoter, and are resistant to the antibiotic G418. The cell lines were generated by transfecting the HEK 293 cells with a plasmid construct encoding the hSK4 sequence and a neomycin resistance gene contained within the mammalian expression vector pcDNA 3.0 (Invitrogen). G418-resistant colonies were clonally purified and tested for hSK4 expression by patch clamp. The cells were grown in minimal essential medium supplemented with Glutamax, penicillin/streptomycin, sodium pyruvate and 1 mg/ml G418 (Life Technologies Inc.). The cells were grown at 37°C in a water-saturated 5% CO_2, 95% air atmosphere, and passaged 1–2 times weekly. For recording the cells were plated on 35 mm Falcon 3001 plastic Petri dishes 12–24 h prior to recording.

2.2. Electrophysiology

Two variants of the inside-out patch clamp technique were used to record recombinant hSK4 channels constitutively expressed in membrane patches from HEK 293 cells. To determine single channel conductance we used small-tipped pipettes (≈30 MΩ tip resistance), made from thick-wall quartz capillaries for lower-noise recordings [16]. For determination of potency of activation by metal cations we used large-tipped, macropatch pipettes made from thin-walled borosilicate glass (≈3 MΩ tip resistance), and coated with a hydrophobic polymer. Seal resistance was 30–100 GΩ for the micropatches and 10–30 GΩ for the macropatches. A computer-controlled patch clamp amplifier (EPC-9, HEKA [17]) was used to record the channels. Data were sampled at 10 kHz and filtered at 1 kHz (8 pole Bessel). The software (Pulse 8.11, HEKA) controlled stimulation and data acquisition. The data were analyzed offline using Pulse software, as well as TAC v. 3 (Bruxton Corp.). Single channel amplitudes were then determined from Gaussian fits to all-point amplitude histograms from 1 min of recording at each membrane potential. For measuring change in open probability (P_o), the macropatch was held at −100 mV, and cation solutions were applied for 30 s. The potency of SK4 channel activation by metal cations was determined by calculating the electrical charge, i.e. integrating a 10-s segment of membrane current during the cation response, subtracting the charge due to holding current, and normalizing to the charge transduced by maximal activation by calcium, in the same patch. Solutions were applied to the cytoplasmic aspect of the membrane patches using a 500 μm glass tube connected to a micro-manifold of 10 similar tubes. After excision, the membrane patch was placed at the center of the flow out of this tube. A set of valves controlled which solution was flowing through the tube and thus exposed to the patch at any given time. We tested the speed of the perfusion system by monitoring the changes in junction potential following the switch from Ringer to 10% Ringer. The solution changed with a time constant of ∼1 s, achieving steady state within 4–6 s after switching. The cytoplasmic aspect of the membrane patches was constantly perfused with low calcium solution containing 2 mM Mg^{2+}. All measurements were made at room temperature (25°C).

2.3. Solutions

The following metal salts were used: $BaCl_2$, $CaCl_2$, $CdCl_2$, $CoCl_2$, $FeCl_2$, $Pb(NO_3)_2$, $SrCl_2$, and $ZnCl_2$ (Fluka and Sigma Chemicals). The maximal stated contamination of any metal salt by the other metals was 0.05–0.06%. In view of the exquisite sensitivity of SK channels to calcium we were particularly concerned about contamination with this ion. Stock aqueous solutions of these salts were there-

*Corresponding author. Fax: (1) (773) 702-4791.
E-mail: khouamed@midway.uchicago.edu

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fore analyzed for calcium content by inductively coupled plasma atomic emission spectrophotometry (ICP-AES) at Northwestern University Analytical Chemistry Facility. Calcium content ranged from 2.3×10^{-5} to 8.5×10^{-5} (0.0023–0.0085%), yielding a maximum calcium content contributed by the salt in a 100 μM solution of 8.5 nM, which is >10 times lower than required for minimal activation of SK4 channels (data not shown). We therefore concluded that the metal salts were not a significant source of calcium that would interfere with our results. All our solutions were made using Milli-Q water (Millipore). This water contains about 1.2 μM free calcium as determined by ICP-AES. We therefore estimated that, in the absence of a chelator, our calcium contamination would be unacceptably high (≈1.2 μM).

We removed calcium contamination using either fluoride or EGTA as described in [21]. Briefly, for metals (Ba^{2+}, Mg^{2+}, and Sr^{2+}) that bind EGTA with very low affinity (≈10^3 higher dissociation constant), compared to calcium we included 1.59 mM EGTA in the solution to buffer the calcium without appreciably affecting the metal concentration. For metals (Cd^{2+}, Fe^{2+}, Pb^{2+}, Co^{2+}, and Zn^{2+}) that bind EGTA with high affinity we replaced potassium aspartate with fluoride as the main component of the intracellular solution; the fluoride salts of these metals are soluble up to 0.7 mM, while calcium fluoride is insoluble. The dissociation constant for our batch of EGTA and its purity were determined analytically according to [18]. Published values [19] for stability constants were used for other metals. Thus the metals were diluted from aqueous stocks into one of two base intracellular solutions of the composition (in mM): KF, 160; HEPES, 5; pH adjusted with KOH to 7.2, or K aspartate, 160; HEPES, 5; EGTA, 1.59; pH adjusted with KOH to 7.2. Barium binds EGTA very sparingly and has a soluble fluoride. We therefore used this ion with either of our two base intracellular solutions with identical results. Using these procedures we estimated the maximal free calcium content of our intracellular solutions to be <50 nM. This is in agreement with our observations that neither base intracellular solution (KF or K aspartate) activated SK4 channels (see Fig. 2A).

Our protocol involved exposing each patch to two intracellular solutions: a 'low' calcium and a 'high' calcium solution. The low calcium intracellular solution contained (in mM): potassium aspartate 160; $MgCl_2$ 2; EGTA 1.6; HEPES 5; pH adjusted to 7.2 with KOH. This low calcium solution was calculated to have less than 1 nM free calcium. The high calcium intracellular solution had the same composition as the low calcium solution but with the addition of 1.59 mM $CaCl_2$; this solution was calculated to contain >10 μM free Ca^{2+} ions. Every patch was exposed to these solutions multiple times during the experiment (see Fig. 3A); in every case the SK4 channels behaved as expected, i.e. no activation by low calcium, maximal activation by high calcium, and no run-down.

As an additional precaution we regularly washed our perfusion tubing (Teflon and quartz) with 10 mM EDTA- and Chelex-100-treated Milli-Q water.

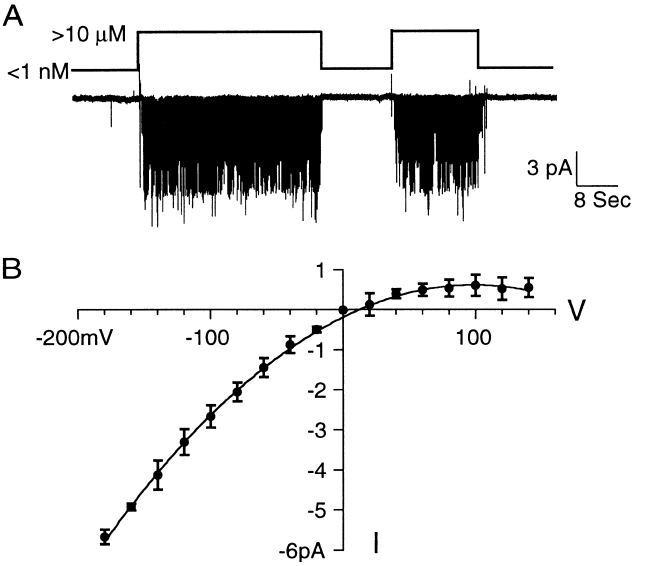

Fig. 1. Activation of recombinant hSK4 channels by intracellular calcium. A: hSK4 channels activated by alternating applications of low (<1 nM) and high (>10 μM) calcium solutions to an inside-out membrane patch voltage clamped at −100 mV. The patch contained at least four channels. B: Current-voltage relation of single hSK4 channels activated by high calcium internal solution. The data were expressed as means ± S.D. (n = 3–6). The solid line is a weighted least-squares fit of a third-order polynomial to the mean values of single channel amplitudes. Note the pronounced inward rectification even though the patch was exposed to symmetrical $[K^+]$.

In addition to the fast perfusion for patches, the recording chamber was constantly perfused with modified mammalian Ringer of composition (in mM): KCl, 147; $CaCl_2$ 2; $MgCl_2$ 1; glucose 5; $NaHCO_3$ 2; HEPES 10 (pH adjusted with KOH to 7.2).

3. Results

3.1. Single channel properties of hSK4 channels

The hSK4 channels were activated by intracellular calcium (Fig. 1A). The channel openings were only seen in patches excised from hSK4-expressing cell lines. More than 80% of

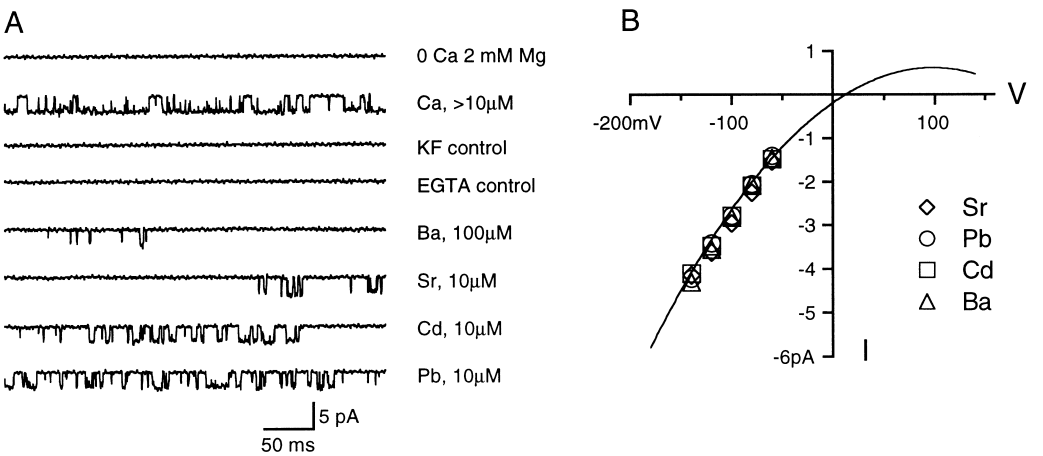

Fig. 2. Activation of hSK4 channels by divalent metal cations. A: The single channel openings induced by Ba^{2+}, Sr^{2+}, Cd^{2+} and Pb^{2+} at the indicated concentration. Channel openings are shown as downward deflections. The holding potential was −100 mV. Note that the control solution with either KF or EGTA failed to induce any channel openings, consistent with the estimation of calcium contamination. B: Single current-voltage relations of hSK4 channel activation by divalent metal cations. Solid line has been transposed from Fig. 1B to illustrate the similarity in conductance of SK4 channels activated by calcium or other metal cations.

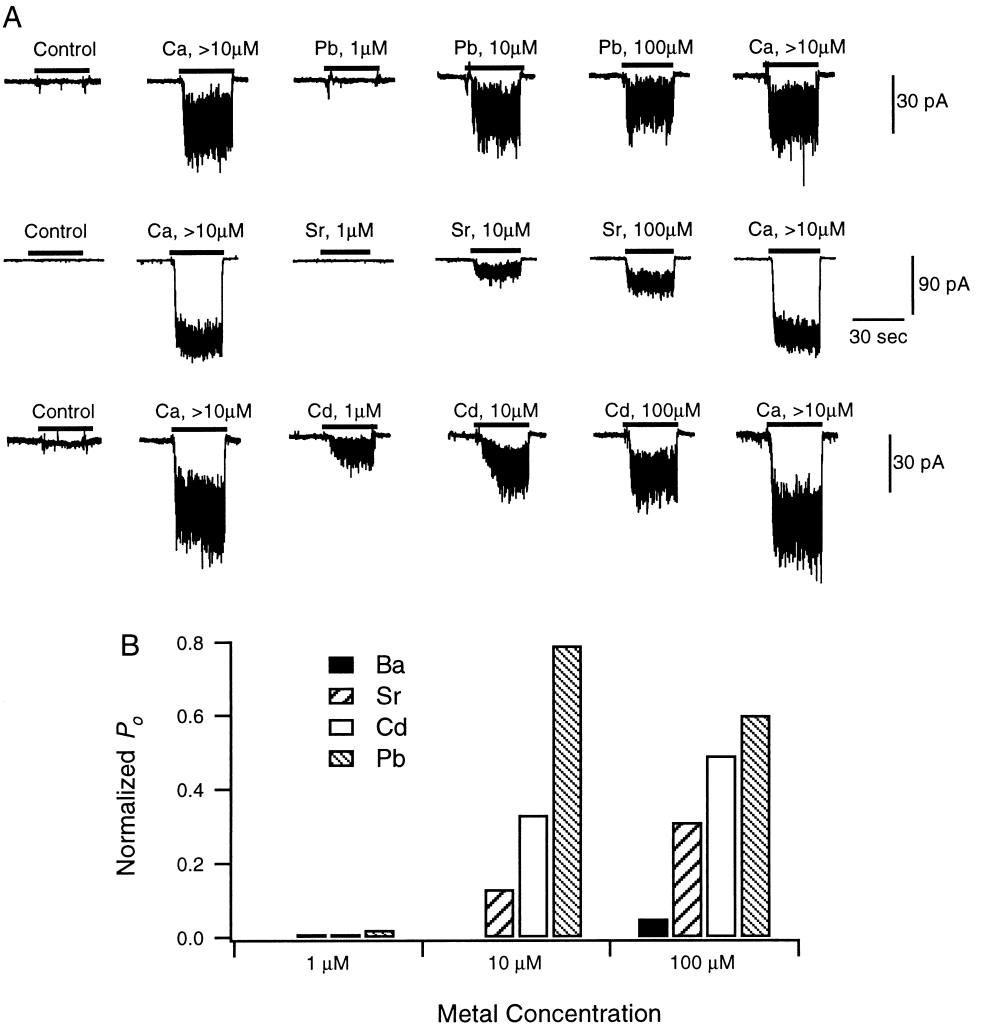

Fig. 3. Concentration-dependent activation of hSK4 channels by metal cations. A: Representative traces recorded from different ion treatments at the indicated concentrations. The patch was first perfused with low calcium solution containing 2 mM Mg^{2+} before any treatment. The treatments were done in the sequence of cation base solution (control), high calcium concentration, 1 μM metal, 10 μM metal, 100 μM metal then returned to high calcium concentration. The background leak current was −3 to −10 pA. The holding potential was −100 mV. SK current is shown downward. B: Relationship between open probability (P_o) and the applied ion concentration. P_o was normalized to high Ca^{2+} Ringer solution (>10 μM) for each patch and then averaged for 3–5 patches.

the patches contained SK4 channels ($n>16$). In contrast, these channels were never observed in patches from non-transfected cells or cell lines expressing the related channel rSK2 (data not shown; $n>10$). The unitary conductance was estimated from linear fits to channel amplitudes over the holding potential −60 mV to −140 mV. In seven patches the unitary conductance was 33.1 ± 3.1 pS (S.D.; see Fig. 1B). The conductance and the pronounced inward rectification at positive potentials are similar to properties observed in channels in erythrocyte and lymphocyte membranes, as well as recombinant hSK4 channels observed in other studies [2,20]. Taken together, the results illustrated in Fig. 1 suggest that the channels observed were recombinant hSK4 channels.

3.2. Activation of hSK4 channels by metal cations

Pb^{2+}, Cd^{2+}, Sr^{2+}, and Ba^{2+}, in addition to Ca^{2+}, activated recombinant hSK4 channels (Figs. 2 and 3). In order to determine whether the channels activated by these metal cations were the same as the hSK4 channels activated by calcium, we compared the conductance of channels opened under different metal treatments. Pb^{2+}, Cd^{2+}, and Sr^{2+} were applied at 10 μM concentration while Ba^{2+} was applied at 100 μM as it did not activate hSK4 channels at 10 μM. The current through open channels was recorded at different applied voltages. We determined the single channels conductance under different metal treatments from single channel current voltage plots as done for Ca^{2+} activation above (Fig. 2). The single channel conductances of SK4 channels activated by metals were 36.1 pS, 33.1, 35.1 pS, and 33.0 pS for barium, cadmium, lead, and strontium, respectively. We conclude that the channels activated by these cations had identical unitary conductance to those activated by calcium.

3.3. Relative potency of hSK4 channel activation by metal cations

Fig. 3 shows SK4 channel currents activated by increasing concentrations of some metal cations. Co^{2+}, Fe^{2+}, and Zn^{2+} at 1, 10, and 100 μM failed to activate hSK4 channels ($n=3$–4 macropatches). Sr^{2+} and Cd^{2+} monotonically increased P_o with concentration, while activation by Pb^{2+} decreased at

higher concentrations (Fig. 3). Ba^{2+} was only seen to activate SK4 channels weakly at the highest concentration tested (100 μM). The rank order of effect at the concentration range of 1–100 μM was $Ca^{2+} > Pb^{2+} > Cd^{2+} > Sr^{2+} > Ba^{2+}$.

4. Discussion

We show that micromolar concentrations of certain metal cations can effectively activate hSK4 channels. The effectiveness with which divalent cations activate SK channels was measured as a normalized open probability, a fraction of the open probability achieved with patch exposure to a super-maximally activating Ca^{2+} concentration (>10 μM). Mg^{2+} failed to activate hSK4 channels as no channel openings were observed either in 2 mM Mg^{2+} contained in low calcium intracellular solution or in 10 μM Mg^{2+} contained in KF solution. Our results confirm and extend previous work on native calcium-activated potassium channels [20–24]. We show for the first time that intracellular strontium can activate SK channels.

There are several reasons for studying the effects of metal cations on recombinant hSK4 channels. First, from the relative potency series of SK4 channel activation by cations we can make comparisons between different SK channel isoforms which may show differences in activation by cations even though calcium activates the subtypes equally well. Second, the relative potency can provide insights into the binding and channel isomerization reactions that underlie the open channel probability. Thus, an ion activates the channel differently from calcium because such ion either binds differently, or following binding the channel gates differently. In other words, from the analysis of the channel gating by different cations we can uncouple the binding from the isomerization steps of channel opening [25]. Related to this is the possibility that there may be two or more different types of calcium binding sites within the channel that can be distinguished by metal cations. Thus, in BK channels a second site can be distinguished because it binds cadmium differently [26]. Furthermore, the relative potency series can be correlated with analogous data obtained from metal binding to protein sites with resolved three-dimensional structures; from such correlation it may be possible to predict some aspects of the interaction between cations and their binding sites on the SK channels [27]. Finally, actions of metal cations on SK channels may be related to the physiological and pathophysiological effects of these cations. It is likely that the hSK4 channel studied here is the Gardos channel of red blood cells [9,11,28]. Metal cations have potent effects on ion transport across red blood cell membranes; for example, the Gardos channel has been reported to be effectively activated by Pb^{2+} [29], in agreement with our results. Lead and cadmium have been shown to compete with calcium for binding sites on signaling and regulatory molecules including calmodulin, troponin C, protein kinase C, and phosphodiesterase [30–33], and strontium can substitute for calcium in triggering transmitter release [34]. Comparison to the better studied BK channels indicates that cations activate BK channels with different orders of potency in different tissues. For example, Pb^{2+} is the most effective ion at activating BK channel from mouse neuroblastoma cells [22–24], but is without effect on skeletal muscle BK channels [21].

It is possible that some of the toxic effects of metal ions [35] may turn out to be mediated through aberrant activation of SK channels. Future studies will delineate the SK channel domains involved in metal binding and activation, as well as determine whether activation by these ions differs amongst the various SK channel isoforms.

After this paper was submitted a report appeared [36] describing a calcium sensing mechanism in SK channels by means of tightly associated calmodulin. Our results complement this paper. An interesting point raised by our data is that although ions which activate SK4 channels also activate calmodulin [31,32], they do so at a different order of potency. It is conceivable that the differences are due to transduction mechanisms downstream of calmodulin binding.

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