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Rapid Report

Small conductance Ca^{2+}-activated K^+ channels formed by the expression of rat *SK1* and *SK2* genes in HEK 293 cells

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The rat *SK1* gene (*rSK1*) does not form functional Ca^{2+}-activated potassium channels when expressed alone in mammalian cell lines. Using a selective antibody to the rSK1 subunit and a yellow fluorescent protein (YFP) tag we have discovered that *rSK1* expression produces protein that remains largely at intracellular locations. We tested the idea that rSK1 may need an expression partner, rSK2, in order to form functional channels. When *rSK1* was co-expressed with *rSK2* in HEK 293 cells it increased the current magnitude by 77 ± 34 % (as compared with cells expressing *rSK2* alone). Co-expression of *rSK1* with *rSK2* also changed the channel pharmacology. The sensitivity of SK current to block by apamin was reduced ~16-fold from an IC_{50} of 94 pM (for SK2 alone) to 1.4 nM (for SK2 and SK1 together). The sensitivity to block by UCL 1848 (a potent small molecule blocker of SK channels) was similarly reduced, ~26-fold, from an IC_{50} of 110 pM to 2.9 nM. These data clearly demonstrate that rSK1 and rSK2 subunits interact. The most likely explanation for this is that the subunits are able to form heteromeric assemblies.

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Small conductance calcium-activated potassium channels (SK channels) are widely distributed throughout the body, occurring in neuronal and non-neuronal tissues. In many neurones SK channels underlie components of the post-spike afterhyperpolarization (AHP) (reviewed in Sah & Faber, 2002). Native SK channels can be identified by their characteristic sensitivity to block by the bee venom peptide toxin apamin. These apamin-sensitive channels can also be inhibited by several small molecule blockers, such as UCL 1848, which, like apamin, are active in nanomolar or sub-nanomolar concentrations (Benton *et al.* 1999; Chen *et al.* 2000; Shah & Haylett, 2000; Faber & Sah, 2002).

Three genes, *SK1, SK2* and *SK3,* code for SK channel α subunits (Kohler *et al.* 1996; Joiner *et al.* 1997; Chandy *et al.* 1998). The rat homologues of *SK2* and *SK3* (*rSK2* and *rSK3,* respectively) produce functional SK channels in heterologous expression systems (Kohler *et al.* 1996; Strobaek *et al.* 2000; Hosseini *et al.* 2001). However, the rat *SK1* gene (*rSK1*) has been reported not to produce detectable SK current in transfected cells (Bowden *et al.* 2001). Information about SK1 has, therefore, been obtained from the human homologue of *SK1* (*hSK1*). However, work with this gene produces results that vary with the expression system used. In *Xenopus* oocytes most hSK1 channels are insensitive to apamin at concentrations up to 100 nM (Kohler *et al.* 1996), although a small apamin-sensitive component exists (Grunnet *et al.* 2001). In mammalian cell lines, *hSK1* produces channels that are mostly apamin sensitive (IC_{50} 3–12 nM) (Shah & Haylett, 2000; Strobaek *et al.* 2000), although occasionally the apamin concentration–inhibition curve also contains an insensitive component (Shah & Haylett, 2000). The pharmacology of native SK1 channels is therefore uncertain.

Since most data concerning native SK channels come from rat tissues, and the behaviour of the rat gene differs from that of the human, it seemed important to re-examine the properties of *rSK1*.

A preliminary account of this work has been reported to The Physiological Society (Benton *et al.* 2003).

METHODS

Constructs

The rat *SK1* and *SK2* genes, subcloned into the pTracer or pcDNA3 mammalian expression vector, were a generous gift of Drs Len Kaczmarek and William Joiner (Yale University). The rat SK1 clone is recorded under Genbank accession number AF000973 and was re-engineered to introduce an optimal Kozak sequence just prior to the start methionine. The rat *SK1* construct was tagged with YFP by subcloning it into the pEYFP vector (Clontech). Constructs were sequenced on an ABI 377 sequencer

using the Big Dye II sequencing kit. Plasmid DNA for transfection was purified using Maxi Prep or Midi Prep kits (Qiagen).

SK1 antibody production

The peptide 'KLPPPWPGPSHLTAA', corresponding to a unique sequence in the C-terminal region of rSK1, was synthesized (Alta Bioscience, Birmingham, UK) and coupled to Keyhole limpet hemacyanin before being used for an initial rabbit immunization (Cocalico, PA, USA). Three subsequent boosts 1 month apart were then administered. Rabbit sera were assayed by ELISA and the IgG fraction of the serum was eventually purified on a protein A Sepharose affinity column prior to use in immunohistochemistry.

Maintenance and transient transfection of cell lines

HEK 293 and rat H4 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum, 2 mM L-glutamine, 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. Cells were plated onto either 35 mm culture dishes for electrophysiology or 18 mm glass coverslips for immunohistochemistry. Transfections were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For initial studies of the pharmacology of homomeric SK channels we used 1 or 2 μg of channel plasmid. For co-expression of *rSK1* with *rSK2* we used 2 μg of the *rSK1* construct and 1 μg of *rSK2*. In addition, cells were co-transfected with 1 μg of QBI plasmid DNA (Qbiogene) which expresses green fluorescent protein (GFP), allowing identification of transfected cells.

Immunohistochemistry

Immunostaining of transfected cells plated on glass coverslips was carried out as described previously (Hosseini *et al.* 2001). Briefly, cells were fixed, permeabilized and blocked, then incubated for 4 h with primary rabbit polyclonal anti-rSK1 antibody (UCL 56) at a concentration of 20 μg ml^{-1}. The cells were washed, then incubated in a 1:200 dilution of a Cy3-conjugated goat anti-rabbit secondary antibody (Chemicon) for 1 h. All antibodies were diluted in blocking buffer. Following a final wash step, the coverslips were mounted onto slides using a small drop of antifade mount (Vector Laboratories Inc.). All staining operations were carried out at room temperature (~22 °C). Stained cells were viewed with a Leica TCS confocal microscope.

Electrophysiology

Currents were recorded from HEK 293 cells using conventional whole-cell voltage clamp methods as previously described (Hosseini *et al.* 2001). The bathing solution contained (mM): NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, glucose 10, Hepes 10, pH 7.4 with NaOH. The pipette filling solution contained (mM): KCl 130, Hepes 10, K_2HEDTA 5 and $CaCl_2$ 1.2 (free Ca^{2+} 1 μM), pH 7.2 with KOH. The pipettes had resistances of 2–4 MΩ when filled with pipette solution. Experiments were conducted at room temperature (20–25 °C).

Membrane currents were recorded with either a List EPC7 amplifier using a Digidata 1320A interface and pCLAMP 8.2 software (Axon Instruments) for acquisition, or a HEKA EPC9 patch clamp amplifier under control of Pulse (HEKA). Data were filtered at 1 kHz and digitized at 5 kHz. Acquired current traces were analysed with either Clampfit 8.2 or HEKA Pulsefit.

Data analysis

For comparison of current levels in cells expressing *rSK2* alone or in combination with *rSK1* the current at −20 mV was normalized against whole-cell capacitance and expressed as current density.

The effect of blocking agents was expressed as the current recorded at −20 mV in the presence of blocker as a percentage of that in its absence. The resulting concentration–inhibition curves were fitted by the Hill equation in the form:

$$\frac{y}{100} = \frac{IC_{50}{}^{n_H}}{[I]^{n_H} + IC_{50}{}^{n_H}},$$

where y is the current in the presence of blocker as a percentage of the control, [I] is the concentration of inhibitor, n_H is the Hill coefficient and IC_{50} is the concentration of blocker that reduces the current to 50 % of the control value.

(A potential source of error in our measurement of current inhibition is that the period of exposure of the cells to blockers (3 or 4 min for UCL1848 or apamin, respectively) might be too short for equilibrium to be reached at the lowest concentrations used (30 and 100 pM). Taking published values of k_{+1} and k_{-1} for apamin binding to SK2 (Strobaek *et al.* 2000), it can be shown that at our lowest concentration (30 pM) this error is likely to be less than 10 % and thus any effect on our estimates of the IC_{50} and n_H value will be small. No kinetic data are available for UCL 1848. The onset and offset of the action of this compound are, however, faster than for apamin and thus errors will be smaller still.)

Curve fitting was performed by the method of least squares with data points weighted by the inverse of their variance, using the curve-fitting routine of Origin 5.0 (Microcal). Where appropriate other values are quoted as the mean ± S.E.M. The significance of differences between means was assessed using Student's unpaired *t* tests.

Drugs and reagents

All materials used for tissue culture were obtained from Invitrogen. UCL 1848 (8,14-diaza-1,7(1,4)-diquinolinacyclotetradecaphanedium ditrifluoroacetate) was synthesized under the supervision of Professor C. R. Ganellin, in the Department of Chemistry, UCL, as previously described (Chen *et al.* 2000). Apamin was purchased from Sigma. Hepes and HEDTA were from Calbiochem. All other reagents were of Analar quality and obtained from VWR. Horse serum, BSA and paraformaldehyde were obtained from Sigma.

RESULTS

Expression of *rSK1*

HEK 293 cells, when transfected with *rSK1* alone, did not exhibit Ca^{2+}-activated K^+ currents whereas transfection with *rSK2* produced a substantial Ca^{2+}-activated K^+ conductance (Fig. 1*A* and *B*). To verify that *rSK1* expression produces a protein in these cells we used an anti-rSK1 antibody. To confirm the antibody's selectivity we expressed a YFP-tagged SK1 subunit and then stained transfected cells with the SK1 antibody. A bright, punctate staining pattern was produced by the antibody fluorescence that closely matched fluorescence from the YFP tag (Fig. 2*A* and *B*). This shows that SK1 protein is made and is specifically recognized by the antibody. Interestingly, this staining (which was not altered when untagged subunits were expressed) did not extend evenly around the circumference of the cell, suggesting that the protein remains primarily in intracellular compartments.

Many ion channel α subunits are known to require other subunits to form functional channels (Krapivinsky *et al.* 1995; Post *et al.* 1996; Ottschytsch *et al.* 2002). Moreover, hSK1 subunits have been shown to co-assemble with SK2 subunits in oocytes (Ishii *et al.* 1997), suggesting that rSK1 may also be able to co-assemble with rSK2 to form functional SK channels.

Co-expression of *rSK1* and *rSK2* leads to a higher current density

Currents in HEK 293 cells transfected with *rSK2* alone or with the same quantity of *rSK2* and a 2-fold excess of *rSK1* were qualitatively similar, having the same reversal potential and an approximately linear current–voltage relationship (Fig. 1*A* and *B*). Currents in co-transfected

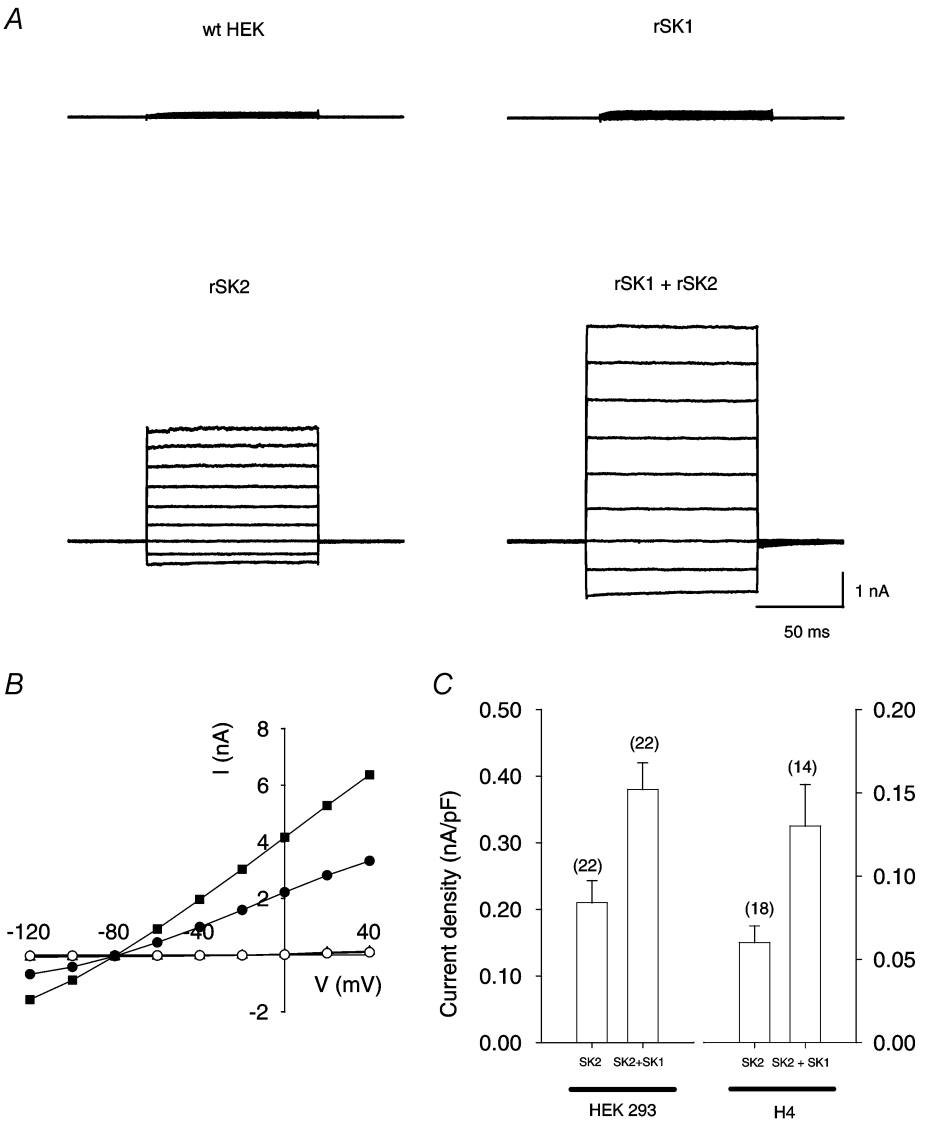

Figure1. Expression of *rSK1* and *rSK2* alone or together in HEK 293 cells

A, typical whole-cell current records obtained from an untransfected (wild-type, wt) HEK 293 cell (top left) and from cells transfected with *rSK1* (top right), *rSK2* (bottom left) or *rSK1* together with *rSK2* (bottom right). The cells were held at −80 mV and 100 ms steps applied from −120 to +40 mV. In all cases, the pipette solution contained 1 μM free calcium to activate SK currents. Currents were recorded 1–2 min after patch rupture. *B*, current–voltage relationships for the currents shown in *A*. ○, wild-type HEK; ▲, rSK1 (obscured beneath wt data); ●, rSK2; ■, rSK1 and rSK2. Currents recorded from wild-type cells or cells transfected with *rSK1* were essentially identical and reversed between −20 and −10 mV. In contrast cells transfected with *rSK2* or a combination of *rSK1* and *rSK2* exhibited large currents with an approximately linear current–voltage relationship, which reversed at approximately –80 mV, close to the predicted value of E_K (−85 mV). *C*, comparison of SK current density in HEK 293 (left) or H4 (right) cells transfected with *rSK2* either alone or with a 2-fold excess of *rSK1*. Histograms represent the mean current density at −20 mV, with the number of cells for each condition shown in parentheses. In both cases co-transfection with *rSK1* caused a significant ($P < 0.05$) increase in current. Error bars indicate S.E.M.

cells were, however, significantly greater than in cells expressing *rSK2* alone: the mean current at −20 mV was 1.9 ± 0.2 nA in cells transfected with *rSK2* and 3.1 ± 0.3 nA in cells transfected with both *rSK1* and *rSK2* ($n = 22$ for each condition, $P < 0.05$). When expressed as a current density this corresponds to a 77 ± 34 % increase (Fig. 1*C*). A similar result was obtained using the rat cell line H4; *rSK2* expression produced SK currents while *rSK1* expression did not, and co-expression of *rSK1* with *rSK2* produced a 123 ± 59 % increase in current density (Fig. 1*C*). If this change in current magnitude occurs because rSK1 and rSK2 form a functional heteromeric channel complex, then the SK channel pharmacology might change. Given the known pharmacology of hSK1, even in its 'apamin-sensitive' form, one would predict that the formation of SK1–SK2 complexes would lead to a reduction in apamin and UCL 1848 sensitivity (Ishii *et al.* 1997; Shah & Haylett, 2000; Strobaek *et al.* 2000).

We thus compared the effects of two selective SK channel blockers, apamin and UCL 1848, on the currents produced by transfection of *rSK2* alone or by co-transfection of *rSK1* with *rSK2*.

Co-expression of *rSK1* and *rSK2* results in the formation of channels with a novel pharmacology

In control experiments, on homomeric rSK2 channels, the apamin dose–response curve was well fitted by the Hill equation with an IC_{50} of 95 ± 8 pM and a Hill coefficient (n_H) of 0.80 ± 0.06 (Fig. 3*A* and *C*). This IC_{50} for apamin is very close to those previously obtained for inhibition of

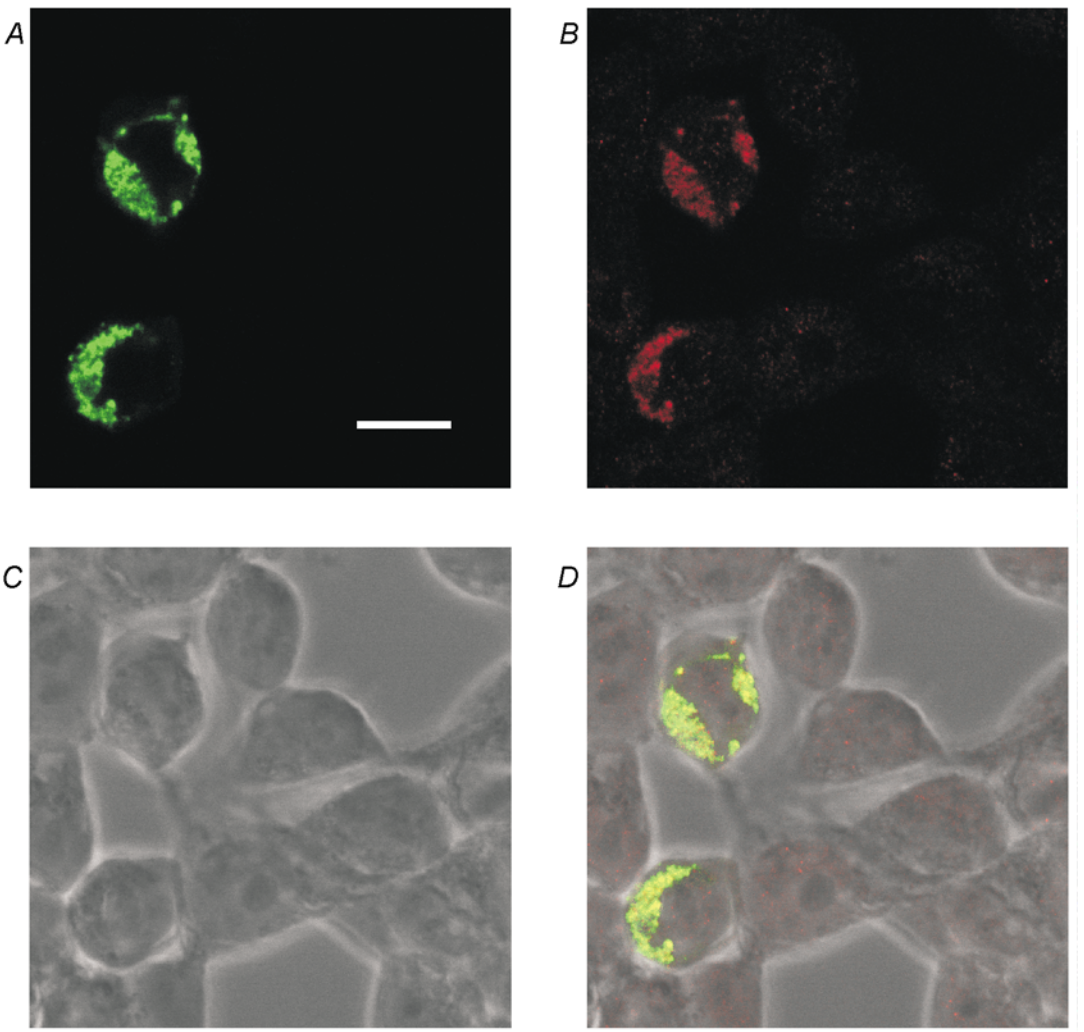

Figure 2. Distribution of rSK1 protein in HEK 293 cells

A, confocal image of HEK 293 cells transiently transfected with a YFP-tagged *rSK1* construct (green filter) showing that the expressed protein is unevenly distributed around the cell and has a punctate staining pattern. *B*, the staining seen with rSK1-specific antibody UCL 56. Comparison with *A* shows that it recognizes the channel protein because the staining coincides well with YFP fluorescence. *C*, bright field image of cells depicted in *A* and *B*. *D*, overlay of images *A*, *B* and *C*. Scale bar is 20 μm.

SK2 current in *Xenopus* oocytes (Kohler *et al.* 1996) and HEK 293 cells (Strobaek *et al.* 2000). In contrast, cells co-transfected with *rSK1* produced a concentration–inhibition curve that was shifted significantly to the right, yielding an IC_{50} of 1.4 ± 0.3 nM and n_H value of 0.6 ± 0.1 (Fig.3*B* and *C*). Thus co-expression of *rSK1* with *rSK2* results in the formation of channels with ~15-fold lower sensitivity to apamin.

We next tested UCL 1848, which has a similar affinity for SK2 to apamin but the advantage of rapid reversibility. For cells expressing *rSK2* alone, UCL 1848 blocked SK current with an IC_{50} of 110 ± 26 pM (n_H value of 0.7 ± 0.1), which is very close to the value of 120 pM previously reported (Hosseini *et al.* 2001). When *rSK2* was co-expressed with *rSK1* the dose–response curve was best fitted with an IC_{50} of 2.9 ± 0.3 nM (n_H value of 0.49 ± 0.04) as shown in Fig. 4*C*. This is qualitatively similar to the result obtained with apamin, although the shift in IC_{50} was rather greater with UCL 1848, a factor of 26. In addition to a change in the IC_{50}, if co-assembly is occurring, then one might expect the dose–response curve to become slightly shallower because it could reflect a number of different populations where various stoichiometries of SK1 and SK2 subunits have formed. Although it is difficult to estimate n_H values accurately, for both UCL 1848 and apamin, in our co-expression experiments, concentration–inhibition curves were best fitted using smaller values of the Hill coefficient. (Our fitting of a single component Hill equation to the SK1–2 data is, therefore, only to aid comparison with the SK2 homomeric expression data.) Taken together, these data show that rSK1 and rSK2 subunits can interact, possibly directly, to form functional channels, with a unique pharmacology.

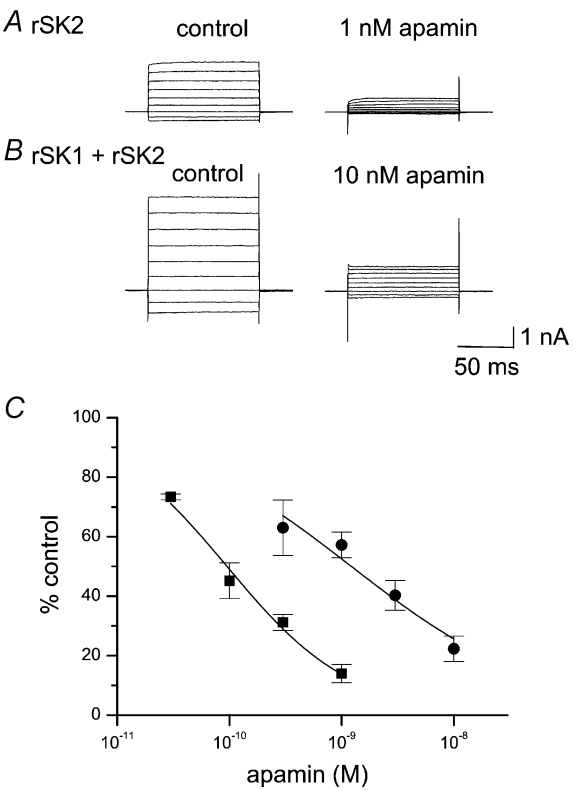

Figure 3. Apamin block of current in cells expressing *rSK2* alone or *rSK1* and *rSK2*

A, typical current traces showing the effect of 1 nM apamin on homomeric rSK2 currents. *B*, typical current traces showing the effect of 10 nM apamin on a cell co-transfected with *rSK1* and *rSK2*. In both *A* and *B*, currents were recorded immediately before and after 4 min exposure to apamin. The scale bar applies to both *A* and *B*. *C*, apamin concentration–inhibition curves for cells transfected with *rSK2* alone (■) or *rSK1* and *rSK2* (●). Continuous lines are fits of the Hill equation to the data, yielding estimates of IC_{50} of 95 ± 8 pM (n_H = 0.80 ± 0.06) and 1.4 ± 0.3 nM (n_H = 0.6 ± 0.1) for rSK2 and rSK1 with rSK2, respectively. Each point is the mean of 4–9 observations and the error bars indicate S.E.M.

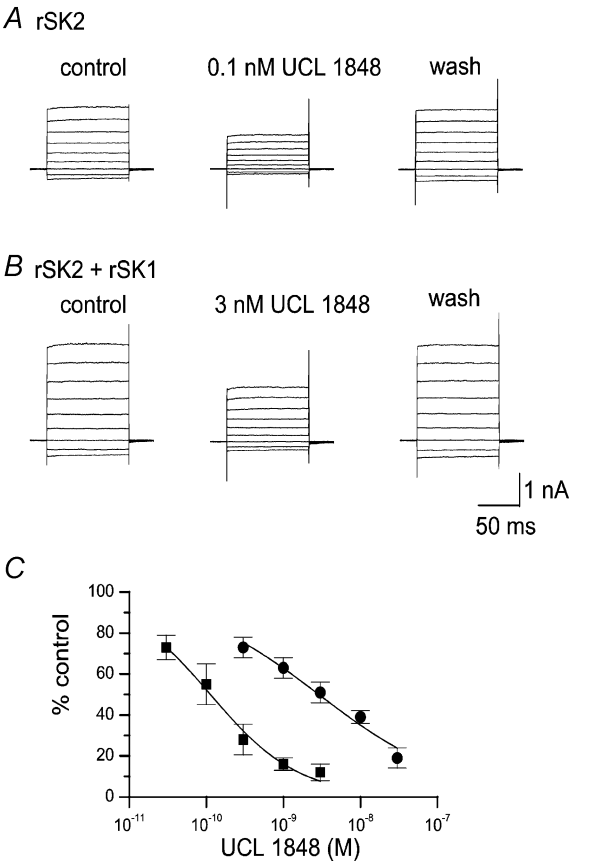

Figure 4. UCL 1848 block of current in cells expressing *rSK2* alone or *rSK1* and *rSK2*

A, effect of 0.1 nM UCL 1848 on rSK2 currents. *B*, effect of 3 nM UCL 1848 on a cell co-transfected with *rSK1* and *rSK2*. In *A* and *B* currents were recorded before and after 3 min exposure to UCL 1848, and 3 min after washout. The scale bar applies to both *A* and *B*. *C*, UCL 1848 concentration–inhibition curves for cells transfected with *rSK2* alone (■) or *rSK1* and *rSK2* (●). Continuous lines show fits of the Hill equation to the data, yielding estimates of IC_{50} of 110 ± 26 pM (n_H = 0.7 ± 0.1) and 2.9 ± 0.3 nM (n_H = 0.49 ± 0.04) for rSK2 and rSK1 with rSK2, respectively. Each point is the mean of 3–5 observations. Error bars indicate S.E.M.

DISCUSSION

We have confirmed that the rat *SK1* gene does not produce functional channels when expressed alone in either human (HEK 293) or rat (H4) cell lines. However, our results with an anti-SK1 antibody and the YFP-tagged *rSK1* construct indicate that a protein is synthesized.

Many potassium channel genes produce 'silent' subunits. These proteins do not form functional channels alone, but can form functional heteromeric channels when co-expressed with related subunits (see for example Krapivinsky *et al.* 1995; Post *et al.* 1996; Ottschytsch *et al.* 2002). The most obvious interpretation of our findings is that rSK1 has a similar function because co-expression of *rSK1* with *rSK2* caused two important changes. Firstly, the currents in cells where *rSK1* was co-expressed with *rSK2* were substantially greater than when *rSK2* was expressed alone. This increase in current was seen in both a human (HEK 293) and a rat (H4) cell line, suggesting that this result is not related to our choice of expression system. Secondly, co-expression of *rSK1* with *rSK2* altered the pharmacology, producing SK currents that were ~15-fold less sensitive to apamin and ~26-fold less sensitive to UCL 1848.

Our observations clearly indicate an important interaction between rSK1 and rSK2. The simplest explanation for this interaction is that rSK1 and rSK2 can form a functional heteromeric complex. Unfortunately, our attempts to confirm this idea by co-immunoprecipitation failed because tagged channel constructs suitable for this purpose did not display normal channel function (data not shown). Thus, an indirect interaction between rSK1 and rSK2 cannot be ruled out. However, it is tempting to speculate that since both human and rat SK1 subunits interact with SK2 and both also interact with SK3 (Ishii *et al.* 1997; Benton *et al.* 2003), the only functional difference between *hSK1* and *rSK1* may be that the human gene can produce a functional channel when expressed alone.

The interaction between rSK1 and rSK2 demonstrated here *in vitro* is particularly intriguing in the light of the reported distribution of *rSK1* and *rSK2* mRNA *in vivo*. A number of *in situ* hybridization studies of the rat brain have revealed that the presence of *SK1* mRNA is nearly always accompanied by *SK2* mRNA (Kohler *et al.* 1996; Stocker *et al.* 1999; Stocker & Pedarzani, 2000). Whether SK1 requires SK2 to produce functional channels *in vivo*, however, remains an open question because recent immunohistochemical data show strong staining for the SK1 subunit where there is only a low level of SK2 protein (Sailer *et al.* 2002).

Interestingly, Stocker, Pedarzani and colleagues (1999) have reported an IC_{50} of 480 pM for apamin inhibition of the current underlying a medium duration AHP (mAHP) in CA1 hippocampal neurons. They point out that this value is considerably higher than would be expected for homomeric SK2 channels and suggest a possible role for SK1–2 heteromers (Stocker & Pedarzani, 2000). (Our IC_{50} of 1.4 nM for apamin block of SK currents produced when *SK1* and *SK2* are co-expressed is somewhat higher but it must be remembered that this value probably depends on the ratio of *SK1:SK2* mRNAs, which are not controlled to be at the ratio of ~1:1, found *in vivo*.) Pedarzani, Stocker and colleagues (2002) have similarly reported that the IC_{50} for block of hippocampal apamin-sensitive channels by the peptide toxin tamapin is ~10-fold higher than expected for homomeric SK2 channels (Pedarzani *et al.* 2002). Again this suggests that the currents may not be carried by homomeric SK2 alone. Based on immunohistochemical experiments, however, others have suggested that the hippocampal apamin-sensitive currents are formed by homomeric SK2 channels (Sailer *et al.* 2002). This conclusion was reached because of a correlation observed between the size of the apamin-sensitive mAHP current and the intensity of SK2, rather than SK1, protein staining. Our data might reconcile these apparently discrepant immunohistochemical and pharmacological findings because if SK1 interacts with SK2 *in vivo*, then the supply of SK2 would be a 'rate-limiting step' in the production of apamin-sensitive channels.

Our data are also interesting with regard to the suggestion that rSK1 subunits are involved in the formation of the channels underlying the apamin-insensitive slow after-hyperpolarization (sAHP) in rat hippocampal pyramidal neurons (Bowden *et al.* 2001). In the present study we have shown that rSK1 does not form a functional channel by itself and that the channels formed when it is co-expressed with SK2 are still apamin sensitive (albeit less so than when SK2 is expressed alone). These findings would seem to weaken the argument that rSK1 forms the sAHP channel by itself.

More work is needed to clarify these issues and to better understand the physiological role(s) of SK1, but our data provide a starting point for examining the functional properties of the rat *SK1* gene, and for comparisons with native tissues.

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