Increased inwardly rectifying potassium currents in HEK-293 cells expressing murine transient receptor potential 4

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Drosophila transient receptor potential (Trp) and its mammalian homologues are postulated to form capacitative Ca^{2+} entry or store-operated channels. Here we show that expression of murine Trp4 in HEK 293 cells also leads to an increase in inwardly rectifying K^+ currents. No similar increase was found in cell lines expressing Trp1, Trp3 or Trp6. Consistent with typical characteristics of inward rectifiers, the K^+ currents in Trp4-expressing cells were blocked by low millimolar concentrations of Cs^+ and Ba^{2+} , but not by 1.2 mM Ca^{2+} , and were only slightly inhibited by 5 mM tetraethylammonium. Single channel recordings of excised inside-out patches revealed the presence of two con-

ducting states of 51 pS and 94 pS in Trp4-expressing cells. The outward current in the excised patches was blocked by 1 mM spermine, but not by 1 mM Mg^{2+} . How Trp4 expression causes the increase in the K^+ currents is not known. We propose that Trp4 either participates in the formation of a novel K^+ channel or up-regulates the expression or activity of endogenous inwardly rectifying K^+ channels.

Key words: capacitative Ca^{2+} entry, K^+ channel, Kir, store-operated channel, Trp4.

INTRODUCTION

In the compound eyes of *Drosophila melanogaster*, transient receptor potential (Trp) and its two homologues, TrpL and Trp γ , form channels that conduct light-induced currents resulting from the activation of G-protein-coupled rhodopsin and the subsequent stimulation of phospholipase C [1–3]. It had long been speculated that, in mammalian cells, capacitative Ca^{2+} entry (CCE), which occurs as a consequence of Ca^{2+} store depletion following the activation of phospholipase C [4,5], might be mediated by channels that share sequence similarity with the *Drosophila* Trp proteins. Indeed, seven Trp homologues have been isolated from mammalian species in recent years, and heterologous expression has revealed a great degree of complexity as to the mode of activation, single-channel conductance and ion selectivity of different Trp homologues [6–14].

Among the seven mammalian Trps, Trp4 has slightly more sequence similarity to the *Drosophila* Trp and TrpL and hence was the first one to be identified by several groups independently using reverse transcriptase coupled to a PCR method. A partial sequence from mouse was first reported [15], which was followed by the full-length cloning of the bovine, rat, mouse and human sequences [7,8,16,17]. The bovine *trp4* was shown to encode a relatively Ca^{2+} -selective channel that was activated in response to Ca^{2+} store depletion when expressed in HEK-293 cells, CHO cells and RBL cells [8,18]. A more recent study indicated that Trp4 participates in the formation of Ca^{2+} -release-activated Ca^{2+} -like channels in bovine adrenal cortex cells [19]. In *Xenopus* oocytes injected with rat *trp4* cRNA, Ca^{2+} -activated Cl^- current was significantly increased after 2 h incubation with a high concentration of thapsigargin (TG) [20]. Thus Trp4 appears to be a component of store-operated channels. However, this view

was challenged by a recent study that showed that murine Trp4 (mTrp4), as well as the closely related Trp5, did not respond to store depletion, but rather to the activation of phospholipase C by cell-surface receptors [21]. In addition, the study also suggested that mTrp4 and mTrp5 form non-selective cation channels instead of Ca^{2+} -selective channels, when expressed in HEK-293 cells. Furthermore, expression of human Trp4 in HEK and in CHO cells led to constitutive Ba^{2+} influx and a non-selective cation conductance without affecting either the store-operated or the receptor-activated Ca^{2+} influx and channel activity [17].

It is unclear why the effect of Trp4 on store-operated Ca^{2+} influx or conductance could be observed in some systems but not in others. Perhaps the differences in the DNA constructs, the cellular environment of the expression systems and the experimental protocols used all contribute to the disparities. More importantly, the overexpression of Trp4 may affect the normal function and expression of a number of other proteins in the host cells, such as the up-regulation of $\text{Ins}(1,4,5)\text{P}_3$ receptor expression in cells expressing Trp3 [12]. In the present study we show that the constitutive and inducible expression of mTrp4 in HEK-293 cells leads to no significant increases in CCE but causes an enhancement of inwardly rectifying K^+ (Kir) currents. Some preliminary data from the present study have appeared in abstract form [22].

EXPERIMENTAL

Materials

Spermine, potassium gluconate, sodium gluconate, calcium gluconate and tetraethylammonium chloride were from Sigma. ³⁵S Express protein labelling mix (10 Ci/l) was purchased from

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; CCE, capacitative Ca^{2+} entry; $[\text{K}^+]_o$, extracellular K^+ concentration; KATP, ATP-sensitive K^+ channel; Kir, inwardly rectifying K^+ ; P_o , open probability; Trp, transient receptor potential; T4cAb, anti-Trp4 C-terminal antibody; T4nAb, anti-Trp4 N-terminal antibody; TG, thapsigargin; mTrp4, murine Trp4.

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Figure 1 Immunodetection of Trp4 in stable HEK cell lines

(A) Immunoprecipitation of [35 S]Trp4 from cell lysates prepared from *mtrp4* transfected G418-resistant clones using an anti-(Trp4 N-terminus) antibody, T4nAb. The names of the stable clones are indicated on the top. One clone (T4-17) did not express Trp4. A stable HEK cell line expressing murine Trp6 (T6-48) was used as a negative control. The filled arrow indicates the band for Trp4; the open arrow indicates a non-specific (NS) band present in all samples. (B) Dose-dependent induction of mTrp4 expression in an inducible cell line by ponasterone A. Equal amount of cells were seeded in wells of a six-well plate. Ponasterone A was added to give the indicated concentrations and cells were incubated at 37 °C for 20 h before [35 S]Met/Cys was added. [35 S]Trp4 was immunoprecipitated as in (A). (C) Immunoblotting of Trp4 in control (Cont) and T4-60 cells by the anti-(Trp4 C-terminus) (T4cAb, left) and the anti-(Trp4 N-terminus) (T4nAb, right) antibodies. (D) Immunolocalization of Trp4 in control (left) and T4-60 (right) cells by confocal microscopy using T4cAb.

NEN Life Sciences. Protein A-Sepharose (4 Fast Flow) was from Amersham Pharmacia Biotech. Oregon Green-conjugated goat anti-rabbit antibodies were from Molecular Probes. LIPOFECTAMINE[®] Plus and all tissue-culture reagents were purchased from Life Technologies. EcR 293 cells, ponasterone A and Zeocin were from Invitrogen. Rabbit polyclonal anti-Trp4 N-terminal peptide (Tyr⁵-Arg¹⁷) antibody (T4nAb) was produced by Quality Controlled Biochemicals (Hopkinton, MA, U.S.A.). Rabbit polyclonal anti-Trp4 C-terminal peptide antibody (T4cAb) was kindly provided by Dr. V. Flockerzi (Universität des Saarlandes, Hamburg, Germany).

cDNA, cell culture, transfection, and cell lines

Full-length cDNA for mTrp4 was isolated from mouse brain as described in [23] and was subcloned into the expression vectors

pcDNA3 and pIND (Invitrogen) for constitutive and inducible expression respectively.

Cell-culture conditions and the procedures for the establishment of stable cell lines constitutively expressing mTrp4 in HEK-293 cells were as described in [23]. The expression of mTrp4 in these cell lines was confirmed by immunoprecipitation following the previously described protocol [24] using T4nAb (Figure 1A). To produce stable cell lines for inducible expression of mTrp4, EcR 293 cells were used. These cells are transformed HEK-293 cells that express modified ecdysone receptor and retinoid X receptor required for inducible expression by ecdysone analogues, e.g. ponasterone A. The cells were maintained in Dulbecco's modified Eagle's medium containing 4.5 mg/ml glucose, 10% heat-inactivated fetal-bovine serum, 50 units/ml penicillin and 50 mg/ml streptomycin supplemented with 400 μ g/ml Zeocin. *mtrp4*/pIND (1 μ g) was transfected into 2×10^5 EcR 293 cells seeded in a 35-mm-diameter dish using LIPOFECTAMINE[™] Plus reagents following the manufacturer's protocol. Transfected cells were selected by 400 μ g/ml G418 in 96-well plates [23], and the expression of mTrp4 was examined by immunoprecipitation after induction by ponasterone A for 20 h. As shown in Figure 1(B), the expression of mTrp4 in one such cell line was dependent on the presence of ponasterone A in a dose-dependent manner, with the maximum induction reached at 5 μ M.

For transient expression, 1 μ g of *mtrp4*/pcDNA3 was co-transfected with 0.2 μ g of pEGFPN1 (Clontech) encoding an enhanced green fluorescent protein into 1×10^5 HEK-293 cells seeded on a 35-mm-diameter dish using LIPOFECTAMINE[™] Plus reagents. Control cells were transfected with pEGFPN1 only. On the second day, cells were harvested, divided into six portions, plated on 35-mm-diameter dishes, and allowed to grow for an additional 20–30 h. Cells expressing the green fluorescent protein were identified under a Nikon epifluorescence microscope at excitation of 480 nm and emission of 510 nm prior to patch-clamp recording.

Immunoblotting and immunocytochemistry

For immunoblotting, crude membrane preparations from untransfected (control) and the constitutive Trp4 cell line, T4-60, were subjected to SDS/PAGE (8% polyacrylamide) and transferred on to nitrocellulose membrane (Bio-Rad). Western blotting was performed as described in [23], and Trp4 was seen in the preparation from T4-60 by the anti-Trp4 N-terminal antibody, T4nAb (Figure 1C, right) and by the anti-Trp4 C-terminal antibody, T4cAb (Figure 1C, left).

For immunolocalization, formaldehyde-fixed cells were incubated with 1:1000 dilution of T4cAb [19], followed by 1:1000 dilution of Oregon Green-conjugated goat anti-rabbit antibodies. Immunofluorescence signals were examined using a Nikon microscope coupled to a confocal laser scanning unit (Bio-Rad MRC-1024) and were found, to a large extent, to be evenly localized to the periphery of the T4-60 cells (Figure 1D, right), consistent with Trp4 being a plasma-membrane protein. Under the same conditions, untransfected HEK-293 cells (Figure 1D, left) or a stable cell line expressing human Trp3 (not shown) showed no immunostaining.

Intracellular Ca^{2+} imaging

Intracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) were measured as described previously [6], with the exception that fura 2-loaded cells were examined on a Nikon Eclipse TE200 inverted micro-

scope, a PTI ImageMaster system was used for controlling the excitation wavelengths of 340 nm and 380 nm from a DeltaRAM monochromator, and emitted fluorescence at 510 nm was recorded by an intensified charge coupled device ('CCD') camera (Photon Technology International). The ratio between the emitted fluorescence intensities from 340 nm and 380 nm excitation for individual cells was calculated after experiments using the ImageMaster software. Averages of ratios for cells from 8 to 24 experiments were made using Microsoft Excel.

Electrophysiological studies

Whole-cell recordings

Recording pipettes were pulled from micropipette glass (Drummond Scientific Co., Broomali, PA, U.S.A.) to a resistance of 2–4 M Ω , filled with a pipette solution typically containing, except where indicated otherwise, (in mM): 140 CsCl, 2 MgCl₂, 10 Hepes, 10 EGTA, 0.3 MgATP₂, and 0.03 GTP, pH 7.20. The normal bath solution contained (in mM): 126 NaCl, 10 CaCl₂, 10 glucose, and 15 Hepes, pH 7.40. Isolated HEK cells were voltage-clamped in the whole-cell mode using an Axopatch 200B Amplifier (Axon Instruments, Foster City, CA, U.S.A.). Once the whole-cell configuration was obtained, the recording solution containing (in mM) 140 potassium gluconate/sodium gluconate (either all K⁺ or all Na⁺, or a mixture of K⁺ and Na⁺ with a final gluconate concentration of 140), 10 glucose, and 15 Hepes, pH 7.40, was applied through fast bath perfusion. Voltage commands were made from the Clampex program of the pClamp 6.0.4 software and currents were recorded into a Pentium II-based computer through a Digidata 1200 PC interface. A voltage ramp of 210 ms to +80 mV after a brief (25 ms) step to –100 mV from holding potential at 0 mV was applied every 2 s. In some experiments, voltage pulses of 384 ms from –100 mV to 80 mV in 15 mV increments from a holding of 0 mV were also used. All experiments were performed at room temperature. The level of K⁺ selective current was determined by subtracting the current recorded in 140 mM sodium gluconate from that recorded in 140 mM potassium gluconate. Data analysis was performed using Clampfit software (Axon Instruments).

Single-channel recordings

The pipettes were pulled from type 7052 glass (Garner Glass Co., Claremont, CA, U.S.A.) to a resistance of 9–11 M Ω and filled with a solution containing (in mM) either 140 sodium gluconate or 140 potassium gluconate, 5 NaCl, 2 CaCl₂ and 10 Hepes. Cells were bathed in normal Ringer's solution containing (in mM): 140 NaCl, 10 KCl, 1 MgCl₂, 2 CaCl₂, and 10 Hepes. Patches were excised to an internal solution containing (in mM) 140 potassium gluconate, 10 Hepes, 2 EGTA and 5 NaCl. MgCl₂ or spermine was added to the potassium gluconate internal solution from the concentrated stock and applied through perfusion as needed. The pH was adjusted to 7.40 for all solutions. Currents were recorded at 5 kHz, filtered at 1 kHz for analysis using pClamp 6.0.4 (Axon Instruments). Event lists were generated by Fetchan and used for analysis by the pSTAT program. Open probabilities (P_o) were determined using an interval of 0.5 s and averaged for a given drug treatment.

RESULTS

Expression of mTrp4 in HEK-293 cells leads to no apparent increase in CCE

In order to study its function, we produced stable HEK-293 cell lines expressing mTrp4 in constitutive and inducible ways

(Figure 1). To test whether overexpression of Trp4 enhances CCE activity, we measured changes of [Ca²⁺]_i by fluorescence video imaging in fura 2-loaded cells using the Ca²⁺ free/Ca²⁺ re-addition protocol described previously [6,24]. Although we consistently detected significant increases in agonist-stimulated Ca²⁺ influx in cells expressing human Trp3, we were not able to detect such an increase in stably and transiently transfected cells expressing mTrp4 (results not shown). Cells were also treated with 0.5 μ M TG in a nominally Ca²⁺-free solution, and 1.8 mM Ca²⁺ was added later to reveal store-operated Ca²⁺ influx. However, no change was found in *mtrp4*-transfected cells as compared with the control untransfected cells (results not shown). Thus it is unlikely that expression of mTrp4 in HEK-293 cells is associated with an increase in CCE.

Expression of mTrp4 in HEK-293 cells leads to an increase in Kir whole-cell currents

To test whether mTrp4 expression enhances store-operated cation conductance, we performed whole-cell voltage clamp experiments for the Trp4 cell line, T4-60. Some spontaneous endogenous currents occurred in both control and Trp4 cells and they were prevented by using gluconate instead of Cl[–] as the main anion in the bath and using Cs⁺ instead of K⁺ as the main cation in the pipette solution. Under basal conditions and in an external solution containing 140 mM Na⁺, currents were very small at all potentials, as revealed by a voltage-ramp protocol. However, when the Na⁺ was replaced by K⁺, a significantly larger inward current developed in the Trp4 cells than in control cells at nearly all negative potentials (Figure 2A). The currents were inwardly rectifying and did not inactivate within a period of 384 ms, as shown by a step-pulse protocol (Figure 2B). The K⁺ specific current was readily observed when as little as 5 mM K⁺ was present in the bath solution (Figure 2C). The reversal potentials under different extracellular K⁺ concentrations ([K⁺]_o) were determined using an internal solution containing 140 mM KCl. The results agreed well with the prediction by the Nernst equation taking K⁺ as the sole charge carrier (Figure 2D). Also, the current amplitude was dependent on [K⁺]_o, following a relationship that $g/g_{\max}$ (the fraction of maximal conductance) is proportional to the square root of [K⁺]_o (Figure 2E). This relationship has been documented for native and cloned Kir channels [25,26]. Therefore, the expression of Trp4 has led to an increased Kir channel activity in HEK-293 cells.

In order to rule out the possibility that the increase in Kir activity was unique to T4-60 cells, we measured the K⁺-selective currents in four other cell lines that constitutively expressed mTrp4 and an inducible cell line after the expression of mTrp4 was induced by 2 μ M ponasterone A for 24–48 h. About 2–4-fold significant increase in K⁺ conductance at –80 mV was found in all cell lines (Table 1). For the inducible cell line, ponasterone A treatment caused a 2-fold increase in the K⁺ current at –80 mV as compared with the uninduced cells, which had the same low level of Kir activity as the untransfected control cells. The K⁺-selective currents were also measured in cells transiently expressing mTrp4 and the enhanced green fluorescence protein. These cells also showed a 2-fold increase of Kir activity over control cells (Table 1). Therefore, the increased Kir activity appears to result from the expression of mTrp4, but not a random selection of cell lines.

In contrast with the Trp4-expressing cells, cells expressing Trp1, Trp3 or Trp6 did not show significant increase in K⁺-selective currents (Table 1), indicating that the enhancement of Kir activity is not common to the expression of Trps but rather is unique to that of Trp4.

Figure 2 Inwardly rectifying whole-cell K^+ currents in T4-60 cells

(A) A voltage ramp to +80 mV after a brief (25 ms) step to -100 mV from holding potential at 0 mV revealed inwardly rectifying currents in T4-60 cells (upper) only in 140 mM potassium gluconate, but not sodium gluconate, bath solution. Non-transfected HEK-293 cells (control) had much smaller K^+ currents (lower). (B) Series of voltage steps (-100 mV to 80 mV in 15 mV increments) from 0 mV showed no current inactivation during the 384 ms pulses. (C) Effect of $[K^+]_o$ on current sizes and reversal potentials of the inwardly rectifying currents in T4-60 cells. 140 mM KCl was used in the pipette solution in place of CsCl, which was used in all other measurements. $[K^+]_o$ used are from the top to the bottom trace 0, 5, 10, 20, 40, 80, 120 and 140 mM. The solutions were prepared by substituting potassium gluconate with equal concentrations of sodium gluconate and were applied through perfusion. Recordings were made as in (A). (D) Reversal potential versus $\log [K^+]_o$. Currents recorded in 140 mM sodium gluconate were subtracted from all traces, and zero current potential was determined. Data are averages $\pm$ range of results obtained from two cells. The continuous line shows the prediction by Nernst equation:

$$E_K = 2.303 \cdot (RT/F) \cdot \log ([K^+]_o/[K^+]_i)$$

where R ($= 8.315 \text{ J K}^{-1} \cdot \text{mol}^{-1}$) is the gas constant, T ($= 295.16 \text{ K}$) is the room temperature, F ($= 96480 \text{ C} \cdot \text{mol}^{-1}$) is the Faraday's constant and E_K is the effect of $[K^+]_o$ on the conductance. $g/g_{\max}$ was calculated by dividing the amplitude of K^+ current at -80 mV in a given $[K^+]_o$ by that in 140 mM $[K^+]_o$. The plot shows that the conductance is approximately linear to the square root of $[K^+]_o$. Results are averages $\pm$ S.E.M. of results obtained from 10–12 cells. Points were fitted by linear regression.

Further study of the Kir currents in T4-60 cells showed that the currents were relatively insensitive to low millimolar concentration of Ca^{2+} and tetraethylammonium, but highly sensitive to extracellular application of low concentrations of Cs^+ and Ba^{2+} (Figure 3 and Table 2), typical features of most known Kir channels [27–29]. The size of the current was not affected by the

holding potential at 60, 0 or -60 mV (not shown), indicating that its activation is not voltage-dependent.

Unlike any store-operated current or any current known to be mediated by Trp channels, the Kir currents found in *mtrp4* transfected cells did not require $\text{Ins}(1,4,5)\text{P}_3$ in the pipette. Furthermore, the Trp4 cells displayed similar size of the Kir

Table 1 Summary of inward K⁺-selective currents at -80 mV in different HEK-293 cell lines

Whole-cell recordings were made in a bath solution containing 140 mM potassium gluconate as in Figure 2(A). T1, T3, T4 and T6 are stable cell lines expressing human Trp1, human Trp3, murine Trp4 and murine Trp6 respectively. Amplitudes for the K⁺-selective currents at -80 mV were determined by subtracting the currents recorded in 140 mM Na⁺ from those recorded in 140 mM K⁺. For the inducible Trp4 cell line, cells were treated with either carrier only (ethanol) or 2 μ M ponasterone A for 24–48 h. Results are averages $\pm$ S.E.M. for numbers of cells as indicated. * $P < 0.0001$ different from control (transiently transfected or a stable cell line expressing a vasopressin receptor [24]); ** $P < 0.005$ different from the uninduced as determined by Student's t test.

Cell name	K ⁺ current at -80 mV (nA)	Number of cells
Control	-0.13 ± 0.02	45
<i>mtrp4</i> transiently transfected	$-0.41 \pm 0.03^*$	19
Constitutive cell lines		
T1-28	-0.10 ± 0.02	4
T3-9	-0.11 ± 0.03	5
T4-1	$-0.48 \pm 0.10^*$	14
T4-3	$-0.41 \pm 0.11^*$	7
T4-20	$-0.49 \pm 0.10^*$	7
T4-60	$-0.59 \pm 0.04^*$	94
T4-82	$-0.48 \pm 0.13^*$	7
T6-48	-0.15 ± 0.03	20
T6-55	-0.15 ± 0.04	10
Inducible Trp4 cell line		
Uninduced	-0.14 ± 0.04	9
Induced	$-0.42 \pm 0.07^{**}$	9

Table 2 Inhibition of Kir current at -80 mV in T4-60 cells by bath addition of Ca²⁺, Ba²⁺, tetraethylammonium (TEA⁺) and Cs⁺

Conditions were the same as in Figure 3. Results are averages $\pm$ S.E.M. for the numbers of cells indicated. ** $P < 0.0005$; * $P < 0.0001$ different from 100% as determined by Student's t test.

Cation	Concn. (mM)	Inhibition (%)	Number of cells
Ca ²⁺	1.2	5 ± 2	7
Ba ²⁺	0.5	$93 \pm 2^*$	7
TEA ⁺	5.0	$26 \pm 4^{**}$	7
Cs ⁺	0.2	$89 \pm 2^*$	6
Cs ⁺	1.0	$93 \pm 2^*$	6

Figure 3 Sensitivity of Kir current in T4-60 cells to bath addition of Ca²⁺, Ba²⁺, tetraethylammonium (TEA) and Cs⁺

Whole-cell recordings were made in a bath solution containing 140 mM potassium gluconate as in Figure 2(A). Inhibitory ions were applied through fast perfusion of the same solution containing ions (in mM) as indicated. The activity was allowed to recover after wash-out and before the application of the next inhibitory ion. Shown are representative traces of ionic inhibition from a typical cell. Currents recorded in 140 mM sodium gluconate were subtracted.

currents in several different pipette solutions, whether or not they contained ATP, GTP, EGTA or Mg²⁺ (results not shown).

Single-channel analysis of Kir activity in Trp4 cells

The presence of the Kir in Trp4 cells was further demonstrated in single-channel recordings of inside-out patches (Figure 4).

With transmembrane potential held at $+60$ mV, current activities were seen upon excision of inside-out membrane patches to an intracellular solution containing potassium. At least two conductance levels were observed for T4-60 cells. Although some patches displayed mostly single conductance as shown in Figure 4(A) for a patch having the higher amplitude and in Figure 4(D), trace i, for another patch having the lower amplitude at $+60$ mV, many cells showed both conducting states in the same patch (Figure 4D, trace ii). An all-point amplitude histogram analysis also indicated the presence of two conductance levels (Figure 4B). Dwell-time analysis showed that the higher conductance state had a mean open time of 3.3 ms (Figure 4C). The K⁺ selectivity of the single channel was demonstrated by the observation that when Na⁺ was used in the pipette, only outward currents were recorded at all membrane potentials (Figure 4E, left), whereas when K⁺ was used in the pipette, both inward and outward currents were observed (Figure 4E, right). Amplitude analyses at different holding potentials for patches placed in symmetrical K⁺ solutions revealed linear current–voltage relationships and slope conductance of 51 and 94 pS for the lower and the higher conducting states respectively. Therefore the channel may have a subconducting state or there may be at least two different channels. Under the same conditions, we have observed single-channel currents in two out of more than 25 patches excised from untransfected control cells. Because of the scarcity of the observation, it is difficult to determine whether the channels are the same as those seen in Trp4 cells. However, if they were the same, then the activity we saw in Trp4 cells would come from the up-regulation of endogenous Kir channels.

The linear current–voltage relationship of the Kir activities seen in inside-out patches of the Trp4 cells is in agreement with the behaviour of a typical Kir channel in this configuration. The presence of Mg²⁺ and polyamines at the cytosolic side of the plasma membrane is mainly responsible for the inwardly rectifying behaviour of Kir in whole-cell configuration [30,31]. We therefore tested the effect of Mg²⁺ and spermine on the outward single-channel current in inside-out patches. Excised patches were held at $+60$ mV and a potassium gluconate solution containing either 1 mM Mg²⁺ or 0.1 or 1 mM spermine was applied to the cytoplasmic side of the membrane through perfusion. In all cases, we did not observe any decrease in the P_o of the channel by Mg²⁺ (0.39 ± 0.13 and 0.56 ± 0.11 for P_o before and after the application of Mg²⁺ respectively. Average $\pm$ S.E.M., $n = 4$). In three out of the four patches, there was a small increase in P_o by Mg²⁺ (Figure 5A). On the other hand, while 0.1 mM spermine caused a small decrease in P_o , application of 1 mM spermine abolished the outward current (Figure 5B; the inhibition of P_o was $98.4 \pm 0.7\%$ for 1 mM spermine, $n = 4$).

Figure 4 Single-channel Kir currents in inside-out patches excised from T4-60 cells

(A) Representative traces of current at +60 mV in symmetrical K⁺ solutions of a patch having predominantly the higher conducting state. (B) An all-point amplitude histogram analysis for the same patch shown in (A) recorded at +60 mV for 20 s. Broken lines show the least-square fits of Gaussian function for zero and two open levels using the Simplex method. (C) Dwell-time analysis of open (upper) and closed (lower) states for the same record as in (B). Continuous curves are single exponential fits by the Simplex method. (D) Representative traces of currents at +60 mV of a patch having predominantly the lower conducting state (trace i) and another patch having both the higher and the lower conducting states (trace ii). (E) Representative traces of single-channel activity at different holding potentials recorded in Na⁺ outside and K⁺ inside configuration (left) or in symmetrical K⁺ solutions (A). Broken lines indicate the closed state. (F) Single-channel current–voltage relationship. Results are averages $\pm$ S.E.M. for three to six patches. Points were fitted by linear regression.

DISCUSSION

In an effort to study the electrophysiological properties of heterologously expressed Trp channels, we accidentally found that there is a significant increase in Kir current in all Trp4-

expressing cells. The K⁺ activities are typical features of Kir channels, including a high sensitivity to extracellular application of Cs⁺ and Ba²⁺ and to the intracellular block by polyamines. Because it is insensitive to intracellular application of 1 mM

Figure 5 Effect of Mg²⁺ (A) and spermine (B) on the outward current in inside-out patches of T4-60 cells in symmetrical 140 mM K⁺

Patches were held at +60 mV. MgCl₂ or spermine was added to the potassium gluconate internal solution and perfused to the cytoplasmic side of the inside-out patch as indicated. Upper traces show the single channel activities of the same patch before and after the addition of Mg²⁺ or spermine. Gaps indicate solution changes, which were less than 15 s. Sections marked (i), (ii) and (iii) are expanded and shown below. Broken lines indicate the closed level.

Mg²⁺ and only blocked by high concentrations of spermine, the Kir channel(s) seems to belong to the group of weakly rectifying Kir channels [32–34]. The single-channel conductance of this channel appears to be higher than that of channels formed by Kir1, Kir2 and Kir3 subfamilies [35–37], but is within the range of that of ATP-sensitive K⁺ channels (KATP) [38,39].

It remains controversial whether Trp4 is involved in CCE. While two groups have demonstrated TG-stimulated Trp4 activity [8,18,20], another group showed that the channel only responded to receptor agonist but not store-depletion by TG [21]. Using Trp4-specific antibodies, we showed that mTrp4 was expressed and was localized at or near plasma membrane of the transfected HEK 293 cells. However, under the same conditions that Trp3-mediated Ca²⁺ influx was clearly revealed, we failed to detect any increase in agonist- or TG-stimulated Ca²⁺ influx. The difficulty of seeing Trp4 function in CCE was also evident in a recent study, where agonist- and TG-stimulated Ba²⁺ influx was not increased in Trp4-expressing cells [17]. Therefore, either Trp4 cannot form a Ca²⁺ influx channel by itself or its overexpression changes the homeostasis of the transfected cells and thus alters the expression and activity of other proteins, some of

which may undermine its effect on CCE. The increased Kir activity may be an example of the latter possibility. It is important to note that Kir activities are not readily noticeable in whole-cell recordings using standard external solutions that contain low or no K⁺. This may be why in previous studies, the effect on Kir activity was not noticed for bovine, human, and rat Trp4 [8,17,18,20]. Moreover, the Kir may not be the only channel affected by Trp4 overexpression. Since the effect of Trp4 on Kir current was only revealed after changing the [K⁺]_o, it is conceivable that under other specific conditions the effect of Trp4 on additional channels may be discovered.

The reason for the increased Kir activity in Trp4-expressing cells is not known. The Trp channels are structurally different from the Kir channels in that each Trp has six transmembrane segments, whereas a Kir subunit has only two, which together with an intervening P-domain form the conducting pore [32–34]. The putative pore region of Trp4 lacks the Gly-Tyr-Gly (or Gly-Phe-Gly as in the case of KATP channels) motif typically required for K⁺ selectivity [34]. Therefore it is unlikely that Trp4 would form a Kir by itself. However, Trp4 might recruit and interact with endogenous Kir subunits in the HEK cells to form

a novel channel, in a manner similar to the octameric structure of KATP, which is made of four Kir (Kir6.1 or Kir6.2) subunits and four sulphonylurea receptor subunits [38,39]. The possibility that Trp4 and Kir6.x co-assemble exists because the cystic-fibrosis transmembrane conductance regulator, which forms a Cl^- channel by itself, could form a Kir with Kir6.1 [40]. Secondly, overexpression of Trp4 could have adverse effects on cellular function, and thus Kir activity may be up-regulated to overcome these effects. The increased basal influx of Na^+ [17] may lead to membrane depolarization, which Kir can correct, and hence stabilize the resting potential of the transfected cells. However, the intrinsic Ca^{2+} and Na^+ influx cannot account solely for the increase in Kir activity, because the increase was not seen in cells expressing Trp1, Trp3 and Trp6, which are partially active under basal conditions [11,24,41,42]. Therefore functions unique to Trp4 must be involved. Thirdly, it could be expected that, during the stabilization process when multiple passages were engaged to allow the selection of transformed cells, the selection pressure could favour the growth of cells with initially higher Kir activity when Trp4 was overexpressed. However, since the Kir activity was readily seen in transiently transfected cells and in induced cells that had expressed Trp4 for less than 48 h, it appears that the enhanced Kir activity is a direct response to Trp4 expression but not a result of selection of cell lines. In transiently transfected CHO cells we also observed 1.5-fold increase in K^+ -selective currents in cells expressing mTrp4 (Z. Zhang and M. X. Zhu, unpublished work). Therefore the enhancement of Kir activity due to Trp4 expression is not limited to HEK-293 cells. Lastly, the transcription and/or translation of the Kir, or its regulatory subunit or activator, may be controlled by a factor that is part of Trp4-mediated signalling. Thus the activity of Trp4 may increase the expression of the Kir or the expression of other proteins that modulate the activity of the Kir.

In summary, we have demonstrated for the first time that the expression of mTrp4 in a mammalian cell line leads to increased Kir activity. This unexpected finding adds a new dimension to the studies on cellular responses modulated by Trp homologues. In addition to mediating Ca^{2+} influx, the non-selective cation channels formed by some members of the Trp family also mediate a substantial amount of Na^+ influx, and thereby initiate membrane depolarization in response to the activation of phospholipase C. By enhancing the activity of Kir, Trp4 helps to stabilize the membrane potential, opposing the depolarizing effect of other Trp proteins, but on the other hand prolonging the duration of an action potential if sufficient depolarizing force is exerted. Whether or not Trp4 itself participates in the formation of Kir channel(s) or how it affects the formation of such channels, as well as the physiological significance for the correlation between Trp4 and Kir, remain to be elucidated.

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