Pergamon

Neuropharmacology 40 (2001) 879–887

NEURO PHARMACOLOGY

www.elsevier.com/locate/neuropharm

Pharmacological modulation of SK3 channels

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Received 13 July 2000; received in revised form 5 January 2001; accepted 26 January 2001

Abstract

Small-conductance, calcium-activated K^+ channels (SK channels) are voltage-insensitive channels that have been identified molecularly within the last few years. As SK channels play a fundamental role in most excitable cells and participate in afterhyperpolarization (AHP) and spike-frequency adaptation, pharmacological modulation of SK channels may be of significant clinical importance. Here we report the functional expression of SK3 in HEK293 and demonstrate a broad pharmacological profile for these channels. Brain slice studies commonly employ 4-aminopyridine (4-AP) to block voltage-dependent K^+ channels or a methyl derivative of bicuculline, a blocker of gamma-aminobutyric acid (GABA)-gated Cl^- channels, in order to investigate the role of various synapses in specialized neural networks. However, in this study both 4-AP and bicuculline are shown to inhibit SK3 channels (IC_{50} values of 512 μM and 6 μM, respectively) at concentrations lower than those used for brain slice recordings. Riluzole, a potent neuroprotective drug with anti-ischemic, anticonvulsant and sedative effects currently used in the treatment of amyotrophic lateral sclerosis, activates SK3 channels at concentrations of 3 μM and above. Amitriptyline, a tricyclic antidepressive widely used clinically, inhibits SK3 channels with an IC_{50} of 39.1±10 μM (n=6).

Keywords: Riluzole; 1-Ethylbenzimidiazolone; Amitriptyline; Bicuculline; Ca^{2+}-activated K^+ channel; 4-Aminopyridine

1. Introduction

In mammalian cells, Ca^{2+} is a ubiquitous signaling ion linked to a number of important cellular functions. In order to regulate and control the intracellular levels of Ca^{2+}, a wide range of membrane proteins respond to changes in Ca^{2+}. As fluctuations in intracellular Ca^{2+} will affect the electrical potential across the plasma membrane, ion channels that react upon changes in intracellular calcium are of crucial importance for maintaining control of the membrane potential. One class of such ion channels is the Ca^{2+}-activated K^+ channels, which structurally, functionally and pharmacologically can be divided into three subfamilies: big-conductance, voltage-dependent Ca^{2+}-activated K^+ channels (BK channels); intermediate-conductance, voltage-independent Ca^{2+}-activated K^+ channels (IK channels); and small-conductance, voltage-independent Ca^{2+}-activated K^+ channels (SK channels). SK channels are gated directly by intracellular Ca^{2+}, a gating mediated via the constitutively bound calmodulin (Xia et al., 1998).

Three mammalian SK channel subtypes have been identified molecularly (Köhler et al., 1996). The SK channels belong to the 6TM K^+ channel family, and share a high degree of homology within the three subtypes. The SK channels are also related to the IK channel, although the homology is significantly lower (40–45%). In contrast, the homology to BK channels is not any different than the homology to other 6TM K^+ channels (<15%). Expression of SK1, 2 and 3 in *Xenopus laevis* oocytes revealed significant differences in the affinity towards apamin (Ishii et al., 1997; Köhler et al., 1996), a toxin isolated from bee venom. These data suggested that SK2 and SK3 channels are highly sensitive to apamin, whereas SK1 channels were reported to be insensitive to 100 nM apamin. This observation could not be repeated after expression in either mammalian

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0028-3908/01/$ - see front matter
PII: S0028-3908(01)00028-4

expression systems (Jager et al., 2000; Shah and Haylett, 2000; Strøbæk et al., 2000) or *Xenopus* oocytes (Grunnet et al., 2001).

In vertebrate neurons, action potentials are followed by an afterhyperpolarization (AHP) that may persist for several seconds. The AHP has several components, a fast, a medium and a slow component (sAHP), all of which have profound consequences for the firing pattern of the neuron. The sAHP is important for spike-frequency adaptation, and the channels underlying the sAHP are the SK channels. Once activated, the SK channels conduct K^+ out of the cell, resulting in hyperpolarization and, thus, an inhibition of firing action potentials.

As SK channels play a fundamental role in most excitable cells and participate in the spike-frequency adaptation, pharmacological modulation of SK channels may be of significant clinical importance. Data on the pharmacology of SK1 and SK2 expressed in mammalian cells has been reported (Shah and Haylett, 2000; Strøbæk et al., 2000), whereas no data have been presented as to the pharmacology of SK3 channels. Here we report a broad pharmacological profile on SK3 channels, including activation of SK3 channels by the neuroprotective compound riluzole, used clinically to treat amyotrophic lateral sclerosis, and inhibition of SK3 channels by amitriptyline, a tricyclic antidepressant.

2. Methods

2.1. Molecular biology

A rat SK3-encoding DNA fragment (GenBank Acc. U69884) from an oocyte expression vector, kindly provided by J. Adelman, was subcloned into a mammalian expression vector (pIRESpuro, ClonTech) between the cytomegalovirus (CMV) major immediate early promoter/enhancer and the synthetic intron sequence. An intron within a mRNA may provide a more efficient nuclear export. An internal ribosome entry site (IRES) from encephalomyocarditis virus (ECMV) follows the intron sequence, which directs the translation of a puromycin resistance gene. Inclusion of both the SK3-coding sequence and a selection gene within the same mRNA provides a highly efficient method for obtaining cell lines stably expressing SK3 channels.

2.2. Stable expression of the SK3 channel in HEK293 cells

HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Life Technology) supplemented with 10% fetal bovine serum (Life Technology) at 37°C in a 5% CO_2 atmosphere. Cells were transfected at a density of approximately 4×10^4 cells/cm^2 with pIRESpuro–SK3 plasmid (2 μg/T75 flask) with lipofectamine (Life Technology) according to the manufacturer's instructions. Cells were selected with 1 μg/ml puromycin (Sigma). After 12 days of selection all control cells were eliminated and more than 100 colonies were observed in the pIRESpuro–SK3 transfected dish. These colonies were pooled and no further selection was performed.

2.3. Electrophysiology

All experiments were performed in whole-cell patch-clamp configuration at room temperature with an EPC-9 amplifier (HELA Electronics, Lambrecht, Germany). Pipettes were pulled from thin walled borosilicate glass (ModelOhm, Copenhagen, Denmark) and had a resistance between 1.5 and 2.5 MΩ. A custom-made perfusion chamber (volume 15 μl) with a fixed AgCl–Ag pellet electrode was mounted on the stage of an inverted microscope.

A coverslip with transfected HEK293 cells was transferred to the perfusion chamber and superfused with a high K^+ solution consisting of (in mM): 144 KCl, 2 $CaCl_2$, 1 $MgCl_2$ and 10 *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES; pH 7.4 with KOH). The only exception was experiments performed at physiological solutions where the extracellular solution consisted of (in mM): 150 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$ and 10 HEPES (pH 7.4 with NaOH).

Pipettes were filled with solutions consisting of (in mM): 144 KCl, 1 or 10 ethylene glycol bis(β-aminoethyl ether)-*N*′,*N*′-tetraacetic acid (EGTA), 0 or 9 nitrilotetraacetic acid (NTA) and 10 HEPES (pH 7.2 with KOH). $CaCl_2$ and $MgCl_2$ were added in concentrations calculated (EqCal; BioSoft, Cambridge, UK) to give a free Mg^{2+} concentration of 1 mM and free Ca^{2+} concentrations of either 0, 100, 300 or 1000 nM, respectively.

No zero current or leak current subtraction was performed during the experiments. Cell capacitance and series resistance were updated before each pulse application. Series resistance values were between 2.5 and 10.0 MΩ, and only experiments where the resistance remained constant during the experiments were analyzed. Current signals were low-pass filtered at 3 kHz and acquired using Pulse software (HEKA Electronics, Lambrecht, Germany).

In the few cases where the baseline was sliding, extrapolation of the baseline during the drug application period was determined and subtracted (Strøbæk et al., 2000).

Data analysis and drawings were performed using IGOR software (WaveMetrics, Lake Oswego, OR, USA) or Graphpad Prism software.

2.4. Vesicle preparation

Approximately 1.5×10^6 transfected or non-transfected HEK293 cells were transferred to ultra centrifuge tubes.

The cell solution was subject to high-speed centrifugation at 212,000*g* for 30 min at 4°C in a Beckman Ti 70.1 rotor. The supernatant was decanted and the pellet resuspended in 10 ml ice-cold 20 mM Tris/HCl, 10 mM NaCl, pH 7.4 and kept on ice. The resuspended supernatant was subject to another high-speed centrifugation at 50,000 rev/min for 30 min at 4°C in a Beckman Ti 70.1 rotor. The supernatant was discarded and the pellet resuspended in 750 μl ice-cold 20 mM Tris/HCl, 10 mM NaCl, pH 7.4. Finally, the vesicle solution was homogenized in a small glass/Teflon homogenizer with 10 strokes at 1000 rev/min and stored at −80°C until further use.

2.5. Preparation of intact HEK293 cells for binding

DMEM medium was removed from transfected HEK293 cells expressing SK3 and grown in cell culture T25 flasks. Cells were washed in cold phosphate-buffered saline before ice-cold buffer consisting of (in mM) 20 Tris/HCl, 10 NaCl and 270 sucrose, pH 7.4, was added. Cell culture flasks were kept on ice for 5–10 min before cells were loosened from the bottom using a rubber policeman. The cells were subsequently used for binding experiments without further treatment.

2.6. Binding studies

In all experiments radiolabeled iodinated apamin (^{125}I-apamin, specific activity 4880 dpm/fmol) was used as ligand. Incubation was carried out in 0.5 ml of a medium consisting of (in mM): 10 KCl, 10 NaCl, 20 Tris/HCl and 0.1% bovine serum albumin (BSA), pH 8.4 (for binding to vesicles), or 10 KCl, 10 NaCl, 20 Tris/HCl, 0.1% BSA and 260 sucrose, pH 8.4 (for binding to intact cells). Incubation in 10 mm×75 mm polystyrene tubes was carried out for 2 h at 4°C. Non-specific binding was defined in the presence of 200 nM non-labeled apamin. All serial toxin dilutions were performed in (in mM) 150 NaCl, 20 Tris/HCl and 0.1% BSA, pH 7.4, and toxin was always added directly to the incubation medium to avoid absorption phenomena. The protein concentration was 3.4 μg protein/sample for vesicle experiments and 16.8 μg protein/sample for binding to intact cells. Protein concentrations were determined according to Bradford using BSA as a standard.

Binding experiments were stopped by addition of 3 ml ice-cold washing buffer consisting of (in mM): 150 NaCl and 20 Tris/HCl, pH 7.4. The solution was filtered rapidly through Toyo Advantec GC 50 glass fiber filters (presoaked at room temperature for at least 1 h in 0.3% (wt/vol) polyethylenimine) on a Millipore 1002530 filter apparatus, followed by two subsequent washings with 3 ml ice-cold washing buffer. In each experiment triplicate assays were routinely performed and the data averaged.

2.7. Data analysis

IC_{50} values were calculated either from equilibrium concentration–response experiments or from single-addition, non-equilibrium experiments (pre-steady state Michaelis–Menten) by an estimate of the kinetic parameters of the block. The IC_{50} values of the individual experiments were subsequently averaged. Normalized data from the concentration–response experiments for apamin block, 4-aminopyridine and bicuculline block were fitted to the one-site competition equation:

$$Y=(\text{top}-\text{bottom})/(1+10^{X}-\log \text{IC}_{50}),$$

where Y=observed relative current, X=log of the concentration of the blocker, top=maximal observed current, bottom=minimal observed current and IC_{50}=half maximum current.

In the pre-steady state Michaelis–Menten fit, the compound-induced decrease in current versus time was fitted to a non-equilibrium Michaelis–Menten equation:

$$I_t=I_0\{1-[C/(C+K_i)\times(1-\exp^{-t(C\times K_{on}+K_{off})})]\},$$

where K_{on} is the on rate (in M^{-1} s^{-1}), K_{off} is the off rate (in s^{-1}), K_i is the inhibitory constant (in M), I_0 is the current measured at time 0 (the unblocked, control current) and I_t is current measured at time t, after addition of the compound at the concentration, C.

3. Results

3.1. Characterization of the SK3 cell line

To characterize SK3 channels with respect to their basic pharmacology, the channels were expressed in HEK293 cells. We have previously used this cell line successfully to generate stably expressed BK channels (Ahring et al., 1997), SK1 and SK2 channels (Strøbæk et al., 2000) and IK channels (Jensen et al., 1998). Fig. 1(A) shows the whole-cell currents in symmetrical K^+ solution of a HEK293 cell expressing SK3 channels. The currents are recorded as a voltage ramp from −100 mV to +100 mV is applied. Just after break-in to the whole-cell mode, a current of maximally 100 pA is measured at −80 mV. As the cell is equilibrated with the pipette solution containing 1 μM free Ca^{2+}, the current measured at −80 mV evolved and reaches a maximum of 11 nA one minute after establishing the whole-cell mode. The whole-cell current is an inwardly rectifying current as previously shown for both the SK1 and SK2 channels (Ishii et al., 1997; Strøbæk et al., 2000). The strong inward rectification of SK3 in the whole-cell measurements is independent of intracellular Mg^{2+} data not shown. When the extracellular high K^+ solution is exchanged for a high Na^+ solution, the reversal potential

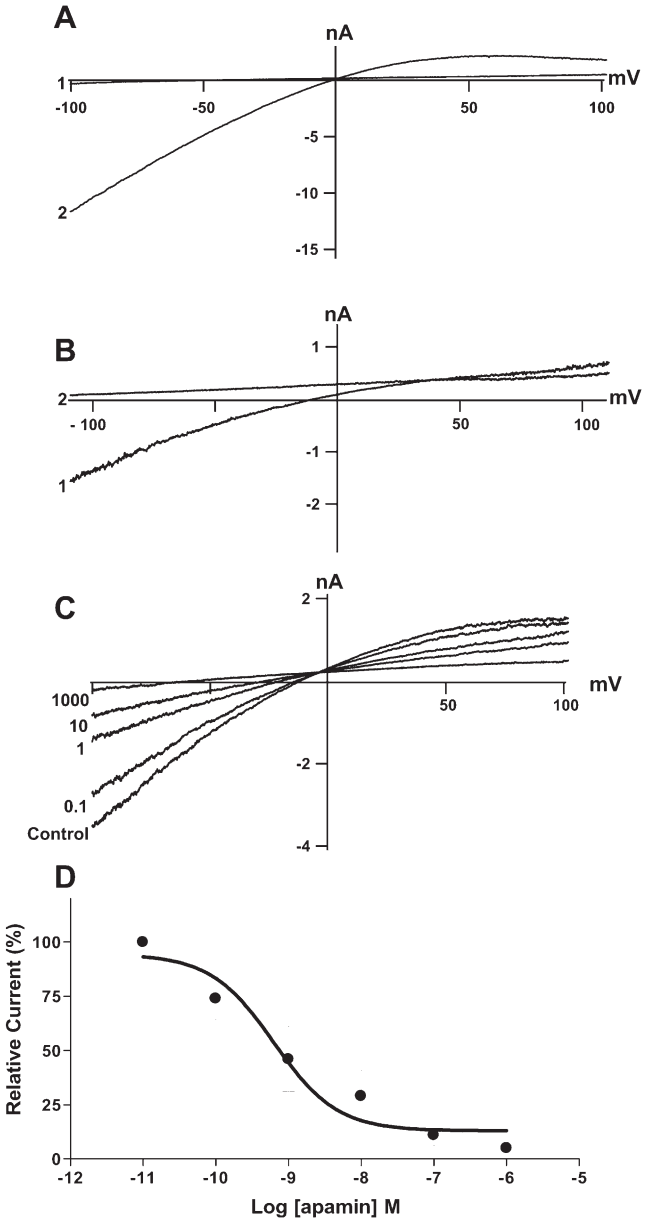

Fig. 1. Expression of SK3 in HEK293 cells. All traces represent whole-cell current recorded on application of voltage ramps from −100 mV to +100 mV. Ramps of 200 ms in duration were applied every 5 s from a holding potential of 0 mV. (A) Ca^{2+} activation of SK3. Traces were recorded just after break-in (trace 1) and after 60 s (trace 2). Both recordings with 1 μM Ca^{2+} in the pipette. (B) K^+ selectivity of SK3. With 300 nM Ca^{2+} in the pipette SK3 activity was allowed to stabilize in symmetrical K^+ (144 mM extracellular K^+, trace 1) before the extracellular solution was changed to a physiological K^+ solution (4 mM extracellular K^+, trace 2). The reversal potential in physiological K^+ solution was approximately −90 mV. (C) Apamin inhibition of SK3. With 300 nM Ca^{2+} in the pipette a stable SK3 activity was obtained before increasing concentrations of apamin were added to the bath. Numbers refer to the apamin concentration in nM. The current was allowed to stabilize before each concentration of apamin was added. (D) Summary of apamin inhibition of SK3. Addition of apamin was cumulative. IC_{50} for the block was calculated to be 630 pM, r^2=0.95 (n=5).

is shifted to the left towards the equilibrium potential for K^+, demonstrating a high K^+/Na^+ selectivity of SK3 [Fig. 1(B)]. Apamin has been widely used as a potent inhibitor of SK channels. Fig. 1(C) shows the effect of apamin on the whole-cell current of a HEK293 cell stably expressing SK3 using a pipette solution with 300 nM Ca^{2+}. After break-through and stabilizing of the current, addition of increasing concentrations of apamin decreases the current concentration-dependently. IC_{50} can be calculated as 630±42 pM (n=5), thus demonstrating that SK3 is potently blocked by apamin (Hill coefficient=0.51).

To further characterize the cell line, the number of SK3 channels was estimated using ^{125}I-apamin binding. ^{125}I-apamin was used at concentrations (41 pM) where binding is saturated after expression in *Xenopus* oocytes (Grunnet et al., 2001). Fig. 2(A) shows the binding of ^{125}I-apamin to total membranes isolated from HEK293 cells stably expressing SK3. The total number of binding sites amounts to 0.40±0.1 fmol/μg protein. Untransfected cells were used as a control and no significant binding of apamin could be demonstrated to these cells [Fig. 2(A), right bars]. These channels are both expressed at the surface as well as intracellularly (in the protein-synthesis and membrane-insertion machinery), and the measurements of ^{125}I-apamin binding were extended to include only surface-expressed SK3 channels. Fig. 2(B) shows the binding of ^{125}I-apamin to the surface of HEK293 cells stably expressing SK3 or to non-transfected cells, respectively. The number of apamin binding sites on the cell surface was measured at 0.27±0.07 fmol/μg protein, corresponding to approximately 65% of the total number of SK3 channels being expressed at the surface [Fig. 2(C)].

3.2. Pharmacological activation of SK3

The cloned IK channel is activated by the benzimidazolone, 1-ethylbenzimidiazolone (EBIO) (Jensen et al., 1998; Pedersen et al., 1999). We tested this compound on the HEK293 cells stably expressing SK3. Fig. 3(A) shows the time course of the whole-cell current using an intracellular concentration of 100 nM Ca^{2+}. Extracellular addition of 50 μM EBIO increased the current approximately 200%. To verify the SK3 current 100 nM apamin was added to the bath, resulting in a complete and reversible block of the current. After wash-out of apamin the activation of the SK3 current by 50 μM EBIO could be repeated. The effect of EBIO was pursued further. Fig. 3(B) shows the whole-cell current measured at ramps from −100 to +100 mV. After break-through and stabilization of the current, increasing concentrations (in μM) of EBIO were added to the bath and resulted in an increase in the whole-cell current measured at all potentials. Fig. 3(C) shows a cumulative dose–response curve for the EBIO effect on SK3 channels. EBIO activates

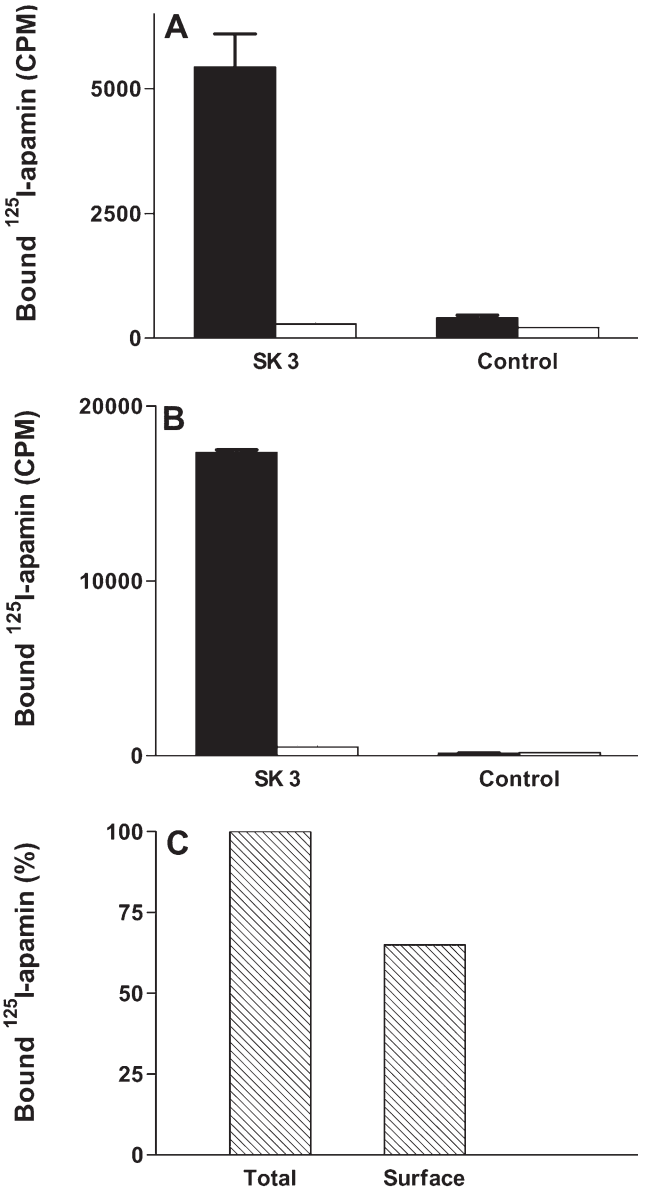

Fig. 2. ^{125}I-apamin binding to HEK cells expressing SK3. (A) ^{125}I-apamin binding to total HEK membranes (intracellular membranes and plasma membranes). Membrane protein (3.4 μg) was incubated for 2 h at 4°C with ^{125}I-apamin (80.000 cpm/sample~41 pM) in the absence (black bars) or presence (open bars) of excess amounts of cold apamin (200 nM). Control experiments were performed with untransfected HEK cells. (B) ^{125}I-apamin binding to intact HEK cells. Membrane protein (16.8 μg) was incubated for 2 h at 4°C with ^{125}I-apamin (80.000 cpm/sample~41 pM) in the absence (black bars) or presence (open bars) of excess amounts of cold apamin (200 nM). Control experiments were performed with untransfected intact HEK cells. (C) Comparison of ^{125}I-apamin binding to total HEK membranes and plasma HEK membranes. Data presented in (A) and (B) were adjusted according to the amount of protein to obtain information about the fraction of SK3 channels present in the plasma membrane.

the SK3 channel concentration-dependently and as little as 1 μM EBIO increases the whole-cell current [Fig. 3(B)]. Fig. 3(C) summarizes the effect of EBIO on SK3 currents and shows the current relative to the control as

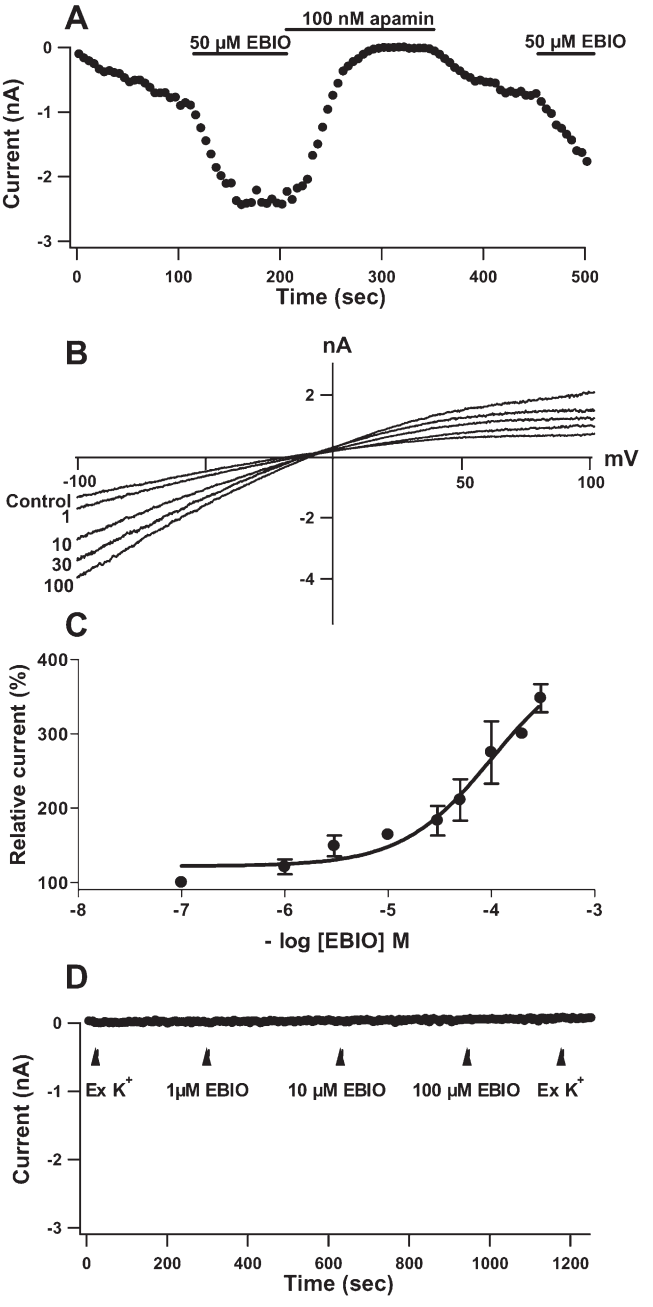

Fig. 3. EBIO activation of SK3. Data based on whole-cell recordings where currents were measured from voltage ramps (−100 mV to +100 mV, 200 ms in duration, 5 s between sweeps). Pipettes contained a high K^+ solution with free Ca^{2+} buffered at 100 nM, the only exception being the data presented in (D) where no Ca^{2+} was present. (A) EBIO activation of SK3 current. Addition of 50 μM EBIO to the bath increased the control current by approximately 200%. The current could be reversibly blocked by addition of 100 nM apamin and the activation by EBIO could be repeated. Currents were measured at −80 mV. (B) After pipette break-through the current was allowed to stabilize (control) before different concentrations (in μM) of EBIO were added to the bath. After each addition of EBIO the current was allowed to stabilize before further addition of EBIO was performed. (C) Summary of EBIO activation of SK3 measured at −80 mV (n=4). (D) Addition of EBIO in the absence of intracellular Ca^{2+}. Time course of whole-cell recordings performed with high intracellular K^+ containing no addition of $CaCl_2$ and 10 mM EGTA (n=4).

a function of the EBIO concentration. Using a low intracellular concentration of Ca^{2+} (calculated at 100 nM), EBIO at low concentrations (100 nM) has no effect on SK3. From 1 μM and above EBIO activates SK3 concentration-dependently, reaching a 3.5-fold increase in current at 500 μM. Using this experimental set-up it was not possible to obtain saturation of the EBIO effect on SK3, since the currents became unstable at EBIO concentrations in excess of 500 μM. The IK channel is activated by EBIO via an obligatory calcium-dependent mechanism (Pedersen et al., 1999). To investigate whether SK3 is activated by EBIO via a similar mechanism, HEK cells expressing SK3 were patch-clamped using an intracellular Ringer's solution that is nominally free of Ca^{2+}. Shown in Fig. 3(D) is the effect of EBIO at three different concentrations on the whole-cell current measured at −80 mV. Not even at a concentration of 100 μM is EBIO able to activate the SK3 channels in the absence of intracellular Ca^{2+}. As is the case for the IK channel, the activation of SK3 channels by EBIO is strictly Ca^{2+}-dependent.

Riluzole is a neuroprotective drug that blocks glutamatergic neurotransmission in the central nervous system (CNS). It inhibits the release of glutamate from cultured neurons, from brain slices, and from corticostriatal neurons in vivo by an effect thought to be on voltage-dependent Na^+ channels. Fig. 4(A) shows the effect of riluzole (3 μM) on the whole-cell current in HEK cells stably expressing SK3 channels. Riluzole elicits a very significant, rapid, stable and reversible stimulation of SK3 channels. The effects of riluzole were concentration-dependent, and riluzole activates SK3 at all potentials as shown by the current–voltage relationship in Fig. 4(B). The activation of SK3 is significant at 3 μM, a concentration of riluzole that causes a 240% increase in the current level compared with the control before addition of riluzole. The activation of SK3 by riluzole is obligatorily dependent on intracellular Ca^{2+} (n=4) results not shown and thus similar to the effect of EBIO on SK3. Concentrations above 10 μM (e.g., 30 μM) result in an unstable voltage-clamp of the cell membrane and, therefore, reliable measurements are not technically possible at these concentrations.

To address the apamin sensitivity of the riluzole-activated currents we conducted a series of experiments with administration of riluzole (5 μM; 3 min duration) followed by administration of riluzole+apamin (100 nM; 5 min duration). Apamin fully blocked the riluzole-induced current in all experiments (n=4), and similar results were found for administration of 50 μM EBIO without and with apamin (n=3) data not shown. In order to investigate the possible effect of the SK channel activators on endogenous currents in HEK cells, 50–100 μM riluzole (n=4) and 100–500 μM EBIO (n=3) were administrated to non-transfected cells; they failed to activate any current data not shown.

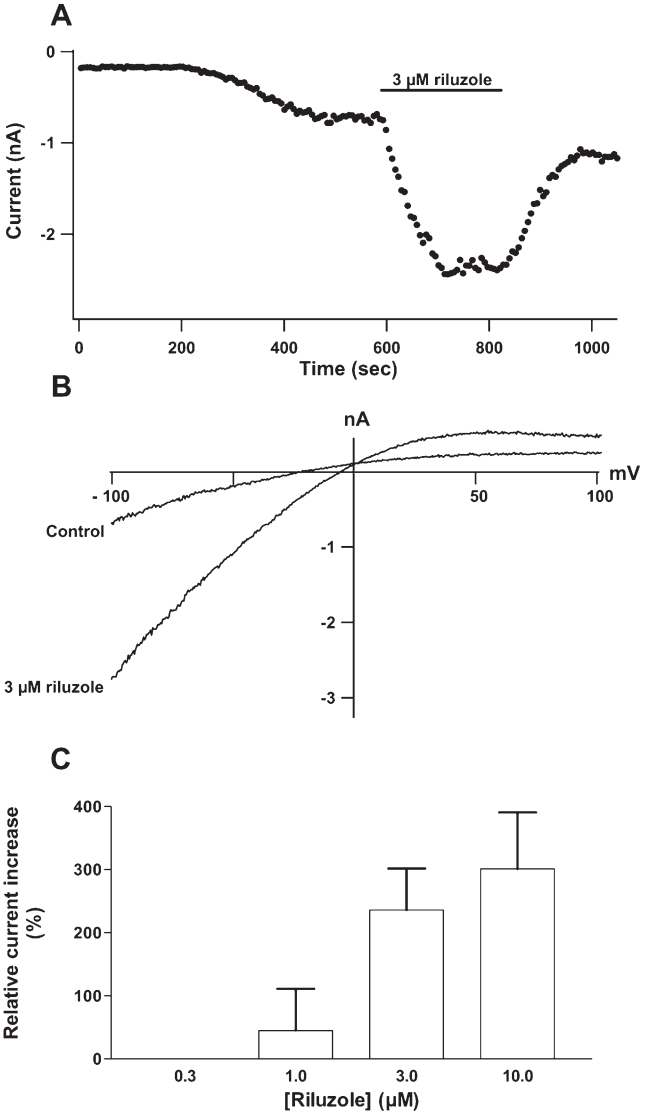

Fig. 4. Effect of riluzole on SK3 channels. Data based on whole-cell recordings where currents were measured from voltage ramps (−100 mV to +100 mV, 200 ms in duration, 5 s between sweeps). Pipettes contained a high K^+ solution with free Ca^{2+} buffered at 100 nM. (A) Time course of whole-cell current. After break-through, the current (which was measured at −80 mV) was allowed to stabilize before 3 μM riluzole was added to the bath. The increase in current was reversed by washing. (B) After break-through current was allowed to stabilize before 3 μM riluzole was added to the bath. Measured at −80 mV, application of riluzole increased the current to approximately 240% of the control value. (C) Activation of SK3 current by riluzole measured at −80 mV (n=4–7 at each point).

3.3. Pharmacological inhibition of SK3

Among the classical inhibitors of the shaker-type potassium channels is 4-aminopyridine (4-AP). 4-AP inhibits various Kv channels with an IC_{50} between 200 and 500 μM. We tested the effect of 4-AP on SK3 channels and found that 4-AP inhibits SK3 channels. Fig. 5(A) shows the whole-cell current measured at −80 mV in the presence of increasing concentrations of 4-AP.

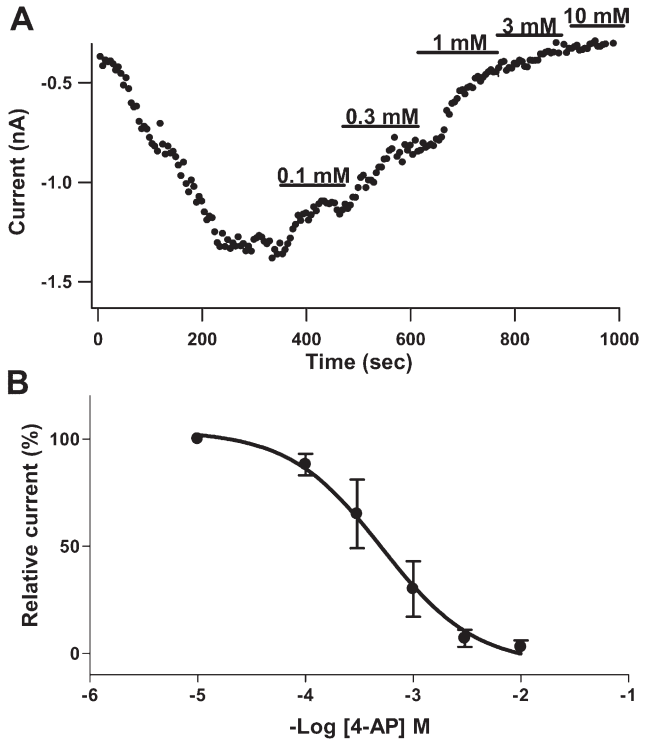

Fig. 5. Inhibition of SK3 by 4-AP. Data based on whole-cell recordings where currents were measured from voltage ramps (−100 mV to +100 mV, 200 ms in duration, 5 s between sweeps). Pipettes contained a high K^+ solution with free Ca^{2+} buffered at 300 nM. (A) Time course of whole-cell current. After break-through the current was allowed to stabilize before different concentrations of 4-AP were added to the bath. (B) Concentration–response relationship of 4-AP block recorded from four individual cells. The addition of 4-AP was cumulative; IC_{50}=512 nM, r^2=0.99.

The SK3 current was recorded using 300 nM Ca^{2+} in the pipette. Once a stable baseline was established, 4-AP was added at increasing concentrations. Already at 100 μM, 4-AP inhibits SK3, and at 10 mM 4-AP, full block of the channel is obtained. 4-AP blocks the SK3 channels concentration-dependently, and an IC_{50} value of 512 μM and a Hill slope of 1.26 can be calculated from the concentration–response curve shown in Fig. 5(B).

Bicuculline methylbromide is a widely used, water-soluble $GABA_A$ antagonist often used in brain slice studies. It was investigated whether bicuculline affects SK3 channel activity. Fig. 6(A) shows the effect of bicuculline methylbromide on the SK3-specific currents at voltages in the interval −75 mV to +75 mV. Bicuculline blocks SK3 potently over the entire voltage range, although the block appears to be voltage-dependent with a larger fraction of the current being blocked at −75 mV than at +75 mV [Fig. 6(A)]. The voltage dependence of the block was not pursued further. The concentration–response relation was investigated by constructing a concentration–response curve from the data presented in Fig. 6(B), which shows the effect of bicuculline in

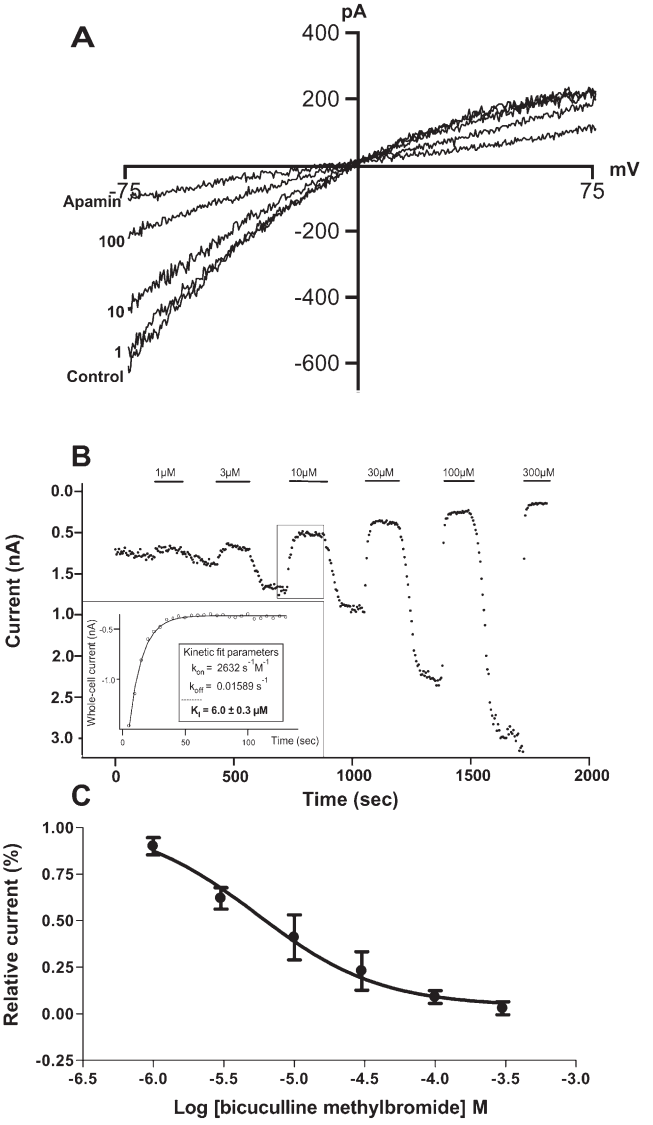

Fig. 6. Effect of bicuculline methylbromide on SK3 channels. The effect of the potential blocker bicuculline methylbromide was investigated in whole-cell recordings where current was measured from voltage ramps (−75 mV to +75 mV, 200 ms in duration, 5 s between sweeps). Pipettes contained a high K^+ solution with free Ca^{2+} buffered at 300 nM. (A) Current measured at control condition was subsequently challenged with increasing concentrations (in μM) of bicuculline methylbromide, which blocked the current in a concentration-dependent manner. (B) Time course of the whole-cell current. Current measured under control conditions could be reversibly blocked by different concentrations of bicuculline methylbromide. The insert shows a kinetic fit to the block by bicuculline methylbromide. IC_{50} estimated from this fit was 6 μM, which is in close proximity to the IC_{50} value determined from the fit of a Hill equation. (C) Summary of bicuculline methylbromide block of SK3 current. Data were fitted to a Hill equation and IC_{50} was calculated to be 6.6 μM, r^2=0.99 (n=3).

increasing concentrations on the whole-cell current at −75 mV as a function of time. Fitting these data to a Hill equation [Fig. 6(C)] gives an IC_{50} of 6.6±0.7 μM (n=3), a value not significantly different from that obtained by using the kinetics of the block to determine

the K_i value [Fig. 6(B), insert]. The pre-steady state Michaelis–Menten fit results in a K_i value of 6.0±0.3 μM (n=11).

Many pharmaceuticals work through several molecular mechanisms, some of which remain unknown. In order to elucidate the pharmacology of SK channels, and to pinpoint the significance of SK channels in the treatment of neurological disease states, a number of pharmaceuticals were tested. The tricyclic amitriptyline is a blocker of SK3 channels and inhibits SK3 channels with an IC_{50} of 39.1±10 μM (n=6) as determined by pre-steady state Michaelis–Menten fits (see Table 1). The block was fast and reversible, although the compound did not wash out easily. Cyproheptadine, an antihistamine H1 antagonist with sedative effects, inhibits SK3 channels with an IC_{50} of 9.2±2 μM (n=3). Similarly, chlorpromazine, a high-dose antipsychotic with sedative effects, is a potent inhibitor of SK3 channels. Chlorpromazine blocks SK3 with a rapid onset and is washed out easily. The kinetics of the block was used to estimate the IC_{50} as 0.6±0.24 μM (n=3).

4. Discussion

Expression of SK3 in mammalian cells gives rise to an inwardly rectifying K^+-selective current. The outward current measured at potentials above 50 mV often had a negative slope, and the ratio of the current measured at −80 mV to the current measured at +80 mV was often 5 or above. The SK3 channel activated as the cytosol was exchanged for the pipette solution due to the increase in intracellular Ca^{2+}. As previously demonstrated after expression in *Xenopus* oocytes, SK3 is sensitive to apamin (Ishii et al., 1997; Köhler et al., 1996; Grunnet et al., 2001). The IC_{50} value for the apamin block of SK3 found in this study (630 pM) is within the same range as the previously reported value on human SK2 (400 pM; Jager et al., 2000) but significantly higher than found on rat SK2 (83 pM; Strøbæk et al., 2000).

The results presented here demonstrate that 4-AP potently blocks SK3 channels at concentrations routinely used in brain slice preparations to inhibit voltage-dependent K^+ channels (Hoffman et al., 1997). The concentrations used are often exceeding 10 mM, a concentration which is sufficient to block most SK3 channels (IC_{50}=512 μM) (see, e.g., Hoffman et al., 1997). As the SK channels represent the K^+ channels underlying the AHP and the spike-frequency adaptation in many neurons, the use of 4-AP in brain slice experiments will affect not only the voltage-dependent K^+ channels but also the SK channels, resulting in profound effects on excitability parameters such as firing frequencies and accommodation. Thus, 4-AP should be used with caution when investigating the molecular mechanisms underlying signal propagation in brain slices. Surprisingly, given the high degree of homology of SK2 to SK3, 4-AP has no effect on SK2 channels at a concentration of 1 mM (Jager et al., 2000).

Amitriptyline is a tricyclic antidepressive used clinically in submicromolar concentrations. Amitriptyline increases the amount of neurotransmitters in the synaptic cleft by inhibition of the re-uptake mechanisms, the serotonine transporter and the noradrenaline transporter. An additional effect of amitriptyline is to enhance the release of transmitters from the presynaptic terminal. This later effect could be mediated via an inhibition of the afterhyperpolarization, which would lead to an increased release of neurotransmitters from the presynaptic terminal due to an increased excitatory input. As the AHP is mediated at least in part by SK channels, blocking these channels would have a significant effect on the release of neurotransmitters.

Riluzole is a neuroprotective drug with anticonvulsant, sedative and anti-ischemic properties, and blocks glutamatergic neurotransmission in the CNS. It is especially effective in preventing spinal cord injury, which is a devastating complication of thoracoabdominal aortic surgery (Lang-Lazdunski et al., 1999). It has also proved very effective in retinal ischemia in models of glaucoma (Ettaiche et al., 1999). Riluzole is used in the treatment of amyotrophic lateral sclerosis and slows down the progression of the disease (Bensimon et al., 1994). Riluzole has protective effects in animal models of Parkinson's disease and in other models of acute neurodegenerative diseases (Bae et al., 2000). The wide range of actions raises the question as to which molecular mechanism riluzole acts through. Riluzole inhibits the release of glutamic acid from cultured neurons, from brain slices, and from corticostriatal neurons in vivo (see, e.g., Doble, 1996), an effect that has been linked to the observation that riluzole inhibits voltage-dependent Na^+ channels use-dependently (Benoit and Escande,

Table 1
Kinetic parameters for the block of SK3 recorded at −80 mV

	On rate (M^{-1} s^{-1})	Off rate (s^{-1})	K_i (μM)	n
Amitriptyline	1005±328	0.0224±0.0084	39.1±10	6
Cyproheptadine	1469±501	0.01728±0.00644	9.18±2.1	5
Chlorpromazine	2996±1560	0.001689±0.0010	0.57±0.24	3

1991). Another molecular mechanism of action that would result in a similar effect is the activation of voltage-independent K^+ channels. This would generate a robust hyperpolarization, possibly both at presynaptic and postsynaptic levels, which in turn would inhibit the release of glutamate. Recently, Lazdunski and co-workers have shown that riluzole activates the background channels TREK-1 and TRAAK (Duprat et al., 2000). This activation is, however, not very pronounced at low concentrations of riluzole (<30 μM). In contrast, SK3 is activated already at 3 μM riluzole. Activation of SK channels in neurons would contribute to a decreased release of glutamate, since the action potential trains would be inhibited if the SK channels are active prior to the arrival of the action potential train. The combined effect of riluzole as a potent, use-dependent Na^+ channel blocker (IC_{50} close to 1 μM) and a potent SK channel activator makes riluzole very well suited for the treatment of convulsions.

Riluzole is the first neuroprotective drug to be marketed. The results presented here provide evidence that SK channels together with Na^+ channels are probably major targets for riluzole, and as such stimulates the development of new neuroprotective molecules acting with high affinity as SK channel openers.

Acknowledgements

This work was supported by the Danish Health Science Research Council, the Velux Foundation, The NovoNordisk Foundation and the Danish Heart Foundation. Pernille O. Hulgaard is gratefully acknowledged for excellent technical assistance.

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