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Maurotoxin: A Potent Inhibitor of Intermediate Conductance Ca^{2+}-Activated Potassium Channels

N. A. CASTLE, D. O. LONDON, C. CREECH, Z. FAJLOUN, J. W. STOCKER, and J.-M. SABATIER

Icagen Inc., Durham, North Carolina (N.A.C., D.O.L., C.C., J.W.S.); and Centre National de la Recherche Scientifique, Unité Mixte Recherche 6560, l'Institut Fédératif de Recherches Jean Roche, Marseille, France (Z.F., J.-M.S.)

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ABSTRACT

Maurotoxin, a 34-amino acid toxin from *Scorpio maurus* scorpion venom, was examined for its ability to inhibit cloned human SK (SK1, SK2, and SK3), IK1, and Slo1 calcium-activated potassium (K_{Ca}) channels. Maurotoxin was found to produce a potent inhibition of Ca^{2+}-activated ^{86}Rb efflux (IC_{50}, 1.4 nM) and inwardly rectifying potassium currents (IC_{50}, 1 nM) in CHO cells stably expressing IK1. In contrast, maurotoxin produced no inhibition of SK1, SK2, and SK3 small-conductance or Slo1 large-conductance K_{Ca} channels at up to 1 μM in physiologically relevant ionic strength buffers. Maurotoxin did inhibit ^{86}Rb efflux (IC_{50}, 45 nM) through, and ^{125}I-apamin binding (K_i, 10 nM) to SK channels in low ionic strength buffers (i.e., 18 mM sodium, 250 mM sucrose), which is consistent with previous reports of inhibition of apamin binding to brain synaptosomes. Under similar low ionic strength conditions, the potency for maurotoxin inhibition of IK1 increased by ~100-fold (IC_{50}, 14 pM). In agreement with its ability to inhibit recombinant IK1 potassium channels, maurotoxin was found to potently inhibit the Gardos channel in human red blood cells and to inhibit the K_{Ca} in activated human T lymphocytes without affecting the voltage-gated potassium current encoded by Kv1.3. Maurotoxin also did not inhibit Kv1.1 potassium channels but potently blocked Kv1.2 (IC_{50}, 0.1 nM). Mutation analysis indicates that similar amino acid residues contribute to the blocking activity of both IK1 and Kv1.2. The results from this study show that maurotoxin is a potent inhibitor of the IK1 subclass of K_{Ca} potassium channels and may serve as a useful tool for further defining the physiological role of this channel subtype.

Peptide toxins from scorpion venoms have proven to be useful tools for defining structural, functional, and expression profiles of ion channel subtypes, in particular sodium and potassium channels (Possani et al., 1999; Garcia et al., 2001).

Maurotoxin (MTX) is a short-chain toxin isolated from the venom of the Tunisian chactidae scorpion, *Scorpio maurus palmatus* (Kharrat et al., 1997). It is a basic, C-terminal amidated, 34-mer peptide cross-linked by four disulfide bridges. At nanomolar concentrations, maurotoxin has been reported to display a variety of pharmacological activities, including inhibition of radiolabeled apamin binding to rat brain synaptosomes (Kharrat et al., 1996, 1997) and blocking insect (Shaker B) or mammalian (Kv1.2, Kv1.1, and Kv1.3) voltage-gated Kv channels heterologously expressed in *Xenopus laevis* oocytes (Kharrat et al., 1996, 1997; Avdonin et al., 2000; Carlier et al., 2000).

In the present study, the effects of maurotoxin on the functional properties of several subtypes of human Ca^{2+}-activated K^+ channels (K_{Ca}) have been examined for the first time. The pharmacology of maurotoxin was evaluated by investigating, under both physiologically relevant and low ionic strength buffers, its potency to block cloned human small conductance SK (hSK1, hSK2, and hSK3) (Kohler et al., 1996; Castle, 1999), intermediate conductance (hIK1, also referred to as SK4 or hIKCa1) (Ishii et al., 1997b; Logsdon et al., 1997), and large conductance Slo1 (also referred to as BK) (Kaczorowski et al., 1996) calcium-activated potassium channels. Maurotoxin was also tested for inhibition of the Gardos channel in human red blood cells (Brugnara et al., 1993), and the K_{Ca} in activated human T lymphocytes (Cahalan et al., 2001).

The studies described show that maurotoxin is a potent inhibitor of intermediate conductance Ca^{2+}-activated potassium channels. In physiological ionic strength buffers, maurotoxin (up to 1 μM) is inactive against small- and large-conductance calcium-activated potassium channel subtypes. Studies that reconcile the absence of functional inhibition of SK channels with previous reports that this toxin inhibits ^{125}I-apamin binding to synaptosomes are described.

Materials and Methods

Materials. Human IK1, SK1, SK2, SK3, Slo1, and Kv1.3 were stably expressed in Chinese hamster ovary (CHO) cells using

Parts of this work have been published in abstract form (Castle et al., 2001).

ABBREVIATIONS: MTX, maurotoxin; SK, small-conductance; IK, intermediate conductance; CHO, Chinese hamster ovary; BSA, bovine serum albumin; RBC, red blood cells; Fmoc, *N*-α-fluorenylmethyloxycarbonyl; TFA. trifluoroacetic acid.

pcDNA3 or pcDNA3.1 (Invitrogen, Carlsbad, CA) and grown in Ham's F12 medium (HyClone, Logan, UT) containing 400 μg/ml G-418 to maintain clonal selection. Mouse Kv1.2 expressed in B82 mouse fibroblasts (Werkman et al., 1993) was a gift from Dr Michael Rogawski (National Institutes of Health, Bethesda, MD) and mouse Kv1.1 stably expressed in a functionally protein kinase A deficient CHO cell line (Bosma et al., 1993) was provided by Dr Bruce Tempel (University of Washington School of Medicine, Seattle, WA). Maurotoxin was synthesized as described below. Other toxins used in this study were apamin, charybdotoxin, iberiotoxin (Sigma, St. Louis, MO), and margatoxin (Bachem, Bubendorf, Switzerland).

Isolation of Human T Lymphocytes. Whole human blood was diluted 1:1 (by volume) with phosphate-buffered saline. Diluted blood was underlayed with an equal volume of Histopaque-1077 (Sigma) by slow addition. The blood was then centrifuged at 400*g* (1400 rpm) for 30 min using a swinging-bucket rotor at room temperature. The mononuclear leukocyte cell band at the Histopaque plasma interface was carefully removed and transferred to a clean tube. Mononuclear cells were washed twice with 15 ml of phosphate-buffered saline, centrifuging each time at 1000*g* (2400 rpm) for 15 min and discarding the supernatant. Finally, cells were diluted to 1.25×10^6 cells/ml in RPMI 1640 medium containing 10% fetal bovine serum and gentamicin. T-cells were activated with 2 μg/ml phytohemagglutinin for 48 h before electrophysiological measurements were made.

^{86}Rb Efflux Studies. CHO cells expressing IK1, SK, or Slo1 Ca^{2+}-activated potassium channels were plated into 96-well plates in Ham's F12 medium with 10% fetal bovine serum and allowed to attach for at least 4 h before being loaded overnight with ^{86}Rb (final concentration, 1 μCi/ml in growth media) at 37°C in 5% CO_2/95% O_2. ^{86}Rb efflux measurements were performed by preincubating cell monolayers for 10 min in the presence and absence of peptide toxins made up in 135 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 10 mM HEPES, and 5 mM glucose, pH 7.4 (buffer A), containing also 0.25 mM Ca and 0.1% bovine serum albumin (BSA). ^{86}Rb efflux from cells expressing IK1 or SK channels was elicited by incubating the cell monolayers with buffer A containing 2 mM Ca, 3 μM ionomycin, and 0.1% BSA. In contrast, ^{86}Rb efflux from cells expressing Slo1 potassium channels was elicited by incubating the cell monolayers with 10 μM ionomycin made up in a buffer containing 135 mM KCl, 20 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM HEPES, 5 mM glucose, and 0.1% BSA. ^{86}Rb efflux measurements in cells expressing Kv1.1, Kv1.2, or Kv1.3 voltage-dependent potassium channels were performed by incubating the cells in a modified buffer A in which 70 or 135 mM K was substituted for the equivalent amount of NaCl for 5 (Kv1.3) or 30 min (Kv1.2, Kv1.1). Buffer containing ^{86}Rb was counted by measuring Cerenkov light in a Trilux Microbeta 96-well liquid scintillation counter (PerkinElmer Wallac, Gaithersburg, MD). The ^{86}Rb remaining in the cells was determined by lysing cells in 0.1% SDS for 10 min and counting the lysate. ^{86}Rb efflux was normalized to the total radioactivity present in the cells at the beginning of the experiment.

^{86}Rb Influx into Human Red Blood Cells. ^{86}Rb influx into red blood cells was studied in heparinized human blood obtained from Biological Specialty Corp (Colmar, PA). Whole blood was initially diluted 1:1 with buffer consisting of 140 mM NaCl, 5 mM KCl, 10 mM Tris, and 0.1 mM EGTA, pH 7.4. The blood was centrifuged at 1000 rpm and the pellet, consisting predominantly of red blood cells (RBCs), was washed three times with buffer. The RBCs were preincubated in test compound for 10 min. Influx of ^{86}Rb was initiated by raising RBC intracellular calcium levels by addition of $CaCl_2$ and ionomycin (final concentrations, 2 mM and 5 μM, respectively) made up in buffer containing 5 μCi/ml ^{86}Rb and incubated at room temperature for 15 min. About 10 min into the incubation period, the RBC/^{86}Rb/test compound mixture was layered over 400 μl of dibutyl phthalate in 1.5-ml microcentrifuge tubes. At the 15 min time point, the tubes were centrifuged in a microcentrifuge at 15,000 rpm for 15 s. The aqueous layer and butyl phthalate was aspirated, and the pellet was resuspended in 400 μl of deionized water to lyse cells. The resuspended RBCs were transferred to clean tubes and 400 μl of chloroform/ethanol (1:1) mixture was added to precipitate hemoglobin. Tubes were centrifuged at 15,000 rpm for 30 min to pellet precipitated protein. Upper aqueous layer (300 μl) from each tube was mixed with 700 μl of scintillation cocktail and then counted in the Trilux MicroBeta liquid scintillation counter. The above protocol is a modification of the protocol for measurement of Gardos channel inhibition in RBCs published previously (Brugnara et al., 1993). IC_{50} values were calculated using the OriginLab graphing software logistic function (Origin LabCorp, Northampton, MA).

Electrophysiological Recording. For electrophysiological studies, cells were removed from the culture flask by brief trypsinization and replated at low density onto glass cover slips 24 to 72 h before study.

Coverslips coated with CHO cells stably expressing human IK1, SK3, and Slo1 were placed in a bath on the stage of an inverted microscope and perfused with extracellular solution (138 mM NaCl, 1.8 mM $CaCl_2$, 5.4 mM KCl, 0.8 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4). For electrophysiological measurements in human T lymphocytes, a high-potassium extracellular solution (145 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4) was employed. Pipettes were filled with an intracellular solution [130 mM KCl, 5 mM K^+, 2·ATP, 4.7 $CaCl_2$ (free Ca^{2+} concentration, 1 μM), 5.7 EGTA, 1 $MgCl_2$, 10 HEPES, pH 7.4, osmolarity, 295 mOsmol] for recombinant IK1 and SK potassium channels and endogenous currents in human T lymphocytes. For hSlo measurements, the calcium and EGTA concentrations in the above solution were adjusted (using WebMaxC; http://www.stanford.edu/~cpatton/webmaxcS.htm) to give a free Ca^{2+} concentration of 20 μM. For electrophysiological measurements of voltage-gated potassium current (i.e., Kv1.3 and Kv1.2) pipettes were filled with 100 mM KF, 40 mM KCl, 5 mM NaCl, 5 mM EGTA, 2 mM $MgCl_2$, and 10 mM HEPES, pH 7.4, osmolarity, 295 mOsmol. Patch electrodes had a resistance of 1 to 3 mΩ. All recordings were made at room temperature (22–24°C) using an Axopatch 200B amplifier and pCLAMP 6 or 8 software (Axon Instruments, Union City, CA). Potassium currents were measured using the whole-cell configuration of the patch-clamp technique. Series resistance compensation of 90% was routinely achieved; uncompensated series resistance was typically 2 to 5 MΩ. Current records were acquired at 2 to 10 kHz and filtered at 1 to 2 kHz. Toxins were made up in appropriate extracellular buffer containing 0.1% bovine serum albumin. Toxins were applied to cells via a 200-μm glass capillary tube (connected via Teflon tubing to a reservoir) placed ~200 μm from the cell being examined.

^{125}I-Apamin Binding Studies. CHO cells expressing either human SK2 or SK3 were grown to confluent monolayers in 24-well tissue culture plates. Experiments were begun by aspirating the growth media from each well, then adding 200 μl of toxin at 2-fold the final desired concentration made up in a binding buffer comprising 18 mM NaCl, 1 mM $CaCl_2$, 3 mM KCl, 10 mM Tris HCl, 250 mM sucrose, and 0.1% BSA to each well followed by 200 μl of 200 pM ^{125}I-apamin (PerkinElmer Life Sciences, Boston, MA) made up in the same buffer. Plates were incubated for 60 min at room temperature before aspirating the binding buffer and rapidly washing each well three times with 2 ml of ice-cold binding buffer. After removing the last wash, 300 μl of 0.1% SDS was added to each well for 10 min to solubilize cell membranes bound with ^{125}I-apamin. The SDS solution was transferred to minivials mixed with 2.7 ml of scintillation cocktail and counted in the Trilux Microbeta liquid scintillation counter.

Chemical Synthesis of Maurotoxin and Its Structural Analogs. Maurotoxin and its structural analogs were synthesized by a solid-phase method (Kharrat et al., 1996, Fajloun et al., 2000a,b) using an automated peptide synthesizer (model 433A; Applied Biosystems, Foster City, CA). Peptide chains were assembled stepwise on 0.25 mEq of *N*-α-fluorenylmethyloxycarbonyl (Fmoc)-amide resin (0.65 mEq of amino group/g) using 1 mmol of Fmoc amino acid derivatives. The side chain-protecting groups used for trifunctional residues were: trityl for Cys, Asn, and Gln; *tert*-butyl for Ser, Thr,

Tyr, and Asp; pentamethylchroman for Arg, and *tert*-butyloxycarbonyl for Lys. The Fmoc-amino acid derivatives were coupled (20 min) as their hydroxybenzotriazole active esters in *N*-methylpyrrolidinone (4-fold excess). The peptide resins (2.0–2.5 g) were treated for 2.5 h at room temperature with a mixture of trifluoroacetic acid (TFA)/H_2O/thioanisole/ethanedithiol (88:5:5:2, v/v) in the presence of crystalline phenol (2.25 g). After filtration of the mixture, the peptide was precipitated and washed by adding cold diethyl ether. The crude peptide was pelleted by centrifugation (3000*g*, 12 min) and the supernatant was discarded. The reduced peptides were then diluted to 2 mM in 0.2 M Tris/HCl buffer, pH 8.3, and stirred under air to allow folding (72 h, 25°C). The folded peptides were purified by reversed-phase high-pressure liquid chromatography (C18 Aquapore ODS 20 μm, 250 $\times$ 10 mm; PerkinElmer) by means of a 60-min linear gradient of 0.08% (v/v) TFA/0% to 35% acetonitrile in 0.1% (v/v) TFA/H_2O at a flow rate of 5 ml/min (λ = 230 nm). The homogeneity (> 99%) and identity of MTX and its analogs were assessed by i) analytical C18 reversed-phase high-performance liquid chromatography, ii) amino acid analysis after peptide acidolysis, iii) Edman sequencing, and iv) molecular mass determination by matrix-assisted laser desorption ionization-time of flight mass spectrometry.

Results

The effect of maurotoxin (see Fig. 1 for amino sequence) on cloned Ca^{2+}-activated potassium channels was initially investigated by measuring the effect of the toxin on ionomycin-activated ^{86}Rb efflux in CHO cells stably expressing human IK1, SK3, or Slo1 Ca^{2+}-activated potassium channels. Figure 2 shows that maurotoxin potently inhibited ^{86}Rb efflux from IK1-expressing cells with an IC_{50} of 1.4 $\pm$ 0.4 nM (n = 7). In contrast to its effect on IK1, maurotoxin produced no inhibition of ionomycin-stimulated ^{86}Rb efflux from CHO cells expressing SK3 or Slo1 calcium-activated potassium channels at up to 300 nM. This differs from the potent inhibition of ^{86}Rb efflux in SK3 cells by apamin (IC_{50}, 1 $\pm$ 0.3 nM, n = 3) and hSlo1 cells by iberiotoxin (IC_{50}, 2.6 $\pm$ 0.5 nM, n = 3).

The selectivity and potency of maurotoxin was confirmed using whole-cell electrophysiological measurements of potassium currents in CHO cells expressing hIK1, hSlo1, or hSK3 channels (Fig. 3). In cells expressing hIK1, elevation of intracellular calcium to 1 μM resulted in a charybdotoxin-sensitive, inwardly rectifying outward current. This current was inhibited in a concentration-dependent manner by maurotoxin with an IC_{50} of 1.1 nM. In contrast, 100 nM maurotoxin produced no significant inhibition of the apamin-sensitive, calcium-dependent, inwardly rectifying current in hSK3 cells (reduction of 5 $\pm$ 3%, n = 3 cells) or the iberiotoxin-sensitive, calcium- and voltage-dependent outwardly rectifying potassium current recorded in hSLO1 cells (reduction of 3 $\pm$ 3%, n = 3 cells).

The absence of inhibition of apamin-sensitive SK channels by maurotoxin seems to be inconsistent with reports in the literature showing inhibition of ^{125}I-apamin binding to rat brain synaptosomes (Kharrat et al., 1996, 1997). To address this question, maurotoxin was examined for its ability to inhibit ^{125}I-apamin binding to hSK2 and hSK3 channels expressed in CHO cells. Figure 4 shows that in contrast to its lack of effect on potassium currents or ^{86}Rb efflux, maurotoxin inhibited ^{125}I-apamin (100 pM) binding to monolayers of CHO cells expressing SK channels with IC_{50} values of 2 and 9 nM for SK2 and SK3, respectively. Under these same experimental conditions, the IC_{50} values for displacement of ^{125}I-apamin by unlabeled apamin were 0.14 nM and 0.8 nM for SK2 and SK3, respectively (Fig. 4).

To investigate the disparity between the ^{86}Rb efflux and electrophysiological observations and ^{125}I-apamin binding data, ionomycin stimulated ^{86}Rb efflux through SK channels was reevaluated using the isotonic low ionic strength buffer used in the ^{125}I-apamin binding studies (Fig. 5, Table 1). In low ionic strength buffer, maurotoxin was found to inhibit ^{86}Rb efflux through all three subtypes of SK channels, exhibiting IC_{50} values comparable with those for inhibition of ^{125}I-apamin binding (see Table 1). In contrast to maurotoxin, the potency of apamin inhibition of efflux through hSK3 was not sensitive to changes in ionic strength, exhibiting similar IC_{50} values in normal and low ionic strength buffers (Fig. 5, Table 1).

The effect of ionic strength on maurotoxin inhibition of hIK1 was also examined. Figure 5 shows that in the low ionic strength "binding" buffer, maurotoxin inhibited ionomycin evoked ^{86}Rb efflux from CHO cell monolayers expressing hIK1 with an IC_{50} of 14 $\pm$ 3 pM (n = 5), showing a 100-fold greater potency than under physiologically relevant ionic strength conditions (IC_{50}, 1.4 nM). The potency of the IK1 blocking toxin charybdotoxin for inhibition of ^{86}Rb efflux through IK1 was also increased in low ionic strength buffer (the IC_{50} was 0.11 nM compared with 6 nM in normal ionic strength buffer) (Fig. 5, Table 1). Interestingly, charybdotoxin exhibited weak inhibition of ^{86}Rb efflux through SK3 channels, suggesting that there is a low-affinity binding site for the toxin on SK channels.

Because IK1 has been reported to encode the Ca^{2+}-activated K^+ channels expressed in human red blood cells and T lymphocytes (Ishii et al., 1997b; Cahalan et al., 2001), maurotoxin was investigated for its ability to inhibit the native channels in these cell types. Effects on Gardos channel activity was assessed by measuring ^{86}Rb uptake into washed human red blood cells that was induced by 5 μM ionomycin (Fig. 6A). Maurotoxin produced a concentration-dependent

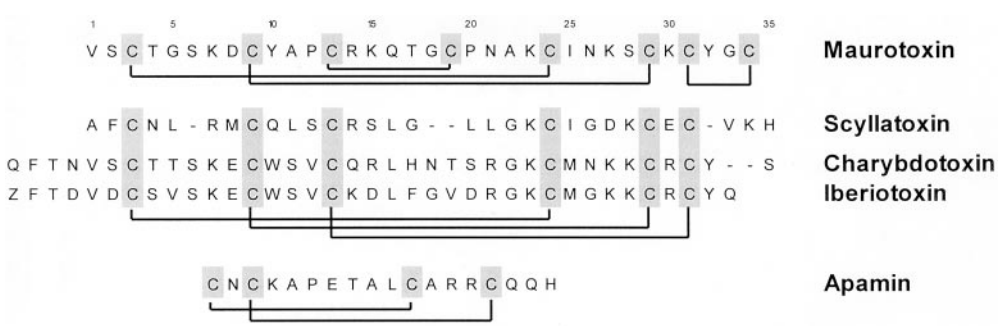

Fig. 1. Amino acid sequence of selected peptide inhibitors of Ca^{2+}-activated K^+ channels. Cysteine amino acid residues are shown in gray and disulfide bridges are indicated by the black lines.

inhibition of ^{86}Rb uptake with an IC_{50} of 1 nM, which is similar to the value obtained with the cloned IK1 channel.

Ca^{2+}-activated K^+ channels are expressed at relatively low levels in resting T lymphocytes. However, after activation with antigenic or mitogenic stimuli, expression of functional calcium-activated potassium channels as well as IK1 mRNA dramatically increases (Cahalan et al., 2001). The effect of maurotoxin on potassium currents recorded in T lymphocytes stimulated with 2 μM phytohemagglutinin for 48 h is shown in Fig. 6B. Currents were elicited in elevated extracellular potassium (145 mM) by 800-ms voltage ramps from −130 mV to +40 mV with 1 μM free calcium in the pipette solution. The currents recorded comprised at least two distinct channel activities. Whereas 200 nM charybdotoxin blocked most of the evoked current, maurotoxin produced a concentration-dependent inhibition of only the voltage-independent inward current between −130 and −60 mV, producing little or no modulation of the voltage-dependent current observed between −50 and +40 mV. The voltage-independent current is carried through intermediate-conductance, calcium-activated potassium channels, whereas the voltage-dependent current is carried through potassium

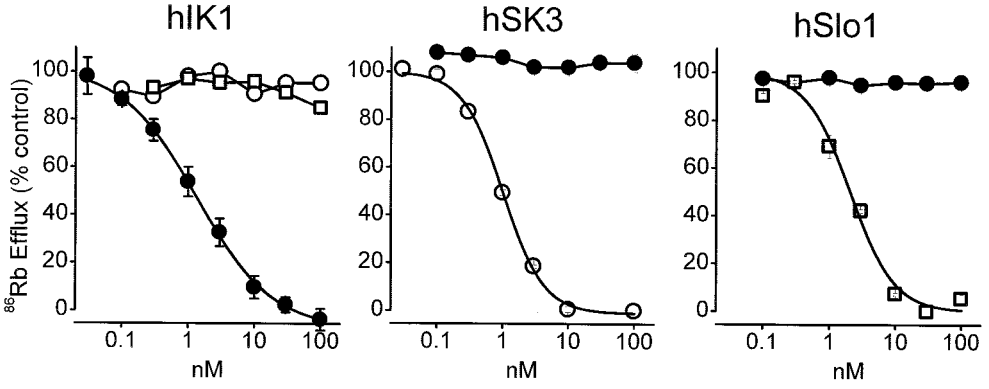

Fig. 2. Maurotoxin selectively inhibits ^{86}Rb efflux through hIK1 channels expressed in CHO cells. Plots show effect of maurotoxin on ionomycin-stimulated ^{86}Rb efflux from CHO cells stably expressing human IK1, SK3, or Slo1 (●). The concentration-dependent inhibitory effects of the SK inhibitor, apamin (○) and Slo1 inhibitor, iberiotoxin (□) are also shown for comparison. Data are the mean ± S.E.M. of three observations (some error bars are smaller than the size of the symbols). Curves are fits of a logistic equation giving IC_{50} values of 1.4 nM for maurotoxin block of IK1, 1 nM for apamin inhibition of SK3, and 2.6 nM for iberiotoxin block of Slo1.

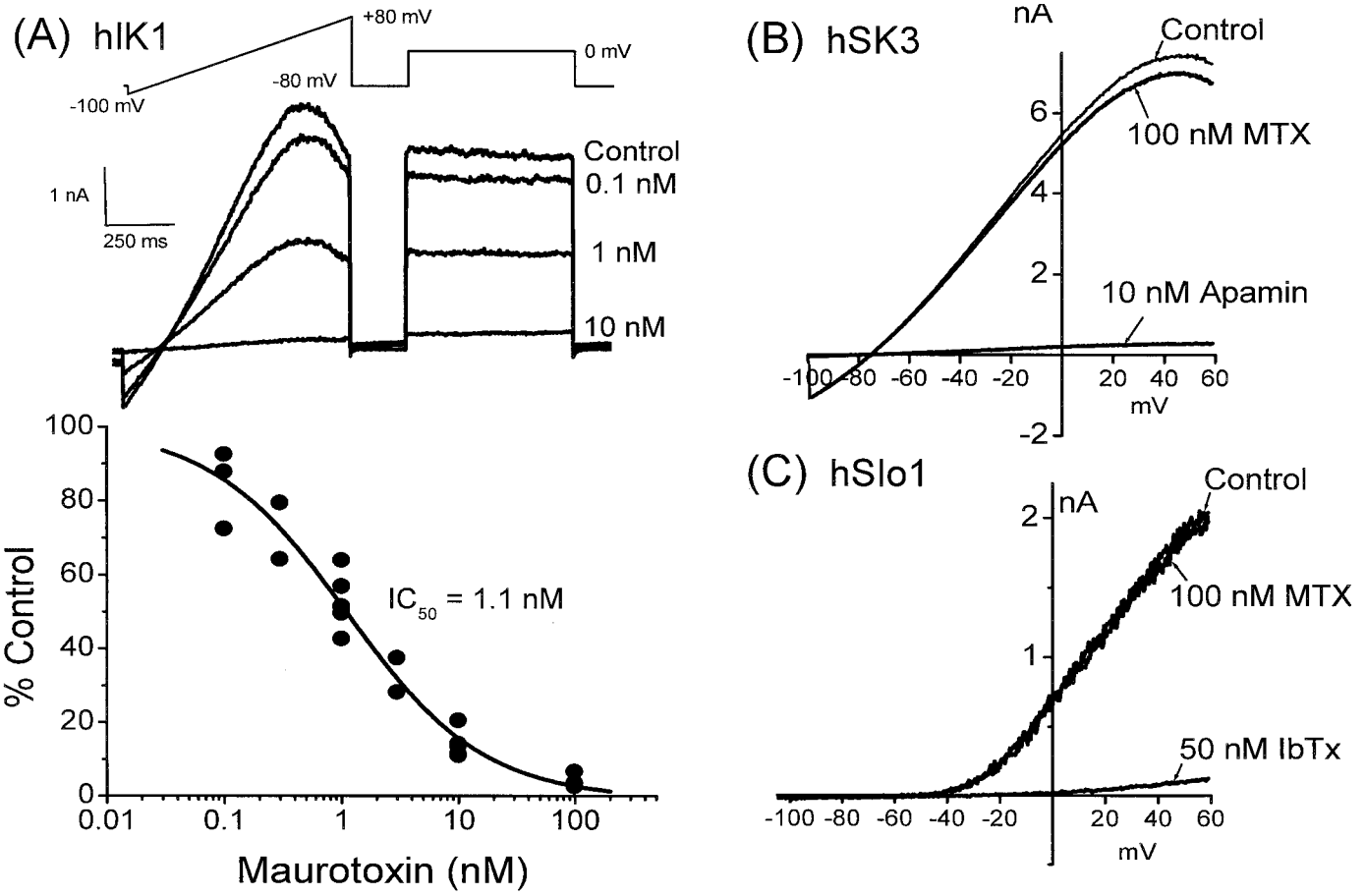

Fig. 3. Maurotoxin selectively inhibits currents through hIK1 channels: A, top, whole-cell current traces recorded in CHO cells stably expressing hIK1 in the absence and presence of maurotoxin. Bottom, concentration response curve for inhibition of IK1 currents measured during a 500-ms step depolarization to 0 mV. B and C, whole-cell current traces recorded in CHO cells stably expressing hSK3 in the absence and presence of 100 nM maurotoxin or the selective channel inhibitors apamin (10 nM) (SK) or iberiotoxin (50 nM) (hSlo).

channels encoded by Kv1.3 (Cahalan et al., 2001). To confirm the absence of maurotoxin-induced inhibition of Kv1.3 channels, 100 nM toxin was applied to CHO cells heterologously expressing human Kv1.3 potassium channels. As shown in Fig. 6B, inset, 100 nM maurotoxin produced no reduction in current amplitude (5 ± 2%, n = 3). In contrast, the current was completely abolished by 10 nM margatoxin, a toxin known to potently inhibit Kv1.3 potassium channels. (Garcia-Calvo et al., 1993). Consistent with its lack of inhibition of Kv1.3 in electrophysiological recordings (Fig. 6), maurotoxin was also without effect on ^{86}Rb efflux from monolayers of Kv1.3-expressing cells up to concentrations of 300 nM (Fig. 7B) (^{86}Rb efflux was potently inhibited by margatoxin, IC_{50}, 0.5 ± 0.2 nM, n = 3).

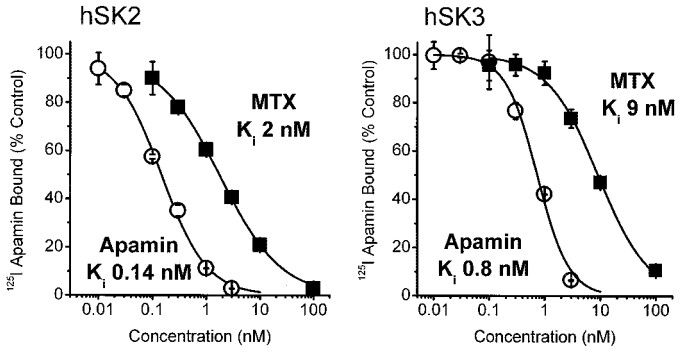

Fig. 4. Maurotoxin inhibits ^{125}I-apamin binding to SK channels. Displacement of ^{125}I-apamin binding by apamin or maurotoxin to monolayers of CHO cells stably expressing human SK2 or SK3 channels grown in 24-well plates. Cell monolayers were incubated in 100 pM ^{125}I-apamin for 60 min at room temperature in isotonic low ionic strength buffer containing 18 mM NaCl, 1 mM $CaCl_2$, 3 mM KCl, 10 mM Tris HCl, 250 mM sucrose, and 0.1% BSA.

Although maurotoxin lacked inhibitory activity against Kv1.3 potassium channels, other voltage-dependent potassium channels have been reported to be sensitive to inhibition by the toxin. In voltage-clamp experiments in oocytes, maurotoxin has been reported to potently inhibit currents through voltage-dependent potassium channels encoded by Kv1.2 and, to a lesser extent, Kv1.1 (Kharrat et al., 1996; Lecomte et al., 2000). Additional experiments were performed to examine the effects of maurotoxin on voltage-dependent Kv1.2 and Kv1.1 potassium channels expressed in mammalian cells. Figure 7A shows that maurotoxin is a potent inhibitor of voltage-activated potassium currents in mouse fibroblast cells stably expressing mouse Kv1.2 potassium channels. The concentration dependence of maurotoxin inhibition of ^{86}Rb efflux through mouse Kv1.2 is shown in Fig. 7B. The IC_{50} for inhibition of Kv1.2 was 120 ± 20 pM (n = 4). In contrast to its effect on Kv1.2, maurotoxin was

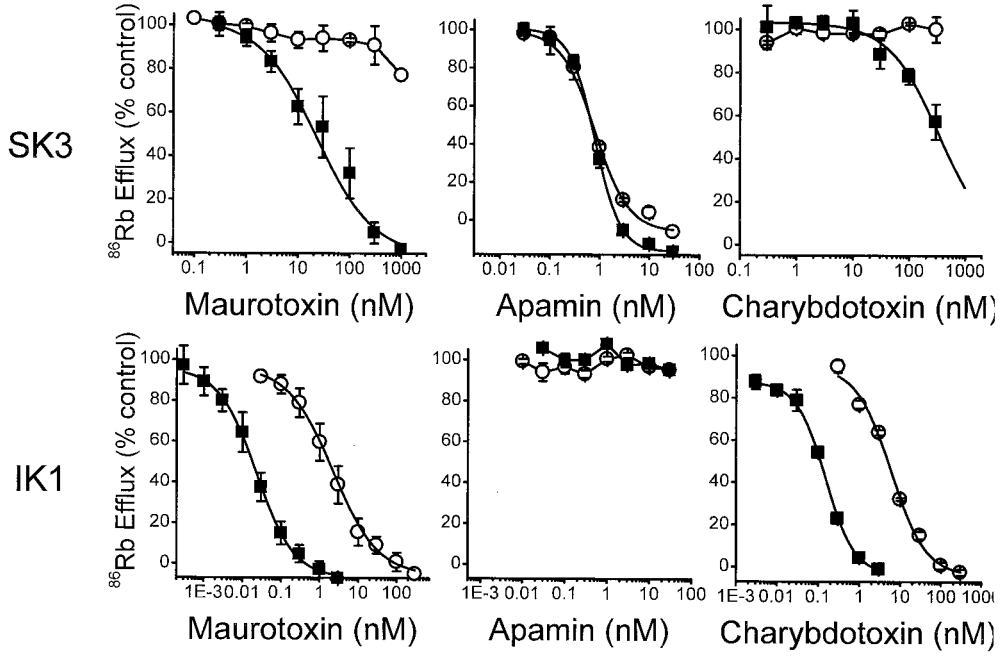

Fig. 5. Potency for maurotoxin inhibition of IK1 and SK3 increases in low ionic strength buffers Graphs show inhibition of ionomycin stimulated ^{86}Rb efflux from monolayers of CHO cells stably expressing human IK1 or SK3 potassium channels by maurotoxin, apamin, and charybdotoxin in normal ionic strength (○) or low ionic strength buffer (■) (see Table 1 for IC_{50} values and buffer composition).

TABLE 1

Effect of ionic strength on inhibition of IK and SK potassium channels

Inhibition of ionomycin-stimulated ^{86}Rb efflux IC_{50}. Normal ionic strength, 135 mM NaCl, 5.4 mM KCl, 2 mM mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM HEPES, 5 mM glucose, and 0.1% BSA. Low ionic strength, 18 mM NaCl, 1 mM CaCl2, 3 mM KCl, 10 mM Tris-HCl, 250 mM sucrose, and 0.1% BSA. Values are averages of two to six separate experiments.

	IK1		SK1		SK2		SK3	
	Normal Ionic Strength	Low Ionic Strength	Normal Ionic Strength	Low Ionic Strength	Normal Ionic Strength	Low Ionic Strength	Normal Ionic Strength	Low Ionic Strength
				nM				
Apamin	>100	>100	1.3 ± 0.1	0.24[a]	0.07 ± 0.02	0.09[a]	0.8 ± 0.1	0.8 ± 0.1
Maurotoxin	1.4 ± 0.4	0.014 ± 0.004	>1000	250 ± 200	>1000	135 ± 65	>1000	45 ± 18
Charybdotoxin	5.9 ± 0.2	0.11 ± 0.03	>1000	N.D.	>1000	N.D.	>1000	370 ± 140

N.D., not determined.
[a] Values are averages of duplicate observations in a single experiment.

without effect on ^{86}Rb efflux from cells expressing Kv1.1 potassium channels, which differs from previous reports of inhibition of this channel by the toxin (Kharrat et al., 1996).

The activity of maurotoxin as an inhibitor of diverse potassium channels such as IK1 and Kv1.2 raises the question of whether similar or different amino acid residues on the toxin contribute to both blocking activities. To examine this, alanine scanning mutation analysis along with other more specific amino acid residue changes was performed. As Fig. 8 shows, sequential replacement of residues 2, 4, 6, 7, and 10 of MTX with alanine produced only minor (less than 10-fold) changes in potency for inhibition of ^{86}Rb efflux through either IK1 or Kv1.2 channels. Replacement of the lysine with a glutamine at position 15 was also without effect. Even converting maurotoxin from a four-disulfide to a three-disulfide bridge toxin ([Abu19,Abu34]-maurotoxin; see Fajloun et al., 2000) did not significantly modify potency for inhibition of IK1 and Kv1.2. In marked contrast to these observations, replacement of amino acid residues with alanine at positions 23 (K23A) and 32 (Y32A) resulted in a loss of activity of more than two orders of magnitude for inhibition of both IK1 and Kv1.2. None of the residue changes described above resulted in increases in potency for maurotoxin-induced inhibition of SK channels, which was more than 1 μM for all analogs tested (Fig. 8A). Because lowering the ionic strength of extracellular buffer was associated with the appearance of measurable inhibition of SK channels by MTX, the MTX mutants described above were also examined for differences in SK channel-blocking activity (Fig. 8B). Like MTX, the modified MTX analogs exhibited similar measurable but weak inhibition of SK channels; potency for block was ~1000-fold less than for inhibition of IK1 in similar low ionic strength buffers. None of the amino acid residue changes improved binding affinity of MTX for SK channels. As with IK1 and Kv1.2, modification of lysine at position 23 and tyrosine at position 32 resulted in complete loss of blocking activity against SK channels.

Discussion

The current studies have shown that the peptide scorpion toxin maurotoxin is a potent inhibitor of intermediate-conductance calcium-activated potassium channels that comprise subunits encoded by IK1 (also termed SK4, IKCa, KCNN4) (Ishii et al., 1997b, Logsdon et al., 1997). In physiologically relevant ionic conditions, maurotoxin is selective for the intermediate-conductance subtype of calcium-activated potassium channel, exhibiting (up to concentrations of 1 μM) no inhibitory activity against the related apamin-sensitive, small-conductance SK family of calcium-activated potassium channels (Castle, 1999). Similarly, maurotoxin had no inhibitory effect against the iberiotoxin-sensitive large conductance voltage- and calcium-activated potassium channel encoded by Slo1 (or KCNMA1) (Kaczorowski et al., 1996) up to concentrations of 300 nM.

The absence of maurotoxin inhibition of SK channels would seem at first to be inconsistent with previous reports that maurotoxin can inhibit ^{125}I-apamin binding to rat brain

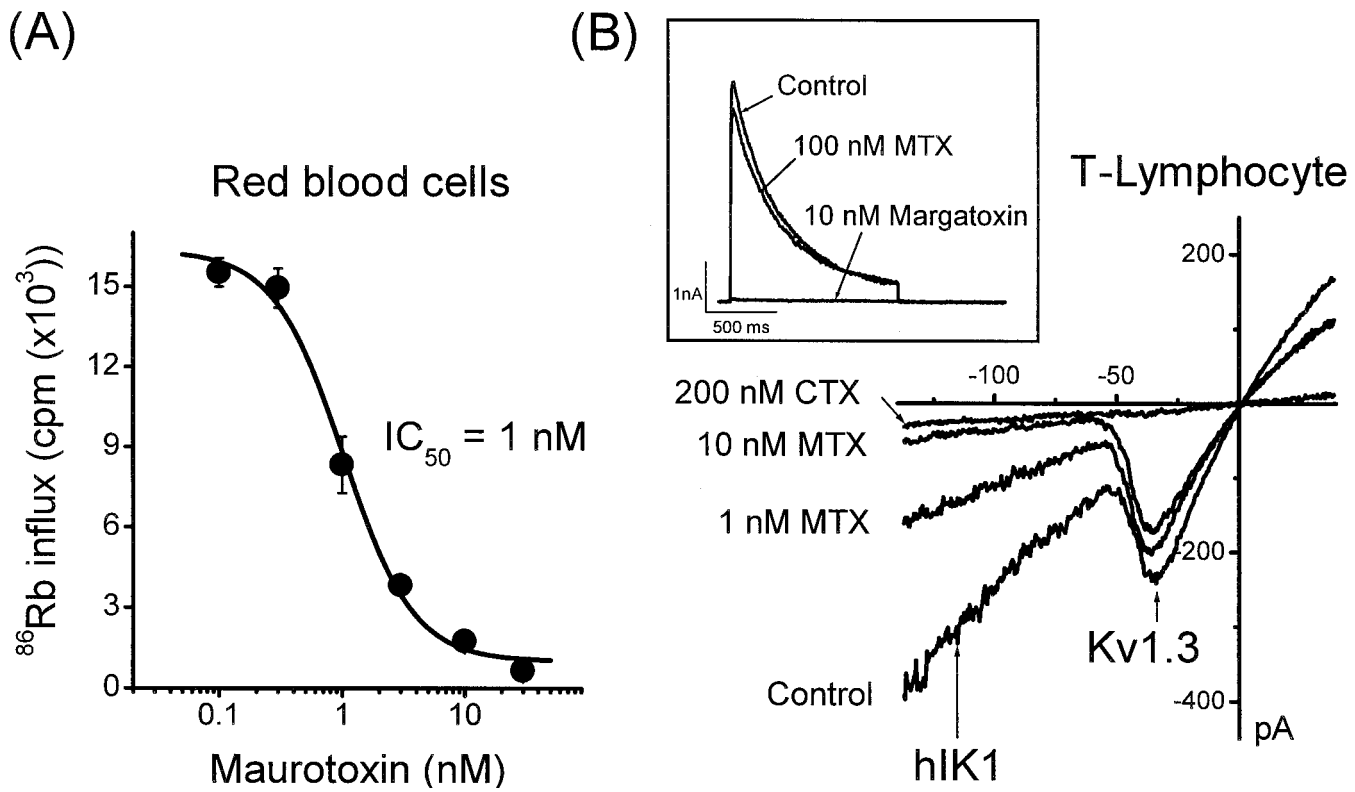

Fig. 6. Maurotoxin inhibits Gardos channel in human red blood cells and IK$_{Ca}$ in activated human T lymphocytes A, inhibition of ionomycin-stimulated ^{86}Rb influx from washed human red blood cells. B, selective inhibition of IK1 component of whole-cell current in a T lymphocyte activated with 2 μg/ml phytohemagglutinin for 48 h. Currents were recorded during an 800-ms voltage ramp from −130 to +40 mV in isotonic KCl with intracellular Ca^{2+} set to 1 μM. The lack of effect of maurotoxin on the Kv1.3 component of the current in lymphocytes was confirmed by the absence of inhibition of Kv1.3 channels stably expressed in CHO cells (inset). Currents were elicited by 1200-ms voltage steps to +20 mV

synaptosomes (Kharrat et al., 1996, 1997). Indeed, the current studies have also shown that maurotoxin can displace ^{125}I-apamin from CHO cells stably expressing human SK2 and SK3 calcium-activated potassium channels. The reason for the apparent disparity between ^{86}Rb efflux and electrophysiology studies and ^{125}I-apamin binding studies is that the latter is performed in a low ionic strength aqueous environment. When ^{86}Rb efflux experiments were performed in a similar low ionic strength environment, efflux through SK potassium channels was inhibited by maurotoxin with a potency that is comparable with the inhibition of ^{125}I-apamin binding. The increase in potency of maurotoxin in low ionic strength buffers was also observed with inhibition of IK1. The IC_{50} for inhibition of ^{86}Rb efflux through IK1 shifts approximately two orders of magnitude, from 1.4 nM to 14 pM, in low ionic strength buffer. A similar shift in potency was also observed for inhibition of IK1 potassium channels by charybdotoxin in this study and has been previously reported for charybdotoxin inhibition of the native intermediate conductance Ca^{2+}-activated K^+ channel in red blood cells (Brugnara et al., 1993). The mechanism for the low ionic strength-mediated increase in potency of maurotoxin has not been investigated in this study, but may result from an increased on rate for binding similar to that reported for the ionic strength dependence of charybdotoxin block of large-conductance, calcium-activated potassium channels (Anderson et al., 1988).

The ability of maurotoxin to potently inhibit intermediate-conductance Ca^{2+}-activated K^+ channels has been confirmed in native cell systems. The Gardos channel in red blood cells exhibits biophysical and pharmacological properties similar to those of heterologously expressed IK1 channels (Christophersen, 1991; Ishii et al., 1997b). The current study has shown that maurotoxin potently inhibits the Gardos channel in red blood cells with an IC_{50} similar to that observed for IK1 channels. Intermediate-conductance, calcium-activated

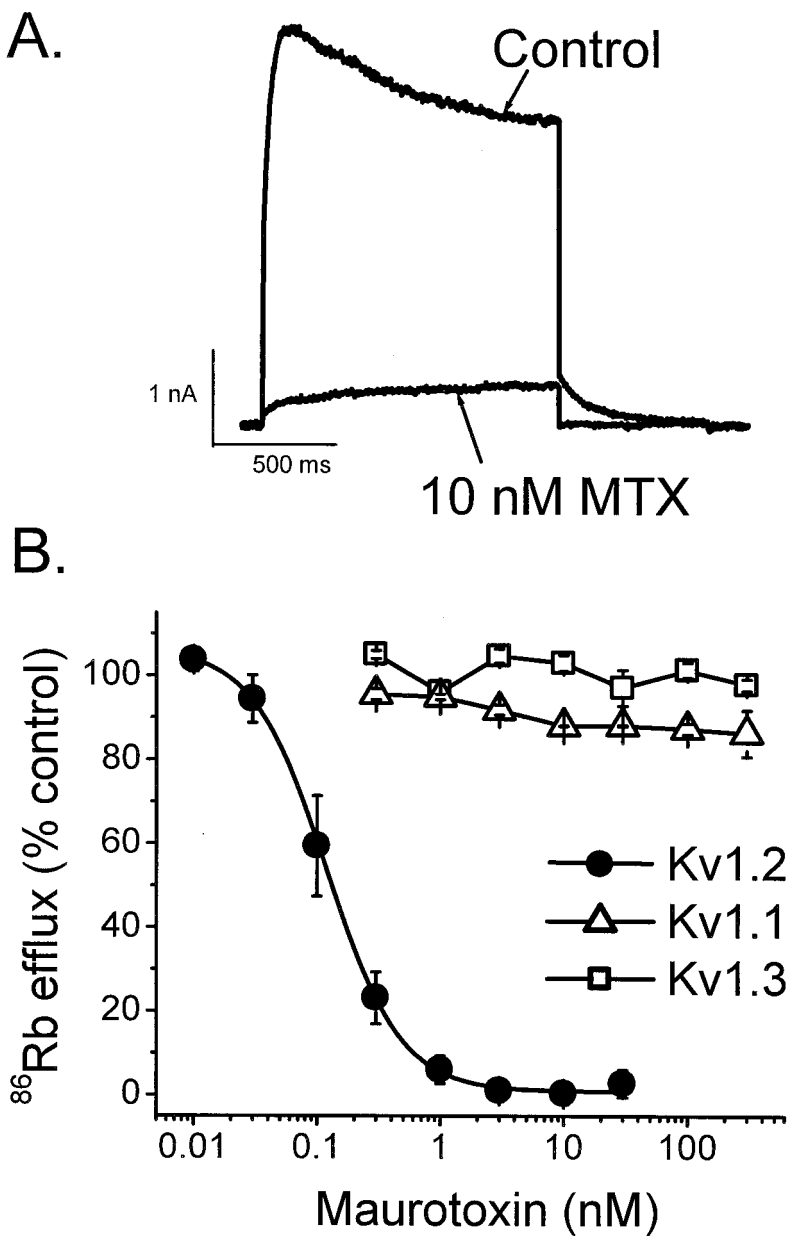

Fig. 7. A, maurotoxin (10 nM)-induced inhibition of voltage-dependent potassium current in mouse fibroblast cells stably expressing mouse Kv1.2. Outward currents were elicited by a 1.2-s voltage step to +40 mV from a holding potential of −80 mV. B, concentration-dependent effects of maurotoxin on KCl-stimulated ^{86}Rb efflux from monolayers of cells stably expressing mouse Kv1.1, mouse Kv1.2, or human Kv1.3 potassium channels. The curve fitted to the concentration-dependent inhibition of Kv1.2 by maurotoxin provided an IC_{50} of 0.12 ± 0.08 nM (n = 4)

potassium channels encoded by IK1 are also expressed in human T lymphocytes, where they are believed to play an important role in the proliferative responses to antigenic and mitogenic stimuli (Cahalan et al., 2001). Indeed, expression of IK1 greatly increases after activation of T lymphocytes (Ghanshani et al., 2000). The current study has shown that maurotoxin potently and selectively inhibits IK1-mediated potassium currents in T lymphocytes, producing no inhibition of the voltage-dependent potassium current, which is encoded by Kv1.3 (KCNA3) in these cells. The absence of inhibition of Kv1.3 potassium currents by maurotoxin in lymphocytes distinguishes it from another scorpion toxin, charybdotoxin, which blocks both IK1- and Kv1.3-mediated potassium currents in these cells (Rader et al., 1996).

The absence of inhibitory activity of maurotoxin against Kv1.3 voltage-dependent potassium channels found in this study was also observed for the related Kv1.1 potassium channel. In marked contrast, maurotoxin was found to be a

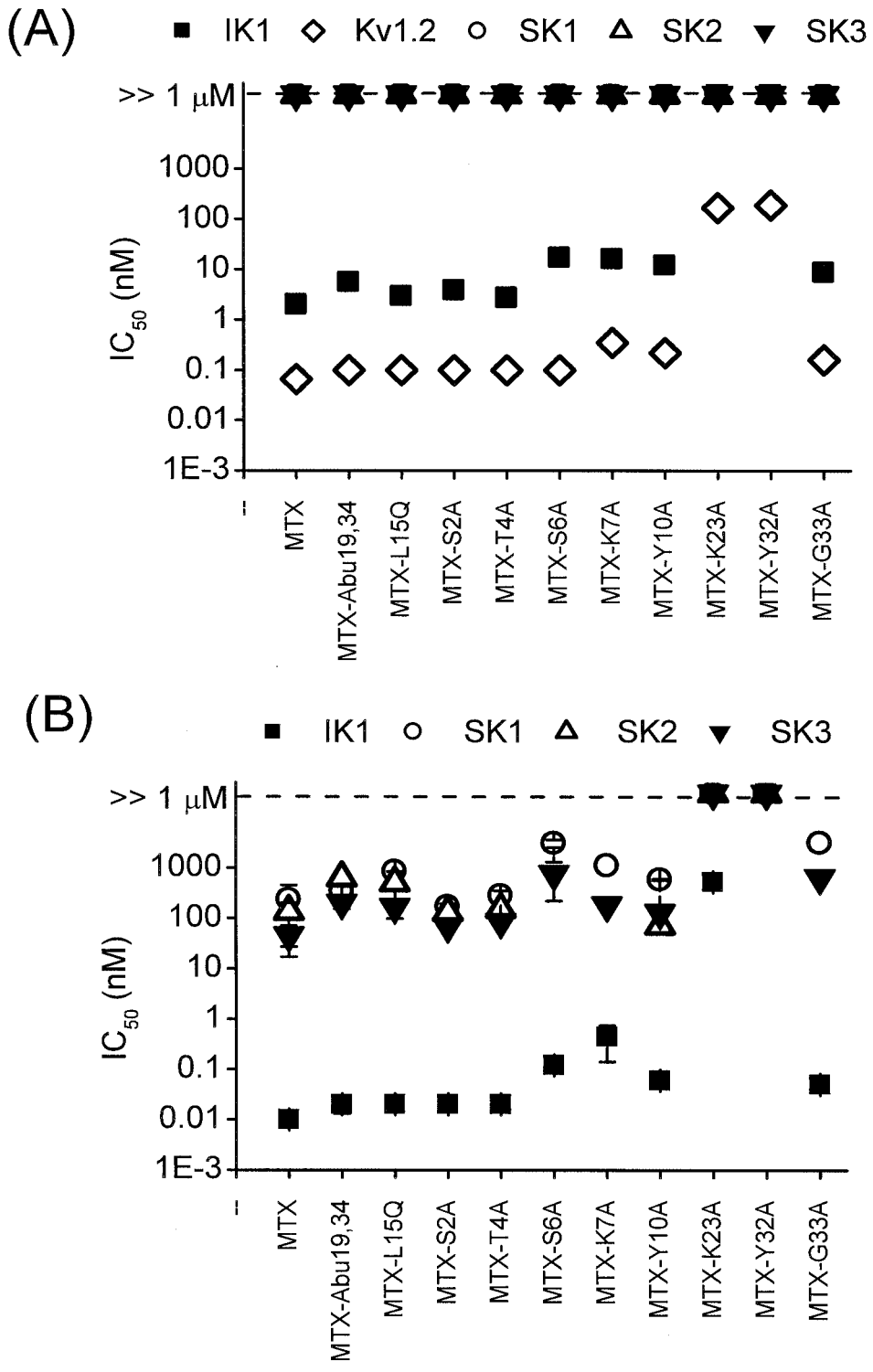

Fig. 8. Effect of changing amino acid residues on blocking activity of MTX. A, IC_{50} values for inhibition of ionomycin-stimulated ^{86}Rb efflux through IK1, SK1, SK2, and SK3 or Kv1.2 in physiologically relevant ionic conditions. B, IC_{50} values for inhibition of ionomycin-stimulated ^{86}Rb efflux through IK1, SK1, SK2, and SK3 in low ionic strength extracellular buffer.

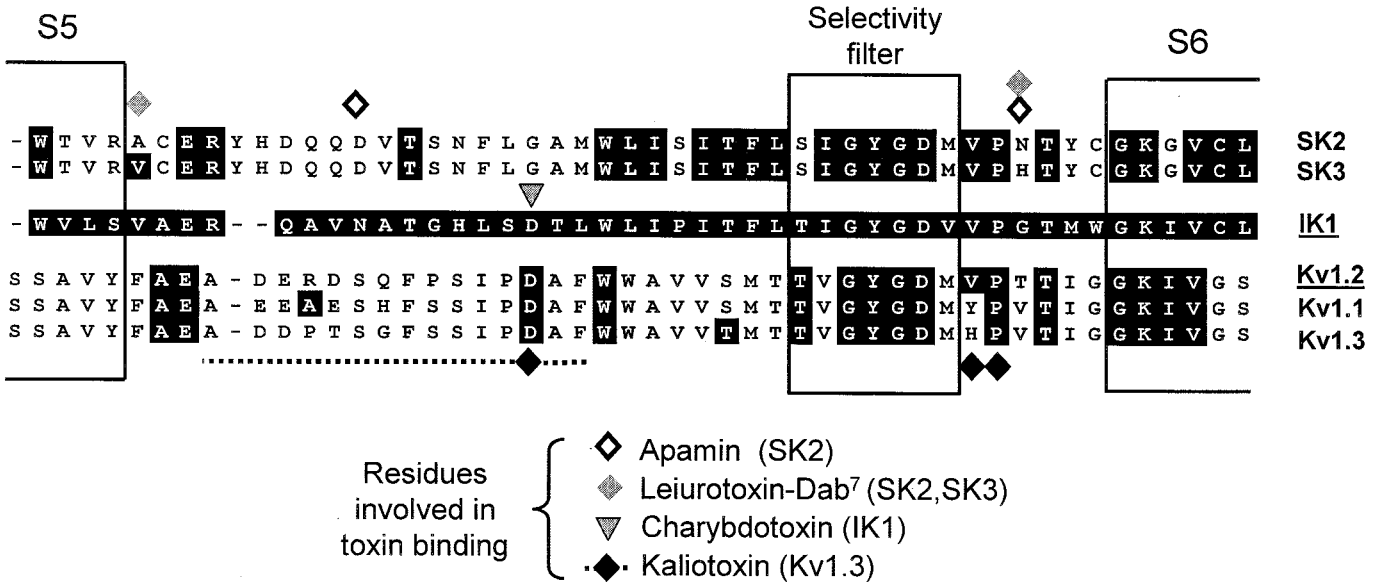

Fig. 9. Comparison of amino acid sequences of pore domain of IK1 with SK and Kv potassium channels. Residues known to be involved in the binding of peptide toxins are indicated. Amino acid sequence identity with IK1 is shaded black.

very potent inhibitor of the Kv1.2 potassium channels expressed in mammalian cells. The inhibition of Kv1.2 is consistent with previous studies performed in oocytes (Kharrat et al., 1996). However, these same studies also observed inhibition of Kv1.1 and Kv1.3 channels by maurotoxin, albeit with a lower potency. The reason for the discrepancy between mammalian cell and oocyte effects of maurotoxin on Kv1.1 or Kv1.3 is unclear, although similar potency differences have been reported for block of these channels by other scorpion toxins (Mourre et al., 1999).

The ability of maurotoxin to inhibit ^{125}I-apamin binding to SK channels (current study) and ^{125}I-kaliotoxin binding to Kv channels (Kharrat et al., 1996, 1997) indicates that the toxin binds to or near the external vestibule of the pore because amino acid residues critical for the binding of apamin and kaliotoxin and other peptide toxins are found in this region of the channel (Aiyar et al., 1996; Ishii et al., 1997a; Legros et al., 2000; Rauer et al., 2000; Shakkottai et al., 2001). Maurotoxin's potent inhibition of both IK1 and Kv1.2 potassium channels suggests that the external vestibules around the pore in both channels exhibit structural similarities. However, structural similarities are not easily inferred from the low amino acid sequence homology of the pore region of IK1 and Kv1.2 channels (Fig. 9). Where homology does exist, similar sequences can be found in maurotoxin-insensitive Kv1.3 and SK3 channels (Fig. 9). From studies of structure-activity relationship for the maurotoxin itself, it seems that single mutations of the toxin at Ser2, Thr4, Ser6, Lys7, Tyr10, Lys15, and Gly33 have modest or no effects on the peptide's ability to inhibit ^{86}Rb efflux through IK1 Kv1.2 or SK channels (Fig. 8). In contrast, substitution of either Lys23 (referred to as the "functional" Lys) or Tyr32 of maurotoxin greatly affects its potency to block IK, SK and Kv1.2 channels, suggesting that these key residues are directly involved in the recognition and high-affinity interaction of the toxin with the all of the potassium channel subtypes tested. In agreement with these experimental data, Lys23 is indeed spatially located near Tyr32 in the three-dimensional structure of maurotoxin (Blanc et al., 1997), suggesting that these two residues are part of the toxin-interacting surface with IK1, Kv1.2, and perhaps SK channels. Note that it was found unexpectedly that the blocking efficacy of the three-disulfide–bridged MTX-Abu19,34 analog toward these channels were similar to that of native maurotoxin, despite some marked differences in three-dimensional structures between the peptides (i.e., reorientation of the helix with regard to the β-sheet) (Blanc et al., 1997; Fajloun et al., 2000a). Together, the data also strongly suggest that the β-sheet structure of maurotoxin is implicated in the interaction of this toxin with IK1 and Kv1.2 channels. Although the involvement of the β-sheet structure of a scorpion toxin in voltage-gated potassium channel (e.g., Kv1.2) recognition is rather well documented (Darbon et al., 1999), nothing about the putative structural domain(s) that might be involved in the recognition of the intermediate-conductance, Ca^{2+}-activated IK1 channel has been documented.

In summary, the studies described have shown that maurotoxin is a potent and selective inhibitor of the intermediate conductance subtype of calcium-activated potassium channels. As such, maurotoxin is likely to be a useful tool for further investigating the role of IK1 in physiological processes. For example, because of its lack of inhibitory activity against Kv1.3, maurotoxin may prove to be an excellent pharmacological agent for better defining the role of IK1 in lymphocyte activation.

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Address correspondence to: Neil A. Castle, Ph.D., Icagen Inc., 4222 Emperor Boulevard, Suite 350, Durham, NC 27703. E-mail: ncastle@icagen.com