The hSK4 (KCNN4) isoform is the Ca^{2+}-activated K^+ channel (Gardos channel) in human red blood cells

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The question is, does the isoform hSK4, also designated KCNN4, represent the small conductance, Ca^{2+}-activated K^+ channel (Gardos channel) in human red blood cells? We have analyzed human reticulocyte RNA by RT-PCR, and, of the four isoforms of SK channels known, only SK4 was found. Northern blot analysis of purified and synchronously growing human erythroid progenitor cells, differentiating from erythroblasts to reticulocytes, again showed only the presence of SK4. Western blot analysis, with an anti-SK4 antibody, showed that human erythroid progenitor cells and, importantly, mature human red blood cell ghost membranes, both expressed the SK4 protein. The Gardos channel is known to turn on, given inside Ca^{2+}, in the presence but not the absence of external K_o^+ and remains refractory to K_o^+ added after exposure to inside Ca^{2+}. Heterologously expressed SK4, but not SK3, also shows this behavior. In inside–out patches of red cell membranes, the open probability (P_o) of the Gardos channel is markedly reduced when the temperature is raised from 27 to 37°C. Net K^+ efflux of intact red cells is also reduced by increasing temperature, as are the P_o values of inside–out patches of Chinese hamster ovary cells expressing SK4 (but not SK3). Thus the envelope of evidence indicates that SK4 is the gene that codes for the Gardos channel in human red blood cells. This channel is important pathophysiologically, because it represents the major pathway for cell shrinkage via KCl and water loss that occurs in sickle cell disease.

Four isoforms (SK1–4) of the small conductance Ca^{2+}-activated K^+ channel have been identified (1–5). These channels, with acronyms small conductance (SK), intermediate conductance (IK), and potassium channel calcium-activated intermediate/small conductance subfamily N (KCNN), are highly conserved Ca^{2+}-activated inward rectifiers (see refs. 1–5). Although KCNN is the notation assigned by GenBank (6), we use herein the SK notation and report studies that deal mainly with the human isoform, i.e., hSK4, recognizing that there is a parallel with the rat, rSK4, and mouse, mSK4, isoforms (7). We are principally concerned with SK4 because of its putative identification as the Ca^{2+}-activated K^+ channel, referred to as the Gardos channel (8), in human red blood cells. Activation of the channel results in a marked hyperpolarization of the membrane accompanied by shrinkage of the cell due to the loss of KCl and water. The main reason the Gardos channel has been assigned to the SK4 gene in human red blood cells is because of parallels in its electrophysiological characteristics (7, 9, 10) between the intact cell and the expressed channel as well as its pharmacological sensitivities; the channel is inhibited by charybdotoxin (11) and clotrimazole (CLT) (12) but not by apamin (13). The SK4 expressed in different cell types shares this inhibitory profile (1, 7). In sharp contrast, expressed SK1, 2, or 3 channels are inhibited by apamin (14–17) but not by CLT (18). Obviously, it is necessary to go beyond the foregoing correlations of channel characteristics to establish which isoform(s) of the SK family are actually found in human red blood cells.

Our approach exploits the use of human erythroid progenitor cells in which we have previously determined the subunit types and isoform composition of the endogenously expressed Na^+ pumps (19, 20). Our particular preparations of the progenitor cells allow us to study purely erythroid developmental stages free of contamination from white cells or platelets. This conclusion is based on "β profiling," by which we mean that the progenitor cells were found to contain the message for the $\beta 2$ and not the $\beta 1$ isoforms of the β subunit of the Na pump in contrast to a white cell and platelet library that contained the message for the $\beta 1$ but not the $\beta 2$ isoforms (20). Our studies using progenitor cells indicate that SK4 is the isoform that subserves the functions of the Gardos channel in human red blood cells. This identification is based on RT-PCR, Northern and Western blotting of progenitor cells, and Western analysis of human red blood cell ghosts. We also found that SK4, when heterologously expressed in Chinese hamster ovary (CHO) cells, displays functional characteristics of the Gardos channel similar to those seen in human red blood cells.

Methods

Reticulocyte and Erythroid Progenitor Cell RNA. This study used samples of RNA taken from the same preparations of reticulocytes and human erythroid progenitor cells purified and cultured as described previously (20). All of these preparations were found to be free of leukocyte and platelet contamination based on the criterion of "β profiling," as previously established (20). RT-PCR was carried out by using the SK isoform specific primers given in Table 1. Note that one primer pair, labeled SK1–3, was designed to detect these three channel isoforms but not SK4. To confirm that primers were capable of amplifying a specific product, RT-PCR was performed with each set of primers and plasmids individually encoding each of the channel isoforms. In each case, a product of the expected size was amplified with >96% sequence identity.

Northern Blotting. Northern blotting was carried out as described before (20). DNA probes were derived from the following gel-purified DNA templates: a 436-bp *Bam*HI–*Hin*dIII fragment of rat SK1 (rSK1); a 582-bp *Eco*RV–*Nar*I fragment of rat SK2 (rSK2); a 710-bp *Sma*I–*Sma*I fragment of rat SK3 (rSK3); and a 723-bp *Sph*I–*Sfi*I fragment of human SK4 (hSK4). These DNA fragments were radiolabeled by using a Prime-It II kit (Stratagene) and ^{32}P-dCTP and purified on G-50 Spin Columns (Roche Applied Science, Indianapolis) to achieve a specific activity of $\approx 10^9$ dpm/μg. As a positive control, probes were also hybridized with a human multiple tissue Northern blot (CLONTECH).

Western Blotting. The anti-mouse SK4 (mSK4/IK_{Ca}) antibody (21) was custom produced by Research Genetics, a division of Invitro-

Abbreviations: CLT, clotrimazole; CHO, Chinese hamster ovary.

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Table 1. PCR primer pair sequences (all designed against human sequences) that were selected to have distinct and high specificity for each of the indicated SK isoforms of the Ca^{++}-activated K^+ channel

Specific primer	Expected size, bp	Primer sequence
SK4	1,767	5′-GCTGACGACCTGCAAGCCACAGTGG-3′
		5′-GGTAACTGAAGCCTCCCAGAATCCTGG-3′
	529	5′-CCTTTCAGACACACTTTGGCTGATCC-3′
		5′-CAGTGCTAAGCAGCTCAGTCAGG-3′
SK3	1,275	5′-GCTCCATCACCCTAATGCCACC-3′
		5′-GCTGCCAATCTGCTTCTCCAGG-3′
	2,226	5′-GCTTGTGCTGCAGGGAATACAGC-3′
		5′-GCAACTGCTTGAACTTGTGTACG-3′
SK2	670	5′-CTCCATTGGTTATGGTGACATGGTACC-3′
		5′-CTAGCTACTCTCTGATGAAGTTGGTGG-3′
	506	5′-GTAGTGGCAAGGAAGCTAGAACTTACC-3′
		5′-GACCGGGACCGCTCAGCATTG-3′
SK1	1,428	5′-CAGGTAGTCATGAACAGCCACAGC-3′
		5′-GAGCGTGCAGCTCCGATACAAGG-3′
	≈150	5′-GGTAGTCATGAACAGCCACAGC-3′
		5′-CCTGTAGAGAGGCACCCAGC-3′
SK1–3	641	5′-GATGACTGGMGNATMGCCATGAC-3′
		5′-GGTSAGCTGAGTRTCCATCATG-3′

In the case of the SK1–3 (i.e., SK1, SK2, and SK3) primer set, M = A or C; R = A or G; and N = A, C, G, or T.

gen. The antibody was directed toward an mSK4-specific peptide with the amino acid sequence RQVRLKHRKLTEQVNSMVD. Pellets of 7- and 13-day-old cultures of human erythroid progenitor cells (see ref. 20) as well as human brain, kidney, and parotid tissues were prepared by previously described methods (see ref. 21). Human red blood cell ghosts were prepared from heparinized peripheral blood by hypotonic lysis, as described (22), and were frozen, thawed, and washed before use.

Approximately10 μg of crude protein was separated by two-phase Tricine polyacrylamide gel electrophoresis (10% T/6% C resolving layer, 4% T/3% C stacking layer) and transferred onto poly(vinylidene difluoride) membrane (BioDyne PVDF, Pall Filtration, General Electric) in buffer containing 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) adjusted to pH 11 and 10% methanol. Blotting was then carried out as described (21). To assess specificity, the mSK4 antibody was preincubated for 1 h with a 50-fold molar excess of competitor peptide corresponding to the epitope recognition sequence before use in blotting.

Stable SK-Expressing Cell Lines. CHO cells stably expressing hSK4 channels as described (1) were used in this study. Another CHO cell line stably expressing rSK3 was established by similar means, except that the cDNA was carried in the plasmid pcDNA 3.1 Zeo (from Guy Moss, University College, London), and Zeocin (Invitrogen) was used to select stable transfectants. Stably transfected survival colonies were sorted by FACS as described (1). A single clonal population of cells was used for each cell line.

Electrophysiology of SK Channels. Single-channel recordings were performed by using the methods described (1). For whole-cell recordings, the standard bath solution (5 mM K_o) consisted of (in mM): 140 NaCl, 1.0 $CaCl_2$, 5 KCl, 29 glucose, 25 Hepes (pH 7.4). Bath composition for ion substitution experiments was made by replacement of 5 mM K_o^+ with 5 mM Na_o^+, resulting in 0 mM K_o^+. It should be understood that the K^+ channels studied in CHO cells were seen not in control cells but only in cells that had been transfected with either the rSK3 or hSK4 isoforms, where they were Ca^{2+}-dependent and displayed the expected unitary conductance characteristics (1).

The pipette solution for whole-cell experiments consisted of (in mM): 30 KCl, 100 K-gluconate, 5 EGTA, and 10 Hepes (pH 7.2). This solution was supplemented with 4.27 or 4.74 mM $CaCl_2$ to achieve free Ca^{2+} concentrations of 1.0 or 3.0 μM, respectively. All whole-cell recordings were performed by using 200-ms ramps from −120 to +80 mV from a holding potential of −70 mV. Current densities were measured for each cell by dividing the current amplitude at 60 mV by the capacitance.

For inside-out patch recordings of stably transfected cells, pipettes were filled with (in mM): 30 KCl, 100 K-gluconate, 1 $MgCl_2$, and 10 Hepes (pH 7.2), and the cytoplasmic side of the membrane was perfused with (in mM): 30 KCl, 100 K-gluconate, 5 EGTA, 10 Hepes (pH 7.2), and 4.27 $CaCl_2$ (1 μM free Ca^{2+}). In some experiments, lyophilized thioredoxin peroxidase (23), i.e., calpromotin (24, 25), was dissolved in the latter solution at a concentration of 10 μM and used to perfuse excised inside-out patches. All patch recordings were made over a period of 30–60 s at a holding potential of −80 mV, first at 25°C and subsequently, after rapidly switching (<10 s), at a controlled bath temperature of 35°C in a temperature-controlled 35-mm tissue culture cup. Electrical heating was mediated via a Pt/Ir oxide film on the outside of the cup (Bioptechs, Butler, PA).

Net K_o^+ and $^{86}Rb^+$ Fluxes. Blood was drawn into heparin from normal volunteers and used without delay. Net K^+ effluxes were measured by incubating red cells in a low K_o^+ medium for various time periods. The Gardos channel (i.e., Ca^{2+}-activated K^+ channel) was activated, in the presence of Ca_o^{2+}, by use of either the divalent ionophore, A23187, or energy depletion or both, as indicated below. The experimental protocols used were variations on those described by others (26, 27). Because details of the protocols varied, we have adopted abbreviations for some constituents that were common to many of the solutions used. Thus, H is Hepes buffer, C is $CaCl_2$, M is 0.2 mM $MgCl_2$, A is 10 μM A23187, V is 1.0 mM orthovanadate, I is 10 mM inosine, IA is 6.0 mM iodoacetamide, and CLT is 10 μM chlotrimazole. It should be understood that V is used to inhibit the Ca^{2+} pump, thereby allowing for the accumulation of (Ca_i^{2+}) necessary to activate the Gardos channel. The combination of I plus IA is used to deplete the cells of energy (ATP) that could interfere with the activation of the Gardos channel. In all experiments, final incubations were carried out in the absence and presence of CLT, and all samples taken from suspensions where CLT was absent were mixed before centrifuging with a stop solution containing CLT. This procedure provided the time resolution for rapid fluxes. Hematocrits for net K_o^+ measurements varied from 11% to 20%. The pH of all solutions was ≈7.4 at 37°C. Changing the temperature from 37 to 27°C lowers the pH by ≈0.1 pH unit, without appreciable effect on the K^+ flux (28). For Table 2, the following experimental conditions were used. Experiment A: cells were washed with a solution containing (in mM) 40 NaCl, 90 NaSCN, 2 KCl, M, and 20 H. The cells were then energy depleted by incubation in this solution together with 1.3 mM C, I, IA, and V for 25 min at 37°C. The suspension was then split, with half incubated at 27°C and the other half at 37°C. After 3 min, samples were taken at 0, 15, 30, 45, and 60 min. Experiment B: cells were washed in the presence or absence of SCN^- in solutions containing (in mM): either 40 NaCl + 90 NaSCN or 130 NaCl together with 0.5 KCl, 30 H, and M. After washing, the cells were suspended, respectively, in each of these solutions together with the additions of 1.3 mM C, I, IA, and V. The cells were then energy depleted by incubation in these solutions for 25 min at 37°C. Then each solution was split, with half incubated at 27°C and the other half at 37°C. After 3 min, aliquots were taken over a 30-min period for determination of K_o^+. Experiment C: cells were energy depleted by incubation for 3 h in a solution

Table 2. The effect of temperature on the net efflux of K^+ from human red blood cells after activation of the Gardos channel by various means

Experiment	Main anion	K_i^+	K_o^+	Flux protocol	$^\circ k_K^{\Delta CLT}$ in hr^{-1} 27°C	37°C
A	SCN^-	93	≈2.4	Net K^+	2.1	0.77
B	SCN^-	94	≈1.2	Net K^+	1.3	0.15
C	SCN^-	93	≈0.5	Net K^+/A23	31.8	17.5
D	SCN^-	90	120	^{86}Rb	0.33 ± 0.03	0.27 ± 0.03

The effluxes of K^+ were measured at either 27 or 37°C (see *Methods* for details) and estimated from the rate of increase in K_o^+ from cells incubated in a low K_o^+ medium. The cells were treated with V (except Experiment C) to inhibit Ca^{++} efflux via the Ca^{++} pump. In addition, the cells were energy depleted in Experiments B and C but not in A, in which they were normal. K^+ efflux from cells were begun in Experiments A and B after a 3- to 5-min equilibration at their respective temperatures, and in Experiment C, after addition of the ionophore A23187 (A23). The K_i^+ (mmol/liter red blood cells) and K_o^+ (mM) values refer to time zero. The effluxes of K_i^+ were also estimated from the rate of appearance of medium ^{86}Rb from cells preloaded with ^{86}Rb (Experiment D). The cells were treated with V to inhibit Ca^{++} efflux via the Ca^{++} pump. In addition, the cells were energy depleted. ^{86}Rb effluxes, from cells having been washed in the cold, were begun after a 3- to 5-min equilibration of the cell suspensions at their respective temperatures. All effluxes were carried out in the absence and presence of CLT and presented as the CLT-sensitive flux (ΔCLT). The CLT-insensitive efflux was in all cases <5–10% of the total flux. Because the net K_i^+ efflux (Experiments A–C) appeared to be exponential in all cases, the outward rate constant, $^\circ k_K^{\Delta CLT}$ in hr^{-1} was estimated from the initial rate, as explained in *Methods*. The values of $^\circ k_{Rb}^{\Delta CLT}$ in Experiment D represent the means ± SEM, where $n = 4$.

containing (in mM): 10 NaCl, 50 KCl, 60 KSCN, 1.0 Na_2HPO_4, 30 H, I, IA, and V. The cells were washed and resuspended in the following solution (in mM): 60 NaCl, 60 NaSCN, 0.4 KCl, 30 H, M, I, IA, and V together with 50 μM C. After 3 min at either 27 or 37°C, the flux was initiated by addition of A with samples taken at 0, 45, 90, 135, and 180 s. Experiment D: Cells loaded with ^{86}Rb by incubation for 3 hr in (in mM): 30 KCl, 90 KSCN, 20 H and M. Cells were then resuspended in this solution together with 1.3 mM C, I, IA, and V and incubated at 37°C for 15 min before washing in the cold. Cells were resuspended at 27°C and 37°C and after 3 min samples were taken at 0, 5, 10, 15, and 20 min.

Calculation of ^{86}Rb rate constants were the same as previously described (29). Hematocrits (Hcts) were calculated by using the equation, $Hct = (S_{WB} - S_o)/(S_i - S_o)$, where S_{WB} is the whole blood, cellular (S_i), or supernatant (S_o) concentration of K^+. The outward rate constants (hr^{-1}) calculated for the results in Table 2 were from the expression $^\circ k_K^{\Delta CLT} = [(1 - Hct)/(Hct \times \% H_2O)] R_o/[K^+]_i$, where R_o (in mM/unit time) is the initial rate assuming the curves are single exponentials, and $[K^+]_i$ is in mmol/l cell water. The percent water is taken from the difference in wet and dried weights of packed cells. CLT, inosine, and iodoacetamide were obtained from Sigma; A23187 from Calbiochem; and 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetate or -tetraacetic acid–acetoxymethyl ester (BAPTA-AM) from Molecular Probes. All other chemicals, wherever possible, were of reagent grade.

Results

Our prime aim in undertaking the studies reported here was to identify the gene that codes for the Gardos channel (or Ca^{2+}-dependent K permeability) of human red blood cells. We first screened, by RT-PCR, our previous preparations of RNA extracted from human reticulocytes (19) by use of the primer sets listed in Table 1, to establish which isoforms of the SK (or KCNN) channel family were present. As is evident in Fig. 1, the isoform SK4 is present in these preparations. We also obtained

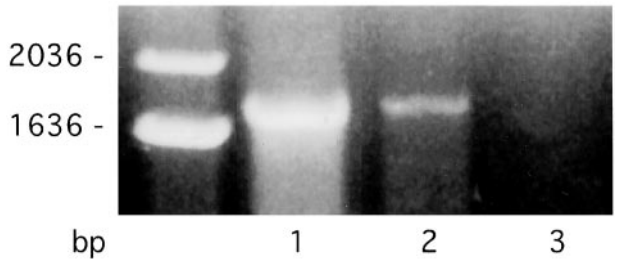

Fig. 1. The SK4 isoform of the Ca^{2+}-activated K^+ channel is present in human reticulocytes. PCR products were obtained with the isoform-specific primers defined in Table 1. Single-stranded cDNA derived from reticulocytes (see *Methods*) was used as template. Lanes 1 and 2 had, respectively, 25- and 4-μl samples applied to the gel. Lane 3 is a water control. The mass ladder (in base pairs) is shown at left (Life Technologies, Grand Island, NY). The expected product size was 1,767, and a product of approximately this size was found in lanes 1 and 2. The product was sequenced in this and other analyses and was shown to have >98% identify with the expected sequence for SK4.

analogous results with the second set of SK4 primers given in Table 1 (data not shown). Use of any of the other isoform primers listed in Table 1 for SK1, 2, or 3 was negative with regard to their specific presence in these RNA preparations. When products did appear, they were neither of the expected size nor did they display any sequence homology with the isoform being tested. Because we used preparations of reticulocytes that were free from white cell and platelet contamination by the criterion of "β profiling" (20), the results strongly indicated that SK4 was the isoform responsible for the Gardos channel in human erythrocytes. However, there is a caveat to this interpretation, because SK4 has also been identified as a constituent of human lymphocytes (3). Thus, although contamination of our preparations is improbable, it must remain a possibility.

We next turned to our preparations of human erythroid progenitor cells, because, as shown before (20), an erythroid "β profiling" was also a characteristic of these differentiating cells. Fig. 2 shows the results of Northern analysis of these progenitor cells from days 7–12 of culture. The evidence presented in Fig. 2 shows that SK4 is present, and there is a clear tendency for its expression level to increase with increasing maturation. The positive control blots are given on the right side of Fig. 2 and made use of mRNA from human placental tissue, which is known (17) to contain the SK4 isoform (1, 17). Although we saw no evidence by Northern analysis for SK1 or SK3, some blots indicated that hSK2 might be present. This was not pursued because of failure to find the SK2 isoform by RT-PCR and because the Gardos flux in human erythrocytes is not inhibited by apamin (13). Thus we conclude from Northern analysis that

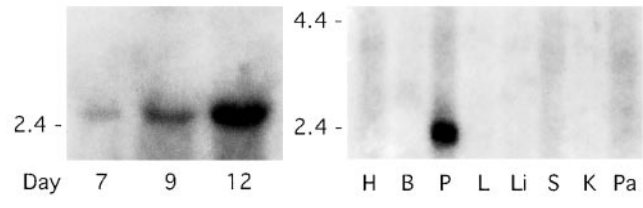

Fig. 2. Northern blots probed for the mRNA encoding the Ca^{2+}-activated K^+ channel isoform, SK4, using RNA prepared from cultured human erythroid progenitor cells at different stages of maturation (see *Methods* for details). Also shown is the positive control blot for SK4 (*Right*), where H is heart; B, brain; P, placenta; L, lung; Li, liver; S, skeletal muscle; K, kidney; and Pa, pancreas, all from human mRNA. These results parallel our previous finding that SK4 is present in reticulocytes. Note that SK4 is present in the progenitor cells at day 7 increasing with differentiation through day 12. The transcript size is indicated on the ordinate.

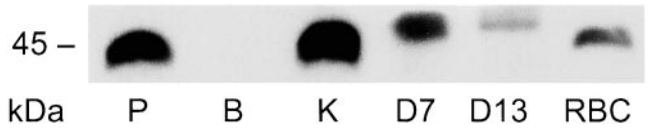

Fig. 3. Western blots showing that the protein for the Gardos channel isoform, SK4, is present in cultured human erythroid progenitor cells and in ghost membranes made from mature human red blood cells. The antibody was prepared against an SK4-specific peptide and used as described in *Methods*. The two positive controls are human parotid gland (P) and kidney (K) with the negative control being brain (B). It is clear that a band of the appropriate molecular weight is present in the human erythroid progenitor cells as they mature from days 7 to 13. It is also evident that the SK4 band is present in human red cell ghost membranes (RBC). The decrease in the blot intensity of the D13 band compared with D7 is primarily due to the decreased protein content (cell number) of cells loaded onto the gel. The slight variation in the molecular weights of SK4 bands seen in the progenitor cells, relative to the other bands, may be due to posttranslational modification or higher salt concentration in the loading mixture. It should also be mentioned that, except in the parotid lane, there are higher molecular weight bands (not shown) that in each case react with the antibody. Importantly, preincubation of the antibody with purified peptide that contains the antigenic epitope produces a complete loss of reactivity in all lanes except in brain, where it is much reduced, and in ghosts, where it is only faintly present in the highest molecular weight bands (data not shown).

differentiating human erythroid progenitor cells expresses only detectable levels of SK4 message.

We also performed a Western blot analysis, the results of which are shown in Fig. 3. By using an antibody directed against a peptide whose sequence is derived from mSK4, we find that a protein of the appropriate molecular weight is detected not only in the human erythroid progenitor cells but also in mature human erythrocyte membranes (21, 30). This signal was completely competed through preincubation of the antibody with the antigenic peptide, demonstrating specificity of the interaction. Another indication of the specificity of the SK4 antibody is that it showed no crossreactivity with brain, a tissue known to express SK1, 2, and 3 but not the SK4 isoform.

We next compared several functional characteristics of the Gardos channel in human erythrocytes with the behavior of SK4 expressed in transfected cells. The first modality to be examined was the effect of external K^+ (K_o^+) on Gardos channel activation. It is known from previous work (9, 31) that for the Gardos channel to open, K_o^+ must be present on the outside of the channel at the time that the cytoplasmic surface of the channel/cell is exposed to internal Ca_i^{2+}. If K_o^+ is absent, the channel stays closed when Ca_i^{2+} is introduced inside and, at least in human red cell ghosts (9, 31), will not open even when K_o^+ is subsequently added. We wished to test whether SK3 or SK4 channels heterologously expressed in CHO cells displayed a similar dependence on K_o^+. To this end, stably transfected CHO cells were preincubated for varying periods of time in the presence or absence of K_o^+. Whole-cell recordings were then made, with Ca_i^{2+} being introduced with a patch pipette, as described in *Methods* and the Fig. 4 legend, with subsequent determination of the current density. As shown in Fig. 4*A*, the current measured in cells expressing SK3 channels remained active and was insensitive to the presence or absence of K_o^+. In contrast, channels in cells expressing SK4 (Fig. 4*B*) were sensitive to the presence of K_o^+, displaying much less activity in cells preincubated without K_o^+. The cells studied in Fig. 4*B* were incubated in the absence of K_o^+ for 3–5 h and were refractory to the addition of K_o^+ after this time (data not shown). We then studied whether the expressed SK4 channel could be activated by K_o^+ after shorter incubation times in its absence. Fig. 4*C* shows the time course of decay in SK4 activity during 2 h incubation in the absence of K_o^+. As shown in Fig. 4*D*, the cells can respond to the addition of K_o^+

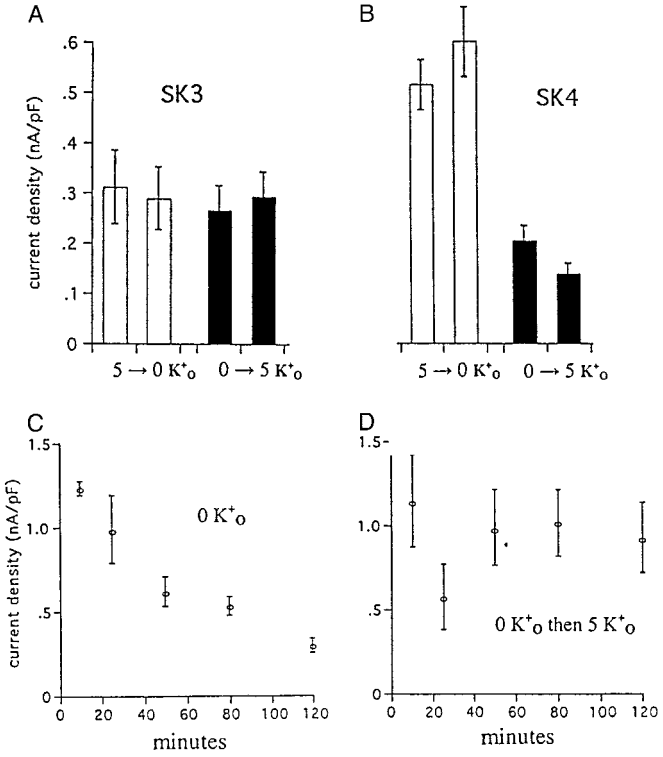

Fig. 4. The effect of external K_o^+ on the activity of heterologously expressed SK3 and SK4 channels in CHO cells. Cells were washed twice in 0 mM K_o^+ and then incubated with 100 μM 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetate or -tetraacetic acid–acetoxymethyl ester (BAPTA-AM) in either 5 mM K_o^+ (open bars) or 0 mM K_o^+ (dark bars) containing media for 3–5 h at room temperature (see *Methods*). At the end of this period, cells were perfused with incubation solution before and during seal formation, breakthrough, and achievement of whole-cell recording mode. Current amplitude at 60 mV was then measured for each cell first during continued perfusion with incubation solution and then after switching from 0 to 5 K_o^+ or vice versa. The whole-cell currents in expressed SK3 channels (*A*) were insensitive to the presence or absence of K_o^+, whereas the currents in expressed SK4 channels (*B*) were markedly diminished by preincubation in 0 mM K_o^+. (*C*) The decrease in whole-cell currents that occurred during the first 2 h of incubation of SK4 expressing cells in 0 mM K_o^+. (*D*) SK4 cells can recover from incubation in 0 mM K_o^+ by reexposure to 5 mM K_o^+ over this time period. SK4 cells exposed for longer periods of time (*B*, dark bars) to 0 mM K_o^+ remain refractory to 5 mM K_o^+. Error bars represent ± SEM, where n = 9–15 separate observations with SK3 (*A*) and 11–15 for SK4 (*B*); in *C* and *D*, n = 3–12.

over this same time period. Thus, SK4 channels expressed in CHO cells behave like the Gardos channel in intact human erythrocytes and ghosts because they stay closed in the absence of K_o^+ but differ from them in that the channels are able to open after the addition of K_o^+. The basis for this difference between expressed SK4 channels and Gardos channels is not known, but it should be remembered that, because these patched CHO cells are attached to the substrate, K_o^+ trapped in the cell/substrate interface may alter the overall response to K_o^+-free solutions. That the whole-cell currents do not fall to zero in K_o^+-free solutions (Fig. 4*B*) may reflect the presence of this residual K_o^+.

The second functional feature we explored was the temperature sensitivity of the Gardos channel. Previous work (32) had demonstrated that the open probability (P_o) of Gardos channels, studied in inside-out patches of human erythrocytes, was remarkably sensitive to temperature: the P_o at 30°C was ≈0.6 falling to ≈0.1 at 37°C. We examined this effect of temperature on Gardos channel-mediated K^+ (Rb^+) flux in intact human erythrocytes as well as the response of the P_o values of SK4 channels expressed in CHO cells to changes in temperature.

PHYSIOLOGY

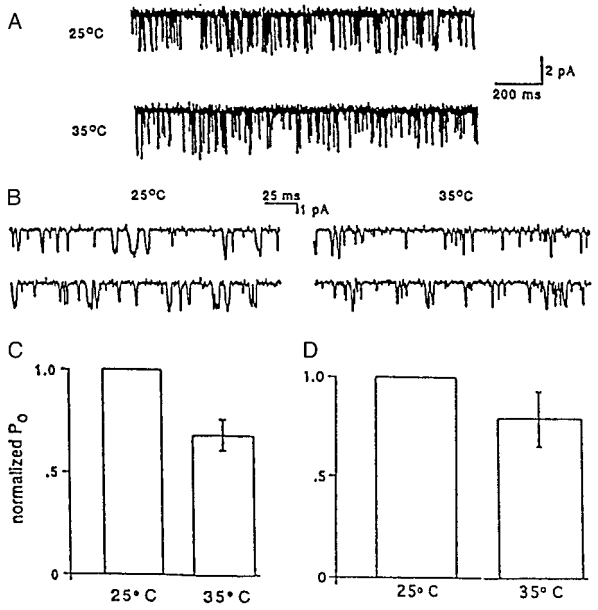

Fig. 5. The sensitivity of hSK4 channels to changes in temperature. Results of single-channel recordings from inside-out patches excised from CHO cells stably expressing hSK4 are presented. The bathing solution contained 1 μM free Ca^{2+} with 130 mM K^+ on both sides of the patch (see *Methods*). Recordings were performed at −80 mV first at 25°C and then, within 10 s, at 35°C. The single-channel activity is shown in *A* and on an expanded time scale in *B*. The bars labeled 35°C in *C* and *D* represent the normalized values at 25°C (taken as 1.0) of the open probability (P_o) of each channel from a given patch. The results presented in *C* represent control characteristics of SK4 channels, whereas in *D*, thioredoxin peroxidase (see *Methods*) has been added to the cytoplasmic bathing medium for reasons explained in the text. The error bars are ± SEM where $n = 6$ in *C* and $n = 4$ in *D*. Lumping the results of *C* and *D* together, the mean difference between the values at 25 compared with 37°C is 0.731 ± 0.072 SEM, with $P < 0.05$.

The results of net K^+ efflux experiments in human erythrocytes are presented in Table 2. The details of the protocols varied for each experiment (see figure legends and *Methods*). The primary aim was to pretreat cells in such a manner that when they were divided and placed at the two different temperatures, the efflux characteristics reflected differences in Gardos channel activity and not differences in their Ca_i^{2+} contents, metabolic states, and possible interfering membrane transport processes during their subsequent incubation. However, this at best is an assumption and is the reason for changing the protocols. When the cells contained Cl^- as the principal anion, the net loss of K_i^+, regardless of protocol design, was the same at 27 and 37°C (data not shown), similar to previous results (33). For instance, net K^+ effluxes were also carried out as part of the same experiment (B in Table 2) but where Cl^- replaced SCN^- in the protocol. The comparable efflux rate constants (hr^{-1}) at 27 and 37°C were 0.33 and 0.35, respectively. Because we thought that the K^+ efflux was rate limited by the membrane's permeability to Cl^-, i.e., $P_K/P_{Cl} \gg 1$, we switched the principal anion to SCN^- because it has been shown that, under the conditions we were using, $P_{SCN} \gg P_K$ (34). In the results shown in Table 2, the efflux of K^+, measured by the rate of net K_i^+ ^{86}Rb release, is clearly greater at 27 than at 37°C. The variation in the flux values seen in the different experiments is due primarily to the different protocols used. An important caveat in these experiments is that there may be heterogeneity in the response of the cell population to Gardos channel activity, because the extrapolated equilibrium end points varied and were not necessarily the same for the two temperatures in most experiments (data not shown). Even so, the temperature dependence of K^+ efflux (Table 2) in intact red cells appears to reflect changes in the P_o of the Gardos channel that are consistent with but less than those obtained in patched red cell membranes (32), because the K^+ fluxes were faster at 27 than at 37°C.

Because of the above results, we were interested in evaluating the temperature dependence of Ca^{2+}-activated K^+ transport through SK4 channels expressed in CHO cells as shown in Fig. 5. Fig. 5 *A* and *B* show single-channel activity on two time scales recorded at 25 and 35°C. The results are summarized in the bar graphs (Fig. 5*C*), where it is clear the P_o value at 35°C is less than that at 25°C, consistent with the previous intact red cell flux studies. The observation that the differences in P_o values are less than expected based on previous results (32) may indicate that the membrane environment surrounding SK4 channels expressed in CHO cells exhibits substantial differences in the temperature-dependent lipid phase transitions as compared with that present in red cells. The differences may also be due to modulators such as calpromotin (24, 25), which is thought to be required for optimum Gardos activity. We tested a purified sample of thioredoxin peroxidase that has been shown to be identical to calpromotin (23). The results presented in Fig. 5*D* indicate that it has no effect on the value of P_o compared with the controls (Fig. 5*C*). In addition, we prepared a hemolysate from red cells according to established procedures (24, 25) that should ensure it contained calpromotin; addition of this lysate to the bathing medium in inside-out patches, as in Fig. 5*D*, was without effect (data not shown).

Discussion

The main result of the work reported in this paper is that the Gardos channel of human red blood cells is coded for by the human SK4 (i.e., KCNN4) gene, as described above in connection with Figs. 1–3. This is based first on analysis of RNA from human reticulocytes and cultured human erythroid progenitor cell, in which we found only the message for SK4 and second, on the use of an antibody directed against SK4. All of these preparations were characterized by "β profiling," indicating that contamination from leukocytes and platelets was not detectable. Because SK4 is found in human leukocytes (3), this also means that caution must be exercised in interpreting studies (e.g., ref. 7) where embryonic stem cells have been analyzed for SK and other isoforms, given that these preparations are likely to be contaminated with nonerythroid forms (see, e.g., refs. 35 and 36). Additional evidence is provided by Western analysis of human progenitor cells and erythrocyte ghosts, where it is clearly shown (Fig. 3) that the SK4 protein is present.

Other support for the conclusion that the SK4 isoform is responsible for the Gardos channel is found in the parallels in function that are displayed in comparisons of human red cells and expressed SK4 channels. This is seen in the effects on Ca^{2+}-activated K^+ channels of preincubation of cells in the presence and absence of K_o^+ (Fig. 4) and in the decrease in P_o of channels when the temperature is raised from 25–27°C to 35–37°C (Table 2 and Fig. 5). It should be emphasized, however, that neither the parallel in the effects of K_o^+ nor the temperature can be considered definitive, because their relative effects fall short of expectations based on intact red cell/ghost results (9, 31) or determination of P_o by patch analyses of red cell membranes (32). On the other hand, previous observations by others, as referred to in the Introduction, have defined critical biophysical and pharmacological properties that implicate the SK4 isoform as being the Gardos channel in human red cells. Thus the single-channel conductance of heterologously expressed SK4 channels as well as their electrical characteristics and pharmacological profiles were essentially the same as that seen for Gardos channels in intact human red cells (7, 9, 10, 13, 32). It is possible that the Gardos channel may have one or more modulators, still to be defined, that are necessary for the hSK4 isoform to display fully the known characteristics of the Gardos channel in human red blood cells. Candidates for such modulators, in addition to the lipid environment, may be cal-

modulin (13, 30, 37, 38), redox systems (39, 40), and/or protein kinases (41, 42).

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