An Apamin- and Scyllatoxin-Insensitive Isoform of the Human SK3 Channel

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ABSTRACT

We have isolated an hSK3 isoform from a human embryonic cDNA library that we have named hSK3_ex4. This isoform contains a 15 amino acid insertion within the S5 to P-loop segment. Transcripts encoding hSK3_ex4 are coexpressed at lower levels with hSK3 in neuronal as well as in non-neuronal tissues. To investigate the pharmacokinetic properties of hSK3_ex4, we expressed the isoforms hSK3 and hSK3_ex4 in tsA cells. Both isoforms were similarly activated by cytosolic Ca\(^{2+}\) (hSK3, EC\(_{50}\) = 0.91 ± 0.4 μM; hSK3_ex4, EC\(_{50}\) = 0.78 ± 0.2 μM) and by 1-ethyl-2-benzimidazolone (hSK3, EC\(_{50}\) = 0.17 ± 0.04 mM; hSK3_ex4, 0.17 ± 0.05 mM, n = 3) and Ba\(^{2+}\) (hSK3, 0.79 ± 0.04, n = 3; hSK3_ex4, 0.8 ± 0.07, n = 3). Ba\(^{2+}\)-tubocurarine blocked both isoforms, and in both cases, the block was strongest at hyperpolarizing membrane potentials. However, the voltage-dependence of hSK3 was stronger than that of hSK3_ex4. The most obvious distinguishing feature of this new isoform was that whereas hSK3 was blocked by apamin (K\(_{a}\) = 0.8 nM), scyllatoxin (K\(_{a}\) = 2.1 nM), and d-tubocurarine (K\(_{a}\) = 33.4 μM), hSK3_ex4 was not affected by apamin up to 100 nM, scyllatoxin up to 500 nM, and d-tubocurarine up to 500 μM. So far, isoform hSK3_ex4 forms the only small-conductance calcium-activated potassium (SK) channels, which are insensitive to the classic SK blockers.

Small conductance calcium-activated potassium channels (SK channels) form a distinct subfamily of potassium channels, which consists of three members, SK1 to SK3 (Köhler et al., 1996). All have a topology that is typical for potassium channels with six transmembrane helices and a P-loop region. They are voltage-independent and can be activated by elevated cytosolic Ca\(^{2+}\) concentrations. The activation of all members of this subfamily is mediated by calmodulin, which constitutively binds to the C-terminal cytosolic region of SK channels (Xia et al., 1998; Fanger et al., 1999; Schumacher et al., 2001). Members of this subfamily are traditionally distinguished by their different sensitivities to apamin (Köhler et al., 1996; Iishi et al., 1997; Grunnet et al., 2001a).

Transcripts of SK1, SK2, and SK3 have been detected in brain tissues, with SK1 expression apparently restricted to brain, whereas SK2 and SK3 are also expressed in non-neuronal tissues (Rimini et al., 2000). SK channels mediate the after-hyperpolarization (AHP) in excitatory cells (Köhler et al., 1996, Stocker et al., 1999). These channels modulate the spike frequency of excitable cells, and therefore they are known to play a crucial role in modulating the firing pattern of these cells. The blockage of currents underlying the AHP leads to a burst of action potentials and an increased dopamine release (Stocker et al., 1999; Pedarzani et al., 2001; Savic et al., 2001). In the rat, it has been shown that SK3 channels function as pacemakers in dopaminergic neurons (Wolfart et al., 2001), which outlines SK3 to play a key role in

ABBRVIATIONS: SK channels, small-conductance calcium-activated potassium channels; IK, intermediate-conductance calcium-activated potassium channels; AHP, after-hyperpolarization; 1-EBIO, 1-ethyl-2-benzimidazolone; PCR, polymerase chain reaction; ScTX, scyllatoxin; TEA\(^{+}\), tetraethylammonium; PAC, P1 artificial chromosome; kb, kilobase(s); bp, base pair(s); RT-PCR, reverse transcription-polymerase chain reaction; \(E_{\text{rev}}\), reversal potential.
modulating dopaminergic transmission, which is hypothe-
sized to be affected in schizophrenia.

Extensive alternative splicing, which increases the func-
tional diversity of channels and allows an optimal adjust-
ment of ion currents to the requirements of certain cells, has
been shown for other potassium channels. This was elegantly
shown for maxi-K channels in the chicken cochlea (Navarat-
nam et al., 1997; Rosenblatt et al., 1997; Ramanathan et al.,
1999). Thus far, in the SK/K channels, alternative splicing
has only been shown for SK1 in human (Zhang et al., 2001)
and mouse (Shmukler et al., 2001). In the latter case, the
calmodulin binding site is affected, which indicates that the
gating of the channels is altered between SK1 isoforms. How-
ever, the newly detected isoform reported in this article,
hSK3_ex4, differs from the previously known hSK3 isoform
by an additional extracytosolic loop between the fifth trans-
membrane helix and the P-loop region. These isoforms are
shown to be generated by the alternative skipping of the
fourth coding exon. This insertion is shown to alter the phar-
macokinetic properties of SK3 channels.

Materials and Methods
cDNA Amplification. cDNA was amplified from a human em-
byronic cDNA library containing cDNAs from 6-, 7-, and 8-week-old
human embryos (a gift from Dr Francis Pouillat, Centre National de
la Recherche Scientifique, Paris, France) using the PefLab long-
distance PCR system (PefLab, Erlangen, Germany) and primers P10
(CTTCACCCCTTCTCTTCTCT) and R11 (TGGGGGAGATTATTTA).
PCR products were gel-purified and cloned using the TOPO-TA kit
version D (Invitrogen, Karlsruhe, Germany). Plasmids from 10 clones
were sequenced. One clone, SK3–45.1, was used for generating cDNA
constructs for expression in mammalian cells.

Analysis of the Genomic Structure of the hSK3/KCNN3 Gene. Two cDNA probes, 5′CAG and 3′CAG, on either side of the
CAG repeats were generated by PCR from the clone AAD14
(GenBank accession no. Y08263), using the primers F10/R17
(GGGCACTTGGGTGTCTCTCATC) and F11 (TCTCTCTCACCCTCTTCTCTCTCCTC)
PCR products were gel-purified and cloned using the TOPO-TA kit
version D (Invitrogen). Plasmids from 10 clones were sequenced. One clone, SK3–45.1, was used for generating cDNA
constructs for expression in mammalian cells.

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Concentration-Response of hSK3 Isoforms to Apamin, Scyllatoxin, d-Tubocurarine, TEA\(^+\), and Ba\(^+\). Experiments were carried out using the whole-cell recording mode of the patch-clamp technique (Hamill et al., 1981). Electrodes were pulled from glass capillaries in three stages and fire-polished to resistances of 3.5 to 4 M\(\Omega\) when filled with internal solution (135 mM potassium aspartate, 2 mM MgCl\(\text{\textsubscript{2}}, 10 \text{ mM HEPES, 10 mM EGTA, and 8.7 mM CaCl}\text{\textsubscript{2}}\), and TEA-Ringer were less than 5 mV and were therefore not corrected for.

Membrane potentials were clamped to \(-160\) or \(-120\) mV for 50 ms followed by 400-ms ramps from \(-160\) or \(-120\) to \(+60\) mV and were kept for 5 s between ramps at \(-80\) mV in K-Ringer, \(-20\) mV in K-Ringer, and \(-25\) mV in K30-Ringer (30 mM KCl, 134.5 mM NaCl, 2 mM CaCl\(\text{\textsubscript{2}}, 1 \text{ mM MgCl}\text{\textsubscript{2}}, 5 \text{ mM HEPES, pH 7.4}) as external solution. All potentials caused by the liquid junction potential that develops at the tip of the pipette if the pipette solution is different from that of the bath were less than 5 mV and were therefore not corrected for. TEA\(^+\), Ba\(^+\), apamin, scTX, d-tubocurarine, and 1-EBIO were added to the external solution in increasing concentrations. Curves were fitted using the software Slide Writer Plus version 4.1 (Advanced Graphics Software Inc., Encinitas, CA).

Selectivity of hSK3 and hSK3 ex4 to Monovalent Ions. Patch-clamp recordings were carried out after whole-cell dialysis with internal solution as described above. The membrane potential was clamped to \(-120\) mV for 50 ms followed by a 400-ms ramp from \(-120\) to \(+60\) mV. K-Ringer, TEA-Ringer, K-164.5 mM NaCl, 2 mM CaCl\(\text{\textsubscript{2}}, 1 \text{ mM MgCl}\text{\textsubscript{2}}, 5 \text{ mM HEPES, pH 7.4}) as external solution. All potentials caused by the liquid junction potential that develops at the tip of the pipette if the pipette solution is different from that of the bath were less than 5 mV and were therefore not corrected for. TEA\(^+\), Ba\(^+\), apamin, scTX, d-tubocurarine, and 1-EBIO were added to the external solution in increasing concentrations. Curves were fitted using the software Slide Writer Plus version 4.1 (Advanced Graphics Software Inc., Encinitas, CA).

Activation of hSK3 and hSK3 ex4 by Intracellular Ca\(^{2+}\). Experiments were carried out using the whole-cell mode of the patch-clamp technique. Ringer solution was used that contained Ca\(^{2+}\) as described above combined with 1 mM Ca\(^{2+}\). Light from a 75-W xenon arc lamp was filtered with 10-nm bandwidth filters at wavelengths of 350 nm (F1) and 380 nm (F2) using a filter wheel and a shutter (Lambda 10-2 with fura extension; HEKA Elektronik) under control of the patch-clamp program (Pulseware Inc.) values for K\(_d\), R\(_{\text{max}}\), and R\(_{\text{min}}\) were used as external solutions.

Liquid junction potentials for K\(_0\)-Ringer, Rb-Ringer, Cs-Ringer, and CaCl\(\text{\textsubscript{2}}\)-free of 1 M free Ca\(^{2+}\) were adjusted to [Ca\(^{2+}\)]\(_{\text{lo}}\) = 1 \text{ mM} by whole-cell dialysis with internal solution. N-Ringer (160 mM NaCl, 4.5 mM KCl, 2 mM CaCl\(\text{\textsubscript{2}}, 1 \text{ mM MgCl}\text{\textsubscript{2}}, 5 \text{ mM HEPES, pH 7.4}) as external solution. N-Ringer with 10 mM HEPES, 10 mM EGTA, and 0 mM CaCl\(\text{\textsubscript{2}}\), pH 7.4) were used as external solutions.

Results

Alternative Splicing. We were able to amplify and clone a 2.5-kb transcript of hSK3 from a human embryonic cDNA library derived from human embryos aged 6, 7, and 8 weeks using primers that flank the entire open-reading frame of hSK3 (primers F10 and R11). Ten clones were initially analyzed by end-sequencing and were identified as clones containing cDNAs from hSK3. Sequencing of the entire inserts of two of these clones indicated that they both contained 19 CAGs in the second repeat and a 45-bp insertion between nucleotide positions 1463 and 1464 (Fig. 1) within the codon Arg488, which results in a 15 amino acid change R488S. The insertion does not disturb the open reading frame and codes for the 15 amino acids PESPAQPQGSSSLPAW (Fig. 1) in the extracytosolic region between the fifth transmembrane helix and the P-loop region. Neither AAD11 nor the IMAGE clone 700710, which was used to construct the hSK3 cDNA, contains the 45-bp insertion (Chandy et al., 1998).

Analysis of the Genomic Structure of the Human hSK3/KCNN3 Gene. To confirm that the newly detected hSK3 transcript originated from the hSK3/KCNN3 gene, we looked for the 45-bp insert sequence on genomic DNA clones spanning the hSK3/KCNN3 gene. Screening of the human Pac DNA library using two DNA probes that flanked the CAG repeats revealed 13 overlapping PAC clones containing parts of the hSK3 gene. Two PACs, LLNL704G20940Q3 (P12) and LLNL704G23676Q3 (P14), were subcloned, and six subclones, each containing a single PstI fragment, were sequenced. The corresponding genomic sequence of the 45-bp insert, which was found in the PCR product derived from the human embryonic cDNA library, was determined from a cloned fragment of PAC P14 obtained by vectorette PCR. As shown in Fig. 1C, the 45-bp sequence is flanked by typical splice acceptor and donor sequences and was found in the genomic sequence AF336797 extending from position 134161 to 134205. This confirms that the 45-bp insert represents an additional exon, which is spliced between the previously designated third and fourth coding exons (Sun et al., 2001), and we have therefore numbered it exon 4.

hSK3 ex4 Is Expressed Widely. TaqMan quantitative RT-PCR was used to determine the abundance of hSK3 and hSK3 ex4 transcripts in total RNA derived from 29 human tissues. We have arbitrarily divided the expression levels for hSK3 ex4 transcripts into three groups (defined by horizontal lines in Fig. 2), abundant (>10,000 copies/\(\mu\text{L}\) cDNA), intermediate (100–10,000 copies/\(\mu\text{L}\) cDNA), and low (<100 copies/\(\mu\text{L}\) cDNA). Consistent with previous reports (Rimini et al., 2000; Tomita et al., 2003), hSK3 is expressed abundantly in brain, striated and smooth muscle, spleen, thymus, adrenal, thy-
roid, prostate, kidney, and testis. It is expressed at intermediate levels in heart, lymph node, bone marrow, fetal liver, salivary gland, liver, lung, and placenta and at low levels in peripheral lymphocytes (Fig. 2). hSK3_ex4 is present at an intermediate level in most of the brain regions examined, as well as in striated and smooth muscles, thymus, thyroid, testis, bone marrow, spleen, lymph node, and peripheral lymphocytes. It is expressed at lower levels in all of the other tissues studied (Fig. 2). The expression level of hSK3_ex4 tends to parallel that of the hSK3 transcript, and it is generally at a level 0 to 2% of that of hSK3. An exception, however, is found in peripheral lymphocytes, in which the ratio of hSK3_ex4/hSK3 is exceptionally high. This is brought about not by an increase in the absolute abundance of hSK3_ex4, but rather it reflects the exceptionally low expression of hSK3 in this tissue.

Functional Characterization of hSK3 Isoforms. Because the newly detected hSK3 isoform differs in the S5 to P-loop region, we were interested in whether this alteration affects its pharmacological and kinetic properties. Therefore, we tested the effect of blockers, which are known to bind to the outer pore region of SK channels, such as TEA\(^+\), d-tubocurarine, apamin, and ScTX, a toxin isolated from the scorpion *Leiurus quinquestriatus hebraeus*, which is also reported as leiurotoxin I (Chicchi et al., 1988), as well as the effect of Ba\(^2+\), which is known to bind to the inner vestibule of SK channels. We also determined the fractional permeability of the monovalent cations Rb\(^+\), Cs\(^+\), and Na\(^+\) in comparison to K\(^+\) and the activation of the isoforms by 1-EBIO.

**Apamin.** We used apamin as a classic peptidic SK-channel blocker. Apamin is shown to bind to the outer pore region of SK channels (Ishii et al., 1997). Representative experiments for both isoforms are shown in Fig. 3, A and B. In these experiments, the membrane potential was ramped from -120 to +60 mV. In these experiments, an almost linear current/voltage relationship was observed for membrane potentials that were more negative than -40 mV. The slope conductance was determined for the interval between -90 and -70 mV. For cells expressing isoform hSK3, the slope conductance was found to be 0.5 ± 0.5 nS (n = 5) in N-Ringer solution. After changing the external solution to K-Ringer, an elevated inward current was observed at negative potentials. The current/voltage relationship of this current was also linear for membrane potentials more negative than -40 mV. The slope conductance for the above-mentioned interval was elevated to 12.3 ± 22.3 nS (n = 5). Apamin was added to K-Ringer as external solution in increasing concentrations of 0.1, 1, 5, 10, and 100 nM. A voltage-independent but concen-
Fig. 2. Tissue distribution of hSK3 and hSK3_ex4 transcripts. The total number of transcripts in 1 μl of cDNA solution is shown in A (for hSK3) and B (for hSK3_ex4). Expression levels are grouped into abundant (>10,000 copies/μl cDNA), intermediate (100–10,000 copies/μl cDNA), and low (<100 copies/μl cDNA). Horizontal broken lines indicate the threshold between low and intermediate expression levels. C, the ratio of hSK3_ex4 to hSK3 transcripts in percentage of hSK3.
A concentration-dependent blockage of currents was observed, which was carried by hSK3. At a concentration of 100 nM apamin (in K-Ringer as external solution), the remaining whole-cell conductance was found to be $0.8 \pm 0.6 \text{nS}$ ($n = 5$), which corresponds to a reduction of the whole-cell conductance of approximately 94%. The calculated $K_d$ value was found to be 0.8 nM, which is in good agreement with previously published $K_d$ values for SK3 channels of 0.63 nM (Grunnet et al., 2001b) using similar electrophysiological methods. A higher $K_d$ value of 13.2 nM was reported by Terstappen et al. (2001) using fluorescence techniques. In the case of isoform hSK3_ex4-expressing cells, the slope conductances in N-Ringer and K-Ringer solutions were found to be $0.8 \pm 0.8 \text{nS}$ ($n = 6$) and $3.8 \pm 2.5 \text{nS}$ ($n = 6$), respectively. After adding 100 nM apamin to K-Ringer as external solution, the remaining whole-cell conductance was found to be $4.3 \pm 3.0 \text{nS}$ ($n = 6$). Therefore, whole-cell currents, which were carried by hSK3_ex4, remain unaffected by 100 nM apamin. Therefore, hSK3_ex4 channels are more insensitive to apamin than SK1 channels, which were reported to be the most insensitive SK channels with a $K_d$ value of 196 nM (Grunnet et al., 2001a).

**Scyllatoxin.** Representative experiments for both isoforms are shown in Fig. 4, A and B. N-Ringer and K-Ringer were used as external solutions. Membrane potentials were

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**Fig. 3.** Effect of apamin on current through hSK3 and hSK3_ex4. A and B show the whole-cell ramp currents for hSK3 and hSK3_ex4 elicited by clamping the membrane potentials in 400-ms ramps from $-120$ to $+60 \text{mV}$ in N-Ringer and K-Ringer solutions with and without different concentrations of apamin (concentrations given at the left of each trace). C, dose-response curve for experiments similar to those shown in A and B. Whole-cell currents, which were measured in N-Ringer as external solution, were subtracted from currents measured in K-Ringer with and without apamin. The ratio of the whole-cell conductance observed in toxin-containing solution was obtained by measuring the slope of the ramp current between $-90$ and $-70 \text{mV}$, divided by the whole-cell conductance in K-Ringer as external solution and plotted against the apamin concentration. Error bars represent the standard deviation. The solid lines through the data points (C, hSK3; △, hSK3_ex4) are the best fits to the data of a Hill equation according to $g/g_{max} = 1/(1 + [\text{apamin}/K_d])$, assuming a Hill coefficient of 1. Dissociation constants were calculated to be 0.8 nM for hSK3. A $K_d$ value for hSK3_ex4 was not calculated because no reduction of whole-cell conductance can be observed up to 100 nM apamin.
ramped from $-160$ to $+60$ mV for 400 ms. The slope conductance determined for the interval between $-90$ to $-70$ mV with N-Ringer as external solution was $1.9 \pm 1.2 \text{nS} (n = 11)$ for hSK3 and $2.4 \pm 1.4 \text{nS} (n = 9)$ for isoform hSK3_ex4. In K-Ringer solution, the slope conductance increased to $11.4 \pm 3.7 \text{nS} (n = 11)$ and $15.0 \pm 5.7 \text{nS} (n = 9)$ for hSK3 and hSK3_ex4, respectively, when the external solution was replaced by K-Ringer. After scyllatoxin was added in increasing concentrations of 0.1, 1, 5, 10, 50, and 100 nM (and 500 nM in the case of hSK3_ex4) to K-Ringer, the current, which was carried through hSK3 was blocked in a voltage-independent but concentration-dependent manner with a $K_d$ value calculated to be 2.1 nM (Fig. 4C, solid line). This finding is in line with a previous study, which determined a $K_d$ of 1 nM for the

![diagram](https://example.com/diagram.png)

**Fig. 4.** Effect of ScTX on current through hSK3 and hSK3_ex4. A and B, the whole-cell ramp currents for hSK3 and hSK3_ex4 elicited by clamping the membrane potentials in 400-ms ramps from $-160$ to $+60$ mV in N-Ringer and K-Ringer with and without different concentrations of ScTX (concentrations are given at the left of each trace). C, dose-response curve for experiments similar to those shown in A and B. Whole-cell currents, which were measured in N-Ringer as external solution, were subtracted from currents measured in K-Ringer with and without ScTX. The ratio of the whole-cell conductance observed in toxin-containing solution was obtained by measuring the slope of the ramp current between $-90$ and $-70$ mV, divided by the whole-cell conductance in K-Ringer as external solution, and plotted against the ScTX concentration. Error bars represent the standard deviation. The solid (hSK3) and dashed (hSK3_ex4) lines through the data points are the best fits to the data of a Hill equation according to $g/g_{\text{max}} = 1/(1 + ([\text{ScTX}]/K_d))$, assuming a Hill coefficient of 1. Dissociation constants were calculated to be 2.1 nM for hSK3 and 2.6 $\mu$M for hSK3_ex4. The dotted (hSK3_ex4) lines through the data points are the best fits of a modified Hill equation according to $g/g_{\text{max}} = 1 - (a/[1 + K_d([\text{ScTX}])])$, assuming a $K_d$ identical with that found for hSK3 (2.1 nM) and a Hill coefficient of 1. The calculated maximal inhibition of current was found to be $a = 16.7\%$. 


ScTX block of hSK3 (Shakkottai et al., 2001). In contrast, the current carried by isoform hSK3_ex4 remains almost unaffected by scyllatoxin. Only an approximately 12% reduction of whole-cell conductance was observed for a concentration of 500 nM of ScTX (Fig. 4C). A $K_d$ of 2.6 μM was calculated for hSK3_ex4, assuming a blocking mechanism identical with that for hSK3 (Fig. 4C, dashed line). We are aware that this calculation can only be an estimate of the minimal $K_d$ value, because we only measured block of current by ScTX up to 500 nM. On the other hand, we can also describe the data assuming an identical $K_d$ compared with hSK3 but with a maximal inhibition of only 16.7% (Fig. 4C, dotted line). Independent of the inhibition mechanism, it is obvious that the current through this isoform is hardly reduced by the presence of even 500 nM ScTX in contrast to the SK1 channel, which has been shown to be the most insensitive member of the SK channel family, with a $K_d$ for ScTX of 80 (Strobaek et al., 2000) and 325 nM (Shakkottai et al., 2001).

**d-Tubocurarine.** The effect of d-tubocurarine on current through the hSK3 and hSK3_ex4 is shown in representative experiments in Figs. 5, A and B. Membrane potentials were ramped from −120 to +60 mV for 400 ms. The slope conductance calculated for the interval from −90 to −70 mV was found to be $1.4 \pm 1.5$ nS ($n = 4$) and $0.7 \pm 0.4$ nS ($n = 4$) with N-Ringer as external solution for hSK3 and hSK3_ex4, respectively. After changing the bath solution to K-Ringer, an increased whole-cell conductance of $9.6 \pm 9.8$ nS (hSK3) and $3.7 \pm 1.6$ nS (hSK3_ex4) was observed. d-Tubocurarine was

![Fig. 5. Effect of d-tubocurarine on current through hSK3 and hSK3_ex4. A and B, whole-cell ramp currents for hSK3 and hSK3_ex4 elicited by clamping the membrane potentials in 400-ms ramps from −120 to +60 mV in N-Ringer and K-Ringer solutions with and without different concentrations of d-tubocurarine (concentrations are given at the left of each trace). Whole-cell currents, which were measured in N-Ringer as external solution, were subtracted from currents measured in K-Ringer with and without d-tubocurarine. The ratio of the whole-cell conductance observed in toxin-containing solution was obtained by measuring the slope of the ramp current between −90 and −70 mV, divided by the whole-cell conductance in K-Ringer as external solution, and plotted against the d-tubocurarine concentration. Error bars represent the standard deviation. The curves through the data points are the best fits of a Hill equation to the data according to $g/g_{\text{max}} = 1/(1 + [\text{d-tubocurarine}]/K_d)$, assuming a Hill coefficient of 1. Dissociation constants were calculated to be 33.4 μM for hSK3 and 1.4 mM for hSK3_ex4 with the assumption that the blocking mechanism is the same for both isoforms.
added to the bath in increasing concentrations of 10, 50, 100, and 500 μM. Currents carried by the hSK3 isoform were blocked in the presence of d-tubocurarine in a voltage-independent and concentration-dependent manner. In the presence of 500 μM d-tubocurarine, 95% of the current carried by isoform hSK3 was blocked, whereas currents carried by hSK3_ex4 were reduced by only approximately 20%. Concentration-response curves were fitted as described for ScTX (Fig. 5C). The $K_a$ observed for isoform hSK3 was 33.4 μM, which is between those previously observed for SK1 ($K_a = 354.3$ μM) and SK2 ($K_a = 5.4$ μM) (Ishii et al., 1997). Only if identical blocking mechanisms are assumed for both isoforms, a $K_a$ of 1.4 mM can be calculated for isoform hSK3_ex4. It is also possible to assume an incomplete d-tubocurarine block for hSK3_ex4 similar to that one discussed for the ScTX block shown in Fig. 4C.

TEA$^+$ Block. Measurements were carried out with N-Ringer and K-Ringer as external solutions, and membrane potentials were clamped in 400-ms ramps from −120 to +60 mV. An almost linear potassium current was observed for membrane potentials more negative than −40 mV with N-Ringer as external solution; this was elevated as described above when the external solution was exchanged with K-Ringer. TEA$^+$ was added to K-Ringer in increasing concentrations of 0.1, 0.5, 1, 5, and 10 mM. In contrast to the scyllatoxin block, TEA$^+$ has a similar effect on hSK3- as well as hSK3_ex4–carried currents, and the calculated dissociation constants were similar for both isoforms ($K_a = 2.2$ mM for hSK3 and 2.6 mM for hSK3_ex4).

External Ba$^{2+}$ Block. To test whether the additional 15 amino acids also affect the inner vestibule of the pore, we investigated the inhibition of both isoforms by external Ba$^{2+}$ (Fig. 6). For both isoforms, the number of experiments was $n = 3$. K-Ringer and TEA-Ringer were used as external solutions, and BaCl$_2$ was added to K-Ringer in increasing concentrations of 0.1, 0.5, and 1 mM. The membrane potential was clamped in 400-ms ramps from −160 to +60 mV. This is an appropriate protocol because it has already been shown that the Ba$^{2+}$ block is fast compared with the voltage ramp (Hanselmann and Grissmer, 1996). Whole-cell currents with TEA-Ringer as external solution were assumed to be leak currents and were therefore subtracted from the whole-cell currents in K-Ringer with and without Ba$^{2+}$. Relative currents were calculated as fractions of the currents with K-Ringer as external solution. The potassium currents through both isoforms were affected by external Ba$^{2+}$ in a concentration-dependent manner (Fig. 6). However, the blockage was strongest at hyperpolarized potentials and was reversible immediately after washout. To analyze the voltage-dependence of the Ba$^{2+}$ block, the relative currents for each Ba$^{2+}$ concentration were plotted according to the Boltzmann equation $I/I_{\text{max}} = 1/(1 + \exp((E_{\text{rev}} - E)/k))$ with $k$ as the steepness factor of block (Fig. 6, C and D). The calculated half-maximal blocking potentials ($E_{50}$ values) were −160 ± 4 mV (0.1 mM Ba$^{2+}$), −117 ± 2 mV (0.5 mM Ba$^{2+}$), and −91 ± 9 mV (1 mM Ba$^{2+}$) for hSK3 ($n = 3$). For hSK3_ex4 ($n = 3$), the values were calculated to be −290 ± 145 mV (0.1 mM Ba$^{2+}$), −186 ± 17 mV (0.5 mM Ba$^{2+}$), and −146 ± 17 mV (1 mM Ba$^{2+}$). The strongly increased standard deviation, which was calculated for $E_{50}$ at 0.1 mM Ba$^{2+}$ of hSK3_ex4, is caused by the reduced steepness of the voltage dependence of the Ba$^{2+}$ block. The steepness factors calculated for hSK3 and hSK3_ex4 were 32 ± 4 and 61 ± 12 mV, respectively. This corresponds to Ba$^{2+}$-binding sites at fractions of $\delta = 0.42$ from the outside of the electrical field for hSK3 and $\delta = 0.23$ for hSK3_ex4. Therefore, the Ba$^{2+}$-binding site calculated for both isoforms is less deep than the one reported for the apamin-sensitive Ba$^{2+}$ (δ = 0.62) and charybotoxin-sensitive Ba$^{2+}$ (δ = 0.74) potassium channels in Jurkat E6–1 cells and human peripheral T lymphocytes (Hanselmann and Grissmer, 1996). However, the data obtained for hSK3 indicate that Ba$^{2+}$ binds to residues at the inner vestibule of the pore and that the hSK3_ex4 isoform has a different Ba$^{2+}$-binding site, which seems to be shifted toward the outside of the vestibule.

Selectivity of hSK3 Isoforms to Monovalent Cations. Because the altered Ba$^{2+}$-binding site in the hSK3 isoform is approximately halfway into the electrical field of the channel one could assume that the conduction pathway and possibly the selectivity filter might be altered as well. Therefore, we investigated the selectivity of the hSK3 isoforms to different monovalent cations (Fig. 7). The whole-cell current in the presence of TEA-Ringer was assumed to be leak current and was subtracted from the whole-cell currents in the presence of K-Ringer, Rb-Ringer, Cs-Ringer, and K0-Ringer (contains 164.5 mM Na$^+$ without K$^+$. The reversal potential ($E_{\text{rev}}$) was found to be 12 ± 2 mV in K-Ringer, −115 ± 24 mV in K0-Ringer, 6 ± 2 mV in Rb-Ringer, and −33 ± 7 mV in Cs-Ringer for hSK3 (n = 6). For isoform hSK3_ex4 (n = 5), $E_{\text{rev}}$ was found to be 13 ± 4 mV in K-Ringer, −114 ± 39 mV in K0-Ringer, 6 ± 1 mV in Rb-Ringer, and −34 ± 4 mV in Cs-Ringer. The positive reversal potentials found, especially in K-Ringer, are because the currents were not corrected for junction potentials. For both isoforms, the number of experiments was $n = 3$. Because the slope of the current ramps obtained from the experiments in K0-Ringer was very small (slope conductance $g < 0.1$ nS for both isoforms), the estimated $E_{\text{rev}}$ varied strongly, and therefore data were not shown. The relative permeabilities were calculated from $E_{\text{rev}}$ as described previously (Hille, 2001) and are given together with the relative conductances in Table 1 as fractions of the permeability and conductance of both isoforms for K$^+$. However, both isoforms show similar relative permeabilities for Cs$^+$ and Rb$^+$. Only the relative conductance for Rb$^+$ was found to be higher for hSK3 ($g_{\text{fRb}}/g_{\text{fK}} = 1.1$) than for hSK3_ex4 ($g_{\text{fRb}}/g_{\text{fK}} = 0.77$). As already shown for other SK/IK channels (Hanselmann and Grissmer, 1996; Jensen et al., 1998), hSK3 and hSK3_ex4 can carry a significant Cs$^+$ current.”
tance was smaller than that found for K-Ringer (containing 164.5 mM K⁺). After the application of 1 mM 1-EBIO, the slope conductance increased to 25.1 ± 17.0 (n = 7, for SK3) and 19.4 ± 16.8 nS (n = 5, for hSK3_ex4). The concentration-response curves for both isoforms are shown in Fig. 8C. To estimate only the 1-EBIO-activated potassium current, the slope conductance found for K30-Ringer was subtracted from that observed after adding 1-EBIO to the external solution. The slope conductances were normalized for the slope conductance found for 1 mM 1-EBIO and plotted against the 1-EBIO concentration. Because it was not clear whether the activation of hSK3 isoforms reached their maximum in the presence of 1 mM 1-EBIO, the curves were fitted according to the equation \( g/g_{1\text{M 1-EBIO}} = a_{\text{max}} - a_{\text{max}}/(1 + ([\text{1-EBIO}]/EC_{50})^{n_H}) \), with \( a_{\text{max}} \) as maximum relative conductance and \( n_H \) as the Hill coefficient. Best fits for curves of both isoforms were achieved when the Hill coefficient was assumed to be \( n_H = 1.8 \) and \( a_{\text{max}} \) of 1.03 and 1.04 for hSK3 and hSK3_ex4, respectively. However, 1-EBIO showed a similar effect on both isoforms. The half-maximal activation concentrations (EC_{50}) were calculated to be 0.17 mM (for hSK3) and 0.19 mM (for hSK3_ex4). 1-EBIO has already been shown to be an activator of SK/IK channels with an EC_{50} ranging from 74 \( \mu \)M for SK4/IK1 (Jensen et al., 1998) to 650 \( \mu \)M for SK1 and SK2 (Pedarzani et al., 2001). The determined EC_{50} values for both hSK3 isoforms fits the previously published one for SK3 channels with an EC_{50} = 100 \( \mu \)M (Grunnet et al., 2001b). Therefore, the sensitivity of hSK3 channels to

**Fig. 6.** External Ba²⁺ block of currents through hSK3 and hSK3_ex4. A and B, whole-cell currents for both isoforms. Currents were elicited by clamping the membrane potential in 400-ms ramps from −160 to +60 mV in TEA-Ringer (designated “TEA⁻” on the left) and K-Ringer with and without Ba²⁺ (concentrations are given at the left of each trace). C and D, the voltage-dependence of the Ba²⁺ block for hSK3 and hSK3_ex4, respectively. Currents measured in TEA-Ringer were assumed as leak currents and were therefore subtracted from currents measured in K-Ringer with and without Ba²⁺. Relative currents were calculated as fractions of currents with K-Ringer as external solution. The mean relative currents were plotted as the mean of three experiments against the membrane potential for the interval from −160 to −40 mV. Smooth curves represents best fits of the Boltzmann equation \( I_{I_{\text{K-Ringer}}} = I/\left(1 + \exp((E_{0.5} - E)/k)\right) \), with a steepness factor of \( k = 32 \) mV for hSK3 (C) and \( k = 61 \) mV for hSK3_ex4 (D). \( E_{0.5} \) values calculated for both isoforms are given in the text.
1-EBIO is more similar to SK4/IK1 channels than to other SK channels.

**Activation of hSK3 and hSK3_ex4 by Intracellular Ca$^{2+}$.** The experimental procedure allowed the measurement of whole-cell conductance and the internal Ca$^{2+}$ concentration in parallel (Fig. 9). After subtracting the intensity of the autofluorescent light and calculating the ratio for F1 and F2 of the fura measurements as described above, the Ca$^{2+}$ concentrations were calculated according to the equation [Ca$^{2+}$] = 1.63 × (R – 0.21)/(2.2 – R). The initial internal Ca$^{2+}$ concentrations immediately after perfusion of the cell with the pipette solution varied between the cells and were found to be >1.6 μM. Within 5 to 10 min, the Ca$^{2+}$ concentration decreased and reached concentrations ranging between 0.3 and 0.12 μM (Fig. 9, A and B). Saturated maximum whole-cell conductances were observed for Ca$^{2+}$ concentrations larger than 1.5 μM and varied from 3.0 to 11 nS. The minimum conductance was achieved for Ca$^{2+}$ concentrations lower than 0.3 μM, it ranged between 2.5 and 0.1 nS, and it was not subtracted as background conductance. The whole-cell conductance was plotted against Ca$^{2+}$ concentrations for each experiment separately, and curves were fitted according to the equation $g = g_{\text{max}} - a_nR/(1 + ([\text{Ca}^{2+}]/EC_{50})^{n_H})$, where $g_{\text{max}}$ is the maximal conductance, $EC_{50}$ is the half-maximal activating Ca$^{2+}$ concentration, and $n_H$ is the Hill coefficient (Fig. 9, E and F). Because the minimal conductance was not subtracted, $a_n$ was assumed not to be equal to $g_{\text{max}}$. The $EC_{50}$ and $n_H$ values were calculated for each isoform as mean values ($n = 3$) and were found to be 0.91 ± 0.4 μM and 3.3 ± 0.5 for hSK3 and 0.78 ± 0.2 μM and 4.1 ± 0.2 for hSK3_ex4, respectively. The difference between the $EC_{50}$ values was found to not be significant by applying a two-tailed Student’s t test ($p = 0.63$).

![Fig. 7.](image)

**Discussion**

Alternative splicing is a common feature of mammalian potassium channel transcripts. It leads to altered functional properties of potassium currents by generating different isoforms. Therefore, it plays a vital role in the fine-tuning of whole-cell currents and the adjustment of potassium currents to the requirements of a particular cell (Coetzee et al., 1999). Sixteen isoforms, all generated by alternative splicing, were recently described for the SK1 channel in mouse (Shmukler et al., 2001). A similar pattern of alternative splicing was also described for transcripts of the human SK1/KCNN1 gene (Zhang et al., 2001). Because the splicing in those cases predominantly affected the calcmodulin binding site, it was believed that the activation of SK1 channels can be modulated by altering their ability to bind calcmodulin. The hSK3 isoforms described here were generated by the alternative inclusion of the discovered new exon 4. They differ in their amino acid sequence by an insertion of 15 amino acids into the extracytosolic region between the fifth transmembrane helix and the P-loop region of SK3. We were intrigued that perhaps this novel isoform might refine our understanding of the pharmacological as well as physiological properties of the channel. To test whether the pore region of SK3 channels is affected by the 15 amino acid insertion, we tested different blockers that are known to bind at the outer vestibule, such as TEA$^+$ (Bretschneider et al., 1999), d-tubocurarine, apamin (Ishii et al., 1997), and ScTX (Shakkottai et al., 2001). The S5–P-loop–S6 regions of SK2 and SK3 channels differ only at the residues Val485 and His521, which correspond to Val47 and Val95 in the KcsA channel and Tyr415 and Val451 in the Shaker potassium channel. The corresponding residues for SK2 are Ala331 and Asn367. This amino acid variation could be the reason for the different sensitivities of SK2 and SK3 channels to ScTX and might be essential for ScTX binding. Mutant cycle studies indicated that Val485 and His521 are near each other (Shakkottai et al., 2001).

**TABLE 1**

Selectivity of hSK3 and hSK3_ex4 to Rb$^+$ and Cs$^+$. The mean values of three experiments are provided. Errors are given as S.D.

<table>
<thead>
<tr>
<th></th>
<th>hSK3</th>
<th>hSK3_ex4</th>
<th>hSK3</th>
<th>hSK3_ex4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rb-Ringer</td>
<td>-6 ± 1.2mV</td>
<td>-5 ± 2.4mV</td>
<td>0.79 ± 0.04</td>
<td>0.8 ± 0.07</td>
</tr>
<tr>
<td>Cs-Ringer</td>
<td>-45 ± 6.0mV</td>
<td>-46 ± 7.7mV</td>
<td>0.17 ± 0.04</td>
<td>0.17 ± 0.05</td>
</tr>
</tbody>
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Note: $\Delta E = E_{\text{rev}} - E_{\text{rev(K-Ringer)}}$, $PP_{\text{K-Ringer}}$, and $\delta g/K_{\text{Ringer}}$.
al., 2001). The insertion of the additional amino acids in hSK3_ex4 occurs only three amino acids C-terminal from Val485, and it is possible that they separate Val485 and His521 from each other in hSK3_ex4. Such a conformational alteration of the outer vestibule in hSK3_ex4 might well explain the strongly reduced ScTX sensitivity of this isoform as well as its reduced sensitivity to d-tubocurarine and apamin. Interestingly, the TEA⁺ binding sites of hSK3_ex4 would be assumed not to be affected by the insertion. Therefore, we conclude that the inner regions of the vestibule remain unaffected by the additional amino acids in hSK3_ex4. This is also reflected by the similar permeabilities of both isoforms to Cs⁺ and Rb⁺ ions. Surprisingly, the Ba²⁺ block differs between hSK3 and hSK3_ex4. The voltage-dependence of the Ba²⁺ block observed for hSK3 in this report as well as that observed for the Ba²⁺ block of other SK/IK channels (Hanselmann and Grissmer, 1996) indicates that the Ba²⁺ binding site lies roughly halfway through the electrical field of the plasma membrane. The fact that the steep-

Fig. 8. Activation of currents through hSK3 and hSK3_ex4 by 1-EBIO. A and B, whole-cell currents of hSK3 and hSK3_ex4. Currents were elicited by 400-ms ramps from −120 to +60 mV in K30-Ringer with and without 1-EBIO (concentrations are given at the left of each trace). C, concentration-response curves of both isoforms for 1-EBIO. Whole-cell conductances were calculated from the slope of the ramp currents for the interval from −90 to −70 mV. The conductance found for currents in K30-Ringer were subtracted from conductances in K30-Ringer with 1 mM 1-EBIO. Relative conductances were calculated as fractions of conductance in K30-Ringer with 1 mM 1-EBIO and were plotted against 1-EBIO concentrations. Curves were fitted according to $g/g_{\text{max}} = 1/(1 + [(1-EBIO)/EC_{50}]^{n_H})$, where $g_{\text{max}}$ in the maximal relative conductance calculated to be 1.03 for hSK3 and 1.04 for hSK3_ex4. The Hill coefficient was calculated to be $n_H = 1.8$ for both isoforms. For hSK3, the EC_{50} was calculated to be 0.17 mM and for hSK3_ex4 to be 0.19 mM.
ness of voltage dependence of the Ba\(^{2+}\) block is reduced for hSK3_ex4 indicates that the Ba\(^{2+}\) binding site is shifted toward the outside of the electrical field. It is still not clear whether the additional 15 amino acids in hSK3_ex4 create a new Ba\(^{2+}\) binding site at a more exterior position of the vestibule or whether they change the properties of the electrical field at the outer vestibule of hSK3_ex4.

The EC\(_{50}\) values for Ca\(^{2+}\) activation found for both isoforms showed no significant differences, indicating that the insertion of the 15 amino acids did not interfere with the activation of hSK3 channels by Ca\(^{2+}\). Interestingly, the values found for hSK3 were higher than the ones described in previous reports, in which an EC\(_{50}\) ranging from 0.1 to 0.3 \(\mu\)M was described previously (Köhler et al., 1996; Carignani et al., 2002). However, we were able to potentiate SK3 currents through both isoforms with 1-EBIO after whole-cell perfusion with 1 \(\mu\)M free Ca\(^{2+}\). Because 1-EBIO activates SK currents only in the presence of Ca\(^{2+}\) at concentrations lower than those necessary for maximal activation (Pedarzani et al., 2001), this indicates that the 1 \(\mu\)M free Ca\(^{2+}\) used in our experiments does not lead to a maximum activation of whole-cell SK3 currents. Therefore, this result is in line with the unexpectedly high EC\(_{50}\) values found for hSK3 and hSK3_ex4.

SK channels underlie the AHP in excitable cells (Köhler et al., 1996, Stocker et al., 1999; Sah and Faber, 2002). The AHP in the rat CA1 hippocampal pyramidal neurons can be subdivided into scyllatoxin- and apamin-sensitive and -insensitive components (Stocker et al., 1999). The channels that underlie the scyllatoxin- and apamin-insensitive components of the AHP are also recognized to be insensitive to \(d\)-tubocurarine; however, their molecular nature remains unknown. Here, we describe an SK3 isoform that is insensitive to apamin, ScTX, and \(d\)-tubocurarine. If human excitable cells show a scyllatoxin- and apamin-sensitive component of the AHP, like CA1 pyramidal cells in rat, then the hSK3_ex4 isoform might also generate a scyllatoxin- and apamin-insensitive component of the AHP similar to that observed in rat and guinea pigs. The molecular mechanism for the apamin- and ScTX-sensitive AHP component in humans, rats, and guinea pigs, however, seems to be different, mainly for two reasons. First, in rat (Stocker et al., 1999) and in guinea pigs (Martinez-Pinna et al., 2000; Vogalis et al., 2002), the apamin-insensitive component of the AHP was shown to be also TEA\(^{+}\)-insensitive up to 10 mM. Second, a blast search did not reveal an orthologous exon 4 in rat. However, isoforms of SK channels, such like isoform hSK3_ex4, may be good candidates for contributing to the molecular basis of the apamin-insensitive AHP component.

The TaqMan RT-PCR was used to quantify transcripts for both isoforms in different tissues. These experiments showed that both isoforms are coexpressed in most of the examined tissues, but that the hSK3_ex4 transcript is expressed at much lower levels than hSK3 transcripts. The low amount of hSK3_ex4 transcript would suggest a minor role for hSK3_ex4 in vivo. However, the low expression level of hSK3_ex4 transcripts might reflect the fact that hSK3_ex4 is only expressed at higher levels in particular cells of a heterogeneous cell population. Furthermore, the possibility of heteromultimerization with other SK channel subunits might increase the number of SK channels per cell with pharmacokinetic properties similar to those of the hSK3_ex4 isoform.

The fact that hSK3_ex4 exhibits different pharmacological properties compared with hSK3 adds additional molecular support for current views of the site of action of these drugs and raises the possibility that SK3 currents through each isoform might be selectively modulated by specific drugs. This aspect may have heightened importance in view of the fact that the isoform hSK3 has been demonstrated to be a target for antipsychotic drugs (Terstappen et al., 2001).

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