Cell

Does Sumoylation Control K2P1/TWIK1 Background K^+ Channels?

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DOI 10.1016/j.cell.2007.06.012

SUMMARY

A novel model for the regulation of cell excitability has recently been proposed. It originates from the observation that the background K^+ channel K2P1 (TWIK1) may be silenced by sumoylation in *Xenopus* oocytes and that inactivation of the putative sumoylation site (mutation K274E) gives rise to robust current expression in transfected COS-7 cells. Here, we show that only the mutation K274E, and not K274R, is associated with an increase of K2P1 current density, suggesting a charge effect of K274E. Furthermore, we failed to observe any band shift by western blot analysis that would confirm an eventual sumoylation of K2P1 in COS-7 cells and oocytes.

INTRODUCTION

K2P1 (or TWIK1) has been cloned from a human kidney cDNA library (Lesage et al., 1996a). Sequence analysis predicted a unique membrane topology with four transmembrane segments and two pore-forming domains (Lesage et al., 1996a). Following the identification of K2P1, homologous K2P proteins were rapidly isolated from *Drosophila* and mammals (Goldstein et al., 2005; Kim, 2005; Lesage and Lazdunski, 2000; Patel and Honore, 2001; Talley et al., 2003). When compared to other K2P channels, K2P1 displays a couple of unique features. Like its closest homolog TWIK2 (K2P6) (Patel et al., 2000), K2P1 produces currents with a rapidly inactivating component. Because of this inactivation, their steady-state current voltage relationships are much more similar to that of the weak inwardly rectifying ROMK1 current (Lesage et al., 1996a) than those of the other K2P currents that follow the Goldman Hodgkin Katz equation (Duprat et al., 1997; Fink et al., 1996). Another unique feature of K2P1 is the difficulty to record currents from transfected cells and the fact that no native currents corresponding to K2P1 have yet been reported. However, mice deficient for K2P1 have impaired regulation of phosphate transport in the proximal tubule and of water transport in the medullary collecting duct, strongly suggesting that K2P1 is functional and contributes to membrane trafficking/expression of transport molecules in the kidney (Nie et al., 2005). We have shown that K2P1 is mainly localized in the recycling endosomal compartment located at the apical side of transfected kidney cells and native proximal tubule cells (Decressac et al., 2004). In a variety of nonpolarized cells, K2P1 immunoreactivity was detected almost exclusively in the pericentriolar recycling compartment (Decressac et al., 2004). The mechanism that controls surface expression/retrieving of K2P1 is not yet characterized but may be under the dependency of the small G protein ARF6 and its nucleotide exchange factor EFA6 that interacts with K2P1 (Decressac et al., 2004).

Recently, it has been suggested that K2P1 is addressed to the cell surface when expressed in *Xenopus* oocytes and that addition of a small ubiquitin modifier (SUMO) peptide to lysine 274 (K274) is responsible for a block of channel activity (Rajan et al., 2005). From these results, the authors of the study proposed that K2P1 is a plasma membrane channel and that its silencing by sumoylation is the major mechanism explaining the loss of active channel expression in transfected and native cells. This work has gained considerable interest not only because it identifies a novel mechanism of ion channel regulation but also for its general implication in cell biology (Wilson and Rosas-Acosta, 2005). Sumoylation is the posttranscriptional modification of lysine residues in target proteins by covalent attachment of a SUMO peptide moiety at the consensus site ψKxE/D (where ψ is a hydrophobic residue and x is any amino acid) (reviewed in Dohmen, 2004). It is primarily a nucleocytoplasmic phenomenon that mediates protein-protein interactions, nucleocytoplasmic trafficking, and activity of transcription factors, but sumoylation is also known to take place in the cytoplasm. Regulation of membrane excitability by sumoylation of background K^+ channels active at rest would provide a novel level of crosstalk between membrane signaling and some nuclear events through coordination of sumoylated

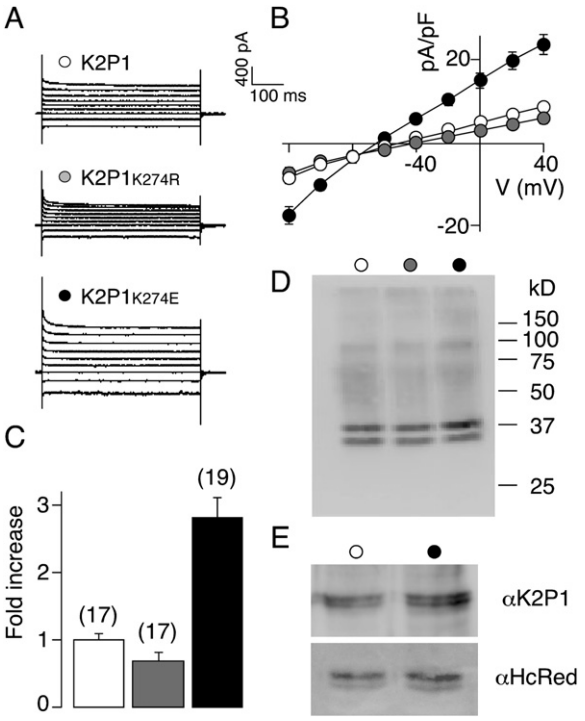

Figure 1. Electrophysiological and Biochemical Characterization of K2P1 and Mutant K2P1K274R and K2P1K274E Channels in COS-7 Cells

(A) Whole-cell current traces from COS-7 cells expressing K2P1, K2P1K274R, and K2P1K274E channels fused to HcRed. Currents were elicited by voltage pulses ranging from −120 mV to 40 mV in 20 mV steps, from a holding potential of −80 mV.

(B) Current density was determined at all test potentials from the steady-state current and whole-cell capacitance. Each value represents the mean ± SEM, n cells for K2P1 (n = 17), K2P1K274R (n = 17), and K2P1K274E (n = 19).

(C) Current density variation for K2P1K274R and K2P1K274E was normalized to the K2P1 value obtained at +40 mV test potential. Values are mean ± SEM.

(D) Western blot analysis of K2P1, K2P1K274R, and K2P1K274E.

(E) Western blot analysis of HcRedK2P1 and HcRedK2P1K274E using anti-K2P1 antibody (as in D) or anti-HcRed antibody. Analysis was carried out as described in Rajan et al. (2005) (lysis buffer containing NEM, an inhibitor of SUMO isopeptidases).

states. However, several aspects of K2P1 sumoylation are highly intriguing. First, the K274 residue in K2P1 does not belong to a classical consensus site for sumoylation ($LK^{274}KF$). Second, if K2P1 is a silenced plasma membrane channel, then it must be kept almost exclusively sumoylated in many different cell types that do not exhibit a robust current following K2P1 expression. This situation has no other example to our knowledge.

Here, we demonstrate that K2P1 is not quantitatively sumoylated in COS-7 cells and *Xenopus* oocytes. In the same expression conditions, the K274E mutation is associated with an increase of K2P1 current density as previously described (Rajan et al., 2005). However, this increase is not observed with the conservative K274R mutation. Taken together these data demonstrate that the increase of current associated with K274E, and absent in K274R, can probably be attributed to a charge effect and that SUMO modification at lysine 274 is not the proposed mechanism for K2P1 silencing.

RESULTS

Expression of K2P1 in COS-7 Cells

In COS-7 cells, K2P1 does not produce macroscopic currents in the majority of the tested cells (not shown). It has been reported that alteration of lysine 274 to glutamate (K274E) leads to functional expression of K2P1 (Rajan et al., 2005). However, we failed to observe any significant current upon expression of K2P1K274E in our batch of COS-7 cells (not shown). We have previously shown that the fusion of the *Heteractis crispa* red (HcRed) fluorescent protein to the amino terminus of K2P1 is associated with a partial expression of the resulting fusion protein at the cell surface of proliferating MDCK cells. In these particular conditions, it was possible to measure K2P1 currents at the plasma membrane (Decressac et al., 2004). The same effect was seen in COS-7 cells where HcRedK2P1 and HcRedK2P1K274E reach the cell surface (not shown) and produce macroscopic currents (Figure 1A). An HcRedK2P1K274E fusion protein produced 2.9 times more current than HcRedK2P1 (Figures 1B and 1C). The current displayed a very fast inactivation component that was previously observed for K2P1 expression in oocytes (Lesage et al., 1996a) and thoroughly characterized for the closely related channel TWIK2 (Patel et al., 2000). The kinetics of K2P1 inactivation was extremely fast and overlapped the membrane capacitive discharge associated with the voltage pulse. However, the fast inactivating peak current was clearly not a stimulation artifact and constitutes a hallmark of the TWIK currents.

We tested another mutant of K2P1, HcRedK2P1K274R, in which lysine 274 is replaced by an arginine residue. This mutation is more conservative than K274E because the positive charge at position 274 is conserved and not substituted by a negative charge. Surprisingly, HcRedK2P1K274R produced almost the same level of current as HcRedK2P1 (Figures 1A–1C). The mutation K274R, unlike K274E, was not associated with an increase of the HcRedK2P1 current. Even though both substitutions are expected to equally prevent the sumoylation of K2P1, these results demonstrate that altering lysine 274 by glutamate or arginine does not have the same effect. Figure 1D shows that K2P1, K2P1K274E, and K2P1K274R transiently expressed in COS-7 cells ran at the same apparent molecular weight (MW) when analyzed by western blot. The 20 kDa shift expected for a protein being covalently bound to a SUMO moiety is not observed for K2P1 (SUMO is only 11 kDa, but it migrates aberrantly at around 20 kDa, even in free form). The apparent MW of the upper band (37–38 kDa) corresponds to the calculated MW of K2P1 and fits the MW of K2P1 previously

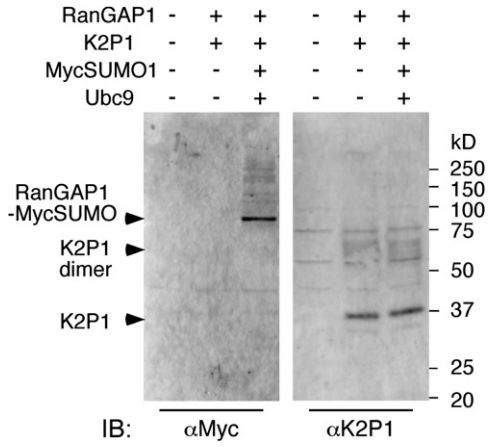

Figure 2. RanGAP1, K2P1, Myc-SUMO1, and Ubc9 Coexpression in COS-7 Cells

Cells were transiently transfected with the indicated plasmids. After straight cell lysis in a denaturating SDS buffer, total proteins were immediately separated by SDS-PAGE and analyzed by western blot. Blots were probed with anti-Myc or anti-K2P1 antibodies as indicated.

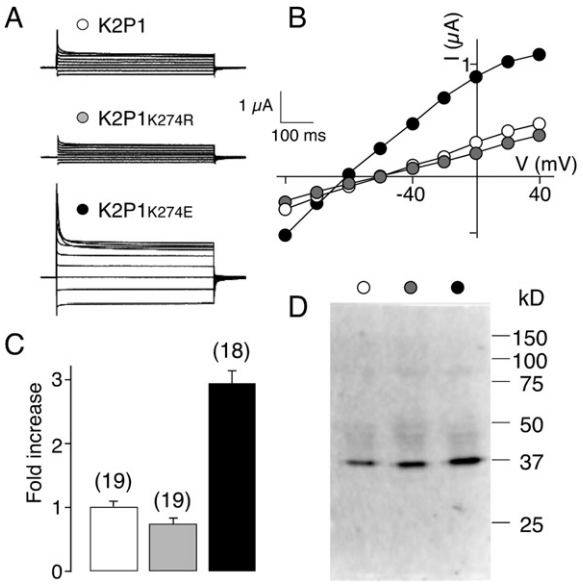

Figure 3. Electrophysiological and Biochemical Characterization of K2P1, K2P1K274R, and K2P1K274E Channels in *Xenopus* Oocytes

(A) Current traces recorded during voltage pulses ranging from −120 mV to 40 mV in 20 mV steps from a holding potential of −80 mV.

(B) Current-voltage relationships deduced from steady-state currents recorded as in (A). Each value represents the mean ± SEM, n cells for K2P1 (n = 19), K2P1K274R (n = 19), and K2P1K274E (n = 18).

(C) Current density variation for K2P1K274R and K2P1K274E was normalized to K2P1 value at 0 mV test potential. Values are mean ± SEM.

(D) Western blot analysis of K2P1, K2P1K274R, and K2P1K274E.

characterized in transfected cells and in native tissues (37–40 kDa) (Lesage et al., 1996b, 1997). The band of lower MW probably corresponds to an immature or degraded form of K2P1 produced by the high levels of overexpression achieved in COS-7 cells. To rule out the possibility that anti-K2P1 antibodies might not bind to a sumoylated form of K2P1, the HcRed-tagged K2P1 and K2P1K274E were detected with either anti-K2P1 or anti-HcRed antibodies. Clearly, no additional band was revealed by anti-HcRed antibodies (Figure 1E). As for the nontagged K2P1 (Figure 1D), two bands were detected for the HcRed-fused proteins that were identical no matter what antibody was used. Their MWs of around 60–64 kDa are compatible with the addition of the 26 kDa HcRed polypeptide to K2P1.

In these experiments, analyzed proteins were first solubilized in a buffer containing a detergent and a SUMO isopeptidase inhibitor (NEM) as described in Rajan et al. (2005). However, and because SUMO isopeptidases are difficult to inhibit (even in the presence of NEM), we conducted a control experiment based on straight SDS lysis of total proteins, immediately followed by western blot analysis. COS-7 cells were transfected with K2P1 and the control Ran-GTPase-activating protein RanGAP1 (Matunis et al., 1996), with or without Myc-SUMO1 and the SUMO ligase Ubc9. In the presence of Ubc9 and myc-SUMO1, a myc-positive band corresponding to the sumoylated form of RanGAP1 was clearly detected as well as other endogenous proteins (Figure 2, left panel). In exactly the same experimental conditions, neither myc-labeling (Figure 2, left panel) nor band shift (Figure 2, right panel) were observed for K2P1, confirming the absence of quantitative K2P1 sumoylation in COS-7 cells.

Expression of K2P1 in *Xenopus* Oocytes

K2P1 sumoylation has been originally characterized in *Xenopus* oocytes (Rajan et al., 2005). In this cell expression system, both biochemical and electrophysiological evidence support K2P1 silencing by sumoylation at lysine 274. These data are in contradiction with the COS-7 cell results presented above. Therefore, the two K2P1 channel mutants were compared to wild-type K2P1 after expression in oocytes. As previously reported (Lesage et al., 1996b), expression of wild-type K2P1 yielded in a modest but detectable current with its characteristic fast inactivating component (Figure 3A). The replacement of lysine 274 by a glutamate resulted in a 2.9-fold increase of the K2P1 current, a value almost identical to that found in COS-7 cells (Figures 3B and 3C). Furthermore, the conservative charge change of lysine 274 by an arginine residue had no stimulatory effect at all (Figures 3B and 3C). Membrane proteins from injected oocytes were also analyzed by western blot to detect an eventual shift of the K2P1 signal due to the addition of a SUMO moiety. Both K2P1K274E and K2P1K274R, which lack the sumoylation acceptor site, and the wild-type K2P1 had the same mobility at the expected MW of 37 KD (Figure 3D). This result clearly ruled out an extensive sumoylation process of K2P1 in oocytes. Taken together, the oocytes data are in agreement

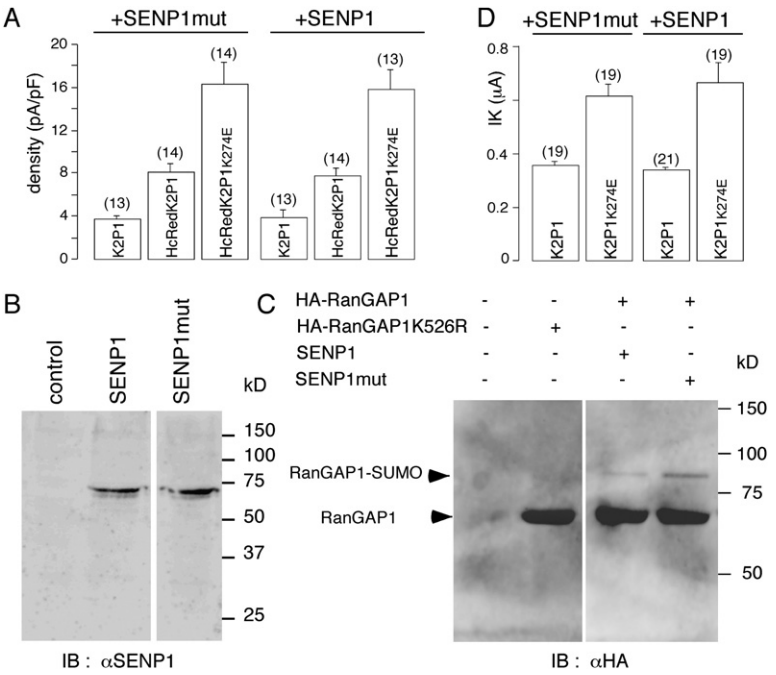

Figure 4. No Effect of SENP1 Protease Coexpression on K2P1 Current Expression and Apparent MW

(A) COS-7 cells were transfected with pIRESCd8K2P1, pHcRedK2P1, or pHcRedK2P1K274E together with pIRESGFPSenp1 or pIRESGFPSenp1 mut. Cotransfected cells were visualized with anti-Cd8 beads or by red fluorescence and by green fluorescence. Current densities were measured at +40 mV. Values are mean ± SEM.

(B) Western blot analysis of SENP1 and SENP1 mut expression in COS-7 cells transfected with pIRESGFPSenp1 and pIRESGFPSenp1 mut.

(C) Western blot analysis of HaRanGAP1 transfected in COS-7 cells with pIRESGFPSenp1 or pIRESGFPSenp1 mut. The mutant HaRanGAP1K526R cannot be sumoylated. The two different forms of RanGAP1 are indicated.

(D) Channel and protease coexpression in *Xenopus* oocytes. Error bars indicate SEM.

with the COS-7 data and do not support K2P1 silencing by addition of a SUMO moiety at lysine 274.

Coexpression of SENP1 and K2P1 in COS-7 Cells and *Xenopus* Oocytes

If sumoylation silences K2P1, SUMO deconjugation is expected to activate the channel. It has been suggested than in heterologous expression systems, the SUMO protease SENP1 was able to activate K2P1 (Rajan et al., 2005). The sequences encoding human SENP1 and its catalytically inactive mutant SENP1 mut (Cheng et al., 2004) were transferred into pIRES-EGFP. From these plasmids, a single transcript codes for both SENP1 (or SENP1 mut) and the green fluorescent protein (GFP). The expression of SENP1 and SENP1 mut was verified by western blot (Figure 4B). As expected, SENP1 induced desumoylation of the control protein RanGAP1 (Figure 4C). In the presence of active SENP1, the percentage of sumoylated RanGAP1 is significantly lower than in the presence of SENP1 mut (2.8 ± 0.9-fold, n = 3). Whole-cell currents were recorded in COS-7 cells expressing simultaneously SENP1 or SENP1 mut (green fluorescent cells) and K2P1 (cells decorated with anti-Cd8 beads) or HcRedK2P1 or HcRedK2P1K274E (red fluorescent cells). As shown previously, HcRed fusion to K2P1 and K274E mutation were associated with a cumulative increase of the K2P1 currents (Figure 4A). However, SENP1 coexpression did not result in any activation of these channels when compared to coexpression of the catalytically inactive SENP1 mut (Figure 4A) or of an empty pIRES-GFP plasmid (not shown). The same SENP1 sequences were transferred into the pEXO plasmid, and SENP1 cRNA was synthetized and injected into *Xenopus* oocytes. No effect of SENP1 was observed in oocytes expressing the protease and K2P1 or K2P1K274E (Figure 4D).

Expression of K2P1 in a Cell-free System

K2P1 and the positive control protein RanGAP1 were cotranslated in a rabbit reticulocyte lysate system (TnT Promega) (Figure 4). As expected, coexpression of SUMO1 and Ubc9 resulted in extensive SUMO modification of RanGAP1, whereas coexpression of SENP1 resulted in complete desumoylation (in this system, RanGAP1 is partially sumoylated even in the absence of SUMO and Ubc9 overexpression; Knuesel et al., 2005). In the same conditions, SUMO1/Ubc9 or SENP1 had no effect on the apparent MW of K2P1.

DISCUSSION

K2P1 has been cloned 10 years ago; however, its functional expression remains challenging. Only modest currents were recorded upon heterologous expression of K2P1 in *Xenopus* oocytes, and no K2P1 currents have been repeatedly measured from transfected and native cells despite extensive attempts in several laboratories. In search of an explanation for this peculiarity, we have previously established that K2P1 is expressed in the endosomal pericentriolar recycling compartment of nonpolarized cells and in the corresponding subapical recycling compartment of polarized epithelial cells. We have also demonstrated that K2P1 interacts with a complex of proteins comprising the small G protein ARF6 and its nucleotide exchange factor EFA6 (Decressac et al., 2004). It is

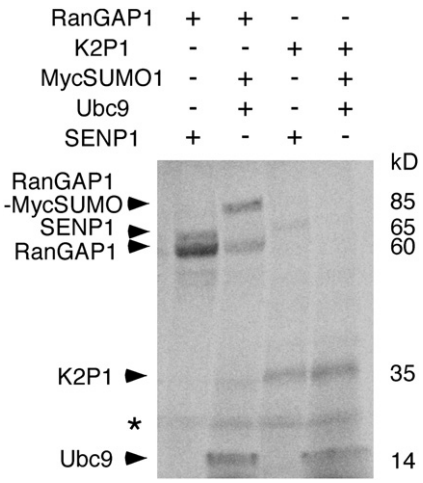

Figure 5. Expression in the TnT T7 Promega Cell-free Transcription/Translation System

Sumoylation of the RanGAP1 control protein is prompted by coexpression of SUMO1 and Ubc9. In the presence of SENP1, only the unsumoylated form of RanGAP1 is present. K2P1 is not affected by SUMO1/Ubc9 or SENP1 coexpression. The apparent MWs of the different proteins are indicated.

well known that ARF6 is actively involved in the recycling of the plasma membrane and membrane proteins (Altschuler et al., 1999). Taken together, these results suggest that the addressing/retrieval of K2P1 at the cell surface is finely tuned. As an additional support for this hypothesis, we have shown that a chimeric channel comprising a fluorescent protein fused to the amino terminus of K2P1 (HcRedK2P1) is able to reach the cell surface and produce macroscopic K2P1 currents in MDCK and COS-7 cells (Decressac et al., 2004). The steric hindrance and/or the masking of retrieval signals resulting from the fused peptide may partially relieve intracellular retention of K2P1 or slow down its retrieval from the plasma membrane.

Intriguing data have recently described K2P1 as a plasma membrane channel kept silent by sumoylation in *Xenopus* oocytes (Rajan et al., 2005). However, the furthering of such a silencing mechanism as a general explanation for the lack of K2P1 current in mammalian cells was very surprising. Sumoylation is the covalent attachment of a SUMO peptide moiety to lysine residues in target proteins. It acts primarily as a nucleocytoplasmic phenomenon that mediates nuclear import/export and activity of transcription factors, but it is also known to take place in the cytoplasm. Several aspects of K2P1 sumoylation are intriguing. The modified lysine at the position 274 in K2P1 does not belong to a classical consensus site for SUMO addition (LK^{274}KF, where F is not the acidic residue usually present in ψKxE/D). If K2P1 is a silenced plasma membrane channel then it must be kept extensively sumoylated in many cell types that do not exhibit a robust current upon K2P1 expression. This implicates that SUMO substrates and conjugating enzymes are present in these cells at high levels since the capacity of this machinery seems never exhausted even in K2P1 overexpression conditions. Finally, the SUMO-conjugating Ubc9 enzyme is relocated at the animal pole in oocytes together with K2P1, suggesting that sumoylation takes place in close vicinity to the plasma membrane (Rajan et al., 2005), though there is no evidence for plasma membrane concentration of Ubc9 in mammalian cells (Wilson and Rosas-Acosta, 2005).

Our data show a total lack of evidence for any sumoylation of K2P1 in COS-7 cells and *Xenopus* oocytes. No macroscopic currents were recorded from transfected COS-7 cells. When HcRed was covalently fused to these channels, macroscopic currents were recorded as expected (Decressac et al., 2004). HcRedK2P1K274E produced larger currents than those of HcRedK2P1. However, this effect cannot be attributed to the loss of a SUMO moiety since the conservative charge mutation K274R failed to produce the same effect as K274E. Furthermore, the three proteins showed exactly the same gel mobility when analyzed by western blot, with no band shifts induced by the replacement of the SUMO-acceptor lysine 274 by glutamate or arginine. The apparent MW of the proteins is around 37–38 kDa, in agreement with the MW calculated from the primary structure (Lesage et al., 1996a), and close to the 38–40 kDa K2P1 protein detected in adult mouse brain (Lesage et al., 1997). These MWs are not compatible with the addition of a SUMO peptide. Obviously, extensive sumoylation of K2P1 does not take place in COS-7 cells. Recent studies have shown that some membrane proteins such as the phosducin and the glutamate receptor mGluR8 undergo sumoylation (Klenk et al., 2006; Tang et al., 2005). However, the sumoylated forms of both proteins in transfected HEK293 or COS-7 cells were minor and only detectable after overexpression of the SUMO-conjugating enzyme Ubc9 and the SUMO1 substrate. All our efforts to force sumoylation in COS-7 cells or in a cell-free system, including coexpression of K2P1 with Ubc9 and SUMO1, failed to lead to any K2P1-sumoylated band (Figures 3 and 5). Rajan and colleagues reported a strong current expression of K2P1K274E in COS-7 cells, but they did not provide any biochemical data that would demonstrate the sumoylation of K2P1 in this cell system.

Our efforts to get evidence of K2P1 sumoylation in *Xenopus* oocytes were also unsuccessful. The stimulatory effect of the K274E mutation was effectively reproduced in oocytes but once again K274R had no effect on channel activity. The lysine 274 is located just downstream of the last membrane-spanning segment (M4) in a cluster of charged residues (K^{274}KFRK). An equivalent cluster of charged residues (K^{301}KTKEE) has been shown to play a crucial role in the gating of TREK1 K_{2P} channels. Any charge modification at this post-M4 site induced a drastic alteration of the TREK1 activity (Chemin et al., 2005). The most likely hypothesis is that the K274E mutation effect in K2P1 relies on the charge modification resulting from the replacement of a positively charged lysine by a negatively charged glutamate. K2P1K274E is more active than K2P1 and K2P1K274R and produces more current when

expressed at the cell surface in oocytes and in COS-7 cells (as a HcRed fusion protein in the latter). This hypothesis is supported by the fact that the current level variations are quantitatively the same in COS-7 and oocytes. When analyzed by western blot, K2P1, K2P1K274E, and K2P1K274R displayed the same apparent MW, again ruling out any SUMO modification of K2P1.

In conclusion, despite intensive efforts we were unable to find any in vivo or in vitro evidence supporting SUMO modification of the background K^+ channel K2P1. We have shown here that K2P1 by itself does produce currents when present at the cell surface. The current increase associated with the K274E modification is likely to be a charge effect unrelated to sumoylation. In the absence of in vivo evidence, sumoylation cannot be considered as a general mechanism of covalent and reversible control of background K^+ channel function.

EXPERIMENTAL PROCEDURES

Molecular Biology

Human K2P1, K2P1K274E, and K2P1K274R were generated by PCR and subcloned into pEXO (Lesage et al., 1996a), pIRESCd8 (Fink et al., 1996), and pHcRed-C1 (Clontech, CA, USA). From pIRESCd8 constructs, a single mRNA coding successively for K2P1 or K2P1K274E and the cell-surface protein Cd8 protein was produced. In pHcRed-C1, channel sequences were fused in frame with the fluorescent protein HcRed. The cDNAs coding for human SENP1 and its catalytically inactive mutant SENP1R360L/K631M were provided by Dr. Yeh (Cheng et al., 2004). The open reading frames were transferred in pIRES-EGFP (BD Biosciences Clontech, CA, USA) and pEXO vectors. All the constructs were verified by sequencing.

Cell Culture and Electrophysiology

COS-7 cells were maintained in Dulbecco's modified Eagle media supplemented with 10 % fetal bovine serum and 100 U/ml streptomycine, 100 U/ml penicillin at 37°C in a humidified 5 % CO_2 atmosphere. Cells were transiently transfected by DEAE-Dextran method using 1 μg DNA of pHcRedC1-K2P1, pHcRedC1-K2P1K274R, and pHcRedC1-K2P1K274E per 35 mm culture dish. Currents were recorded 48 hr after transfection. Recordings were conducted in the whole-cell configuration at room temperature (~22°C) with an EPC 10 amplifier (HEKA Electronic, Germany). The pipette solution contained (in mM) 150 KCl, 0.5 $MgCl_2$, 5 EGTA, and 10 HEPES (pH 7.3). The bathing media was (in mM) 150 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES (pH 7.3). Pipette resistance was 1.5–4 MΩ. Membrane currents were elicited by a 500 ms depolarization ranging from −120 mV to +40 mV in a 20 mV increment, from a holding potential of −80 mV. Only cells with series resistance less than 5 MΩ were used for analysis. Data acquisition and analysis were performed using Patchmaster and Pulsefit (HEKA Electronic, Germany) and IgorPro (WaveMetrics Inc., OR, USA) softwares. pEXO-K2P1, pEXO-K2P1K274R, and pEXO- and pEXO-K2P1K274E were linearized by BamHI enzyme and capped cRNAs were synthetized using the T7 RNA polymerase. Defolliculated *Xenopus* oocytes were injected with cRNAs (15 ng/oocyte) then used for electrophysiological studies 2 to 4 days following injection. In a 0.3 ml perfusion chamber, a single oocyte was impaled with two standard microelectrodes (1–2.5 MΩ resistance) filled with 3 M KCl and maintained under voltage clamp using a Dagan TEV 200 amplifier in standard ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM HEPES, pH 7.4 with NaOH). Stimulation of the preparation, data acquisition, and analysis were performed using pClamp software (Axon Instruments, CA, USA).

Biochemistry

COS-7 cells were transfected with lipofectamine (Invitrogen). After 48 hr, cells were washed and immediately lysed in the SDS-containing Laemmli's buffer or harvested, then resuspended in a buffer containing (in mM) 100 NaCl, 40 KCl, 20 NEM, 1 EDTA, 20 HEPES-KOH (pH 7.4), 10% glycerol, 1% Triton X-100, and complete protease inhibitor tablets (Roche) at 4°C. After centrifugation, solubilized proteins were separated on 10% SDS-PAGE and subjected to western blot analysis using anti-TWIK1 antibodies (1:1000) (Lesage et al., 1996b) or commercial anti-HcRed (1:200, SC-32188, Santa Cruz biotechnology) or anti-SENP1 antibodies (1:200, SC-46634, Santa Cruz biotechnology). cRNA-injected oocytes were ground in lysis buffer without Triton X-100. After three rounds of low-speed centrifugation (1500 rpm, 10 min, 4°C), the supernatant was submitted to high-speed centrifugation to collect membrane proteins. The proteins were resuspended in Laemmli's loading buffer and analyzed by western blot as described above. For in vitro transcription/translation, ^{35}S-labeled proteins were produced by using ^{35}S-methionine and the TnT expression system as specified by the manufacturer (Promega, WI, USA).

ACKNOWLEDGMENTS

We thank E.T.H. Yeh for SENP1 and SENP1 mut, H. Hibino for MycSUMO1, F. Melchior for HaRanGAP1 and HaRanGAP1K526R, and M.J. Lohse for Ubc9. We are grateful to M. Larroque and M. Jodar for excellent technical assistance with oocyte preparation, cell cultures, and molecular biology. This work was supported by "Ligue National Contre le Cancer" (Equipe labelisée to F.L.), by Japan-France Integrated Action Program SAKURA (06980UF to F.L.), and by "Deutsche Forschungsgemeinschaft" (SFB699 to R.W.). S.F. was supported by a fellowship from the "Association de Recherche contre le Cancer" and F.L. was a recipient of a Contrat d'Interface Clinique, Service de Neurologie, CHU de Nice.

Received: October 10, 2006
Revised: March 9, 2007
Accepted: June 4, 2007
Published: August 9, 2007

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