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Sumoylation Silences the Plasma Membrane Leak K+ Channel K2P1

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Summary

Reversible, covalent modification with small ubiquitin-related modifier proteins (SUMOs) is known to mediate nuclear import/export and activity of transcription factors. Here, the SUMO pathway is shown to operate at the plasma membrane to control ion channel function. SUMO-conjugating enzyme is seen to be resident in plasma membrane, to assemble with K2P1, and to modify K2P1 lysine 274. K2P1 had not previously shown function despite mRNA expression in heart, brain, and kidney and sequence features like other two-P loop K+ leak (K2P) pores that control activity of excitable cells. Removal of the peptide adduct by SUMO protease reveals K2P1 to be a K+-selective, pH-sensitive, openly rectifying channel regulated by reversible peptide linkage.

Introduction

Background potassium conductances were recognized to influence resting membrane potential and activity of excitable cells (Goldman, 1943; Hodgkin and Katz, 1949; Hodgkin and Huxley, 1952) long before they were revealed to pass via dedicated portals rather than ill-defined seepage pathways (Goldstein et al., 2001). Molecular identification was presaged by cloning of potassium channels in *S. cerevisiae* and *C. elegans* with two pore-forming P loops in each subunit (Ketchum et al., 1995) and realized in characterization of K2P0 from *D. melanogaster*, which manifests the canonical attributes: a K+-selective pore open across the physiological voltage range that manifests open (Goldman-Hodgkin-Katz type) rectification (Goldstein et al., 1996; Ilan and Goldstein, 2001) and regulated activity (Zilberberg et al., 2000; Zilberberg et al., 2001).

Fifteen animal genes have since been discovered to encode subunits with two P loops and four transmembrane spans, and these are designated K2P channels (Goldstein et al., 2002). As expected for background conductances, K2P channels that show function reveal dynamic regulation by a panoply of chemical and physical stimuli (i.e., kinases, phosphatases, lipids, G proteins, and mechanical stretch) that change open probability (Zilberberg et al., 2000; Zilberberg et al., 2001; Bockenhauer et al., 2001) or forward transport to the plasma membrane (O'Kelly et al., 2002; Rajan et al., 2002). The process of matching K2P subtypes to native currents is now well underway (Kim et al., 1999; Lopes et al., 2000; Sirois et al., 2000; Bockenhauer et al., 2001; Washburn et al., 2002). It appears that each channel pore is formed by a pair of subunits (Lopes et al., 2001) and thus assembly of nonidentical subunits is expected, although it has not yet been seen. Some K2P genes do not produce currents in experimental cells (K2P1, K2P7, K2P12 and K2P15). As synthesis of mRNA in native cells indicates that these are not pseudogenes (Washburn et al., 2002), their silence has been suspected to result from absolute localization to internal membranes or strict downregulation (Orias et al., 1997; Goldstein et al., 1998; Bockenhauer et al., 2000; Rajan et al., 2001).

K2P1, the first mammalian isolate of the K2P superfamily (Lesage et al., 1996), is expressed widely with abundant transcript in heart (ventricle greater than atrium [Wang et al., 1998]), brain (cerebellum and thalamus [Talley et al., 2001]), and kidney (thick ascending loop of Henle and distal nephron [Levy et al., 2004]). While K2P1 was first reported to be a weak, inwardly rectifying potassium channel, and so named TWIK (Lesage et al., 1996), others observed no channel activity (Orias et al., 1997; Goldstein et al., 1998). In this report, K2P1 is shown to be inactive despite residence in the plasma membrane so long as the protein is covalently modified by small ubiquitin-related modifier protein, SUMO (also called sentrin). Sumoylation, the covalent modification of substrate proteins with SUMO, is a reversible, posttranslational modification primarily observed with nuclear proteins and implicated in nuclear import/export, target stability, and transcriptional activity (Muller et al., 2001; Li and Hochstrasser, 2003; Melchior et al., 2003). SUMO subtypes, SUMO-conjugating enzyme, and SUMO proteases are conserved from yeast to humans.

Here, SUMO-conjugating enzyme (Ubc-9) is discovered to be uniformly distributed at the plasma membrane of *Xenopus laevis* oocytes until assembly with K2P1 channels induces polarized expression over the animal pole. Endogenous Ubc-9 sumoylates K2P1 on an intracellular lysine at position 274. Desumoylation by a SUMO protease (SENP-1) activates the mute pore, allowing its characterization. K2P1 is now seen to be a potassium-selective leak channel with a 32 pS unitary conductance that passes larger outward than inward currents under physiological conditions (that is, high internal and low external potassium) due to open rectification. Active at all physiological voltages, K2P1 is sensitive to external pH via protonation of a histidine in the first P loop that is conserved in the TASK (acid-sensitive) K2P channels K2P3 and K2P9 (Lopes et al., 2000, 2001; Rajan et al., 2000). SUMO regulation of K2P1 is also demonstrated in the mammalian renal fibroblast cell line COS-7. K2P1 is thus a canonical leak channel showing tightly regulated activity at the plasma membrane via SUMO modification and removal.

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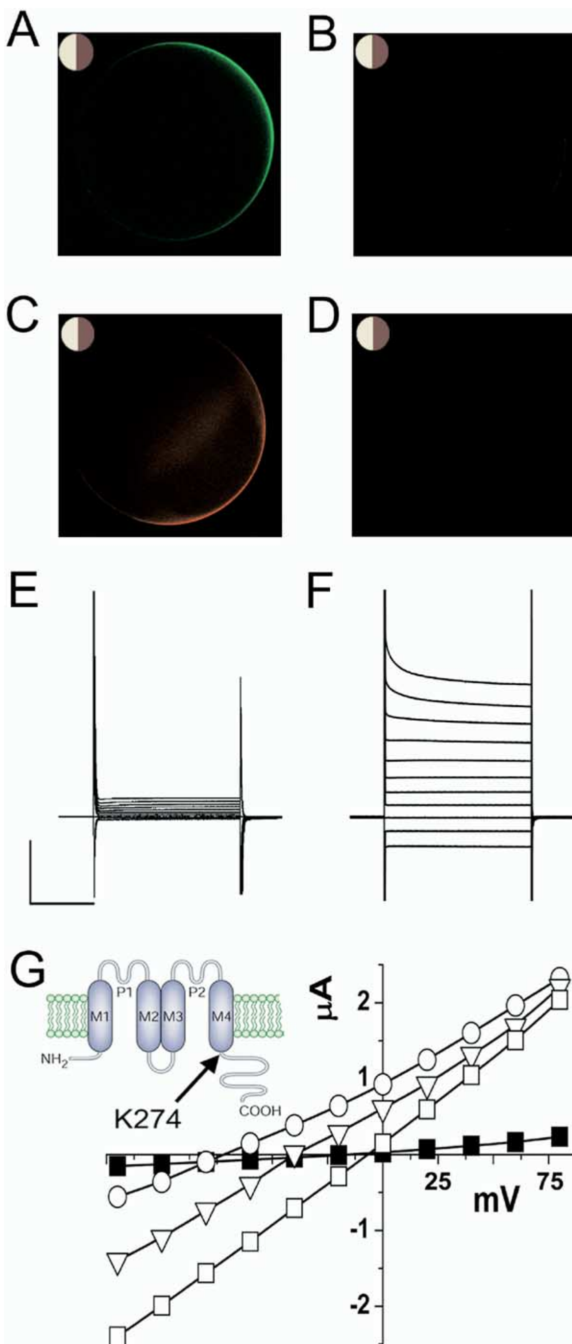

Results

K2P1 Is Electrically Silent Despite Expression at the Plasma Membrane

Expression of K2P1 alone fails to produce potassium currents in oocytes or mammalian tissue culture cells (Orias et al., 1997; Goldstein et al., 1998). Lack of ion channel function can be associated in the first instance with failure of the protein to reach the plasma membrane; thus, K2P7 channels are speculated to operate on intracellular membranes (Salinas et al., 1999). Alternatively, K2P3 channels reach the plasma membrane but surface levels are determined by regulated forward transport (O'Kelly et al., 2002; Rajan et al., 2002). To investigate trafficking of human K2P1, the protein was produced with an N-terminal green fluorescent protein tag (GFP-K2P1) and studied in oocytes by confocal microscopy, as before (Rajan et al., 2002).

Oocytes expressing GFP-K2P1 showed strong fluorescence at the plasma membrane (Figure 1A); this fluorescence was absent in cells with untagged K2P1 (data not shown) or GFP alone (Figure 1B). Of note, surface expression of GFP-K2P1 was polarized to the animal pole of the cells. *Xenopus laevis* oocytes are prototypical polarized cells that display structural and functional asymmetry of the animal and vegetal poles (Ubbels, 1997), with targeting of some membrane proteins to the animal pole a recognized phenomenon. Surface expression in polarized fashion was also seen with K2P1 channels bearing a 16-residue tag at the C terminus (K2P1-1d4) visualized with anti-1d4 monoclonal antibodies (Figure 1C).

Mutation of a Single Residue Yields K2P1 Currents

Surface expression without activity suggested that K2P1 might require a stimulus to operate. However, agents (including long-chain free fatty acids, lysophospholipids, volatile anesthetics, and classical regulators of kinases and phosphatases, data not shown) known to upregulate other K2P channels had no effect. Activity was also not seen when the N and/or C termini of K2P1 were replaced by the regions from three functional K2P channels (K2P2, K2P3, or K2P9, data not shown). Conversely, mutating a single intracellular lysine following the fourth transmembrane span, a region critical to regulation of K2P2 (Bockenhauer et al., 2001), was most informative.

As before (Orias et al., 1997; Goldstein et al., 1998),

Figure 1. K2P1 Reaches the Plasma Membrane but Remains Electrically Silent

The indicated subunits were expressed in *Xenopus* oocytes for 48 hr before surface currents were recorded by two-electrode voltage clamp and surface protein visualized by confocal microscopy.

(A) GFP-K2P1 shows polarized surface expression in oocytes. Light microscopy reveals K2P1 to be at the animal (dark) pole of the cells (data not shown). Inset indicates oocyte position for microscopy; dark side represents animal pole.

(B) Expression of uncoupled GFP in oocytes shows no plasma membrane fluorescence.

(C) K2P1-1d4 expressed in oocytes and visualized by immunostaining with anti-1d4 antibodies also shows polarized expression.

(D) Cells expressing wild-type K2P1 without an epitope tag show no plasma membrane staining with anti-1d4 antibodies.

(E) Wild-type K2P1. Current trace from a representative oocyte. Protocol: holding −80 mV, 1 s test pulses from −120 mV to +80 mV with 2.5 s interpulse interval, sampled and filtered at 5 kHz and 1 kHz, respectively. Bath solution with 4 mM potassium. Scale bars, 1 μA, 200 ms.

(F) K274E-K2P1. Current trace from a representative oocyte studied as in (E).

(G) Average current-voltage relationship for groups of six cells as in (E) with bath potassium levels of 4 mM (■) and (F) with bath potassium levels of 4 (○), 20 (▽), and 100 mM (□) (mean ± SEM). Inset: Predicted topology of K2P1 indicating lysine 274 (arrow), two P loops (P), and the membrane segments (M); N and C termini are intracellular.

human K2P1 expression failed to induce currents in oocytes (Figure 1E). Like wild-type, GFP-K2P1 and K2P1-1d4 channels were electrically silent (data not shown) despite staining consistent with successful targeting to the plasma membrane (Figures 1A and 1C). Conversely, changing the lysine at position 274 to glutamate (K274E-K2P1) produced macroscopic currents by two-electrode voltage clamp (Figures 1F–1H). As expected for a K2P channel, the portal was constitutively active (that is, open at rest) and potassium selective, showing a shift in reversal potential with altered bath potassium of 51 ± 3 mV per 10-fold change. It became clear that lysine had a unique silencing effect when currents were also observed on alteration of the position to glutamine, alanine, cysteine, or arginine, a residue like lysine that is positively charged at physiological pH. This led us to speculate that position 274 was in a receptor site for a regulator that required lysine to bind; the underlying hypothesis was that the regulator suppressed K2P1 function and that mutations interfered with regulator association allowing the channels to open.

K2P1 Assembles with Native SUMO-Conjugating Enzyme at the Plasma Membrane

Evaluating the region of K2P1 with lysine 274 for known motifs revealed a variant SUMO modification site, -L$\underline{K}^{274}$KF-. SUMO-1, -2, and -3 are ~100 amino acid, soluble, intracellular proteins, found in all eukaryotic cells, that act via covalent linkage to the ε amino group of lysine on an acceptor protein to be regulated. Sumoylation proceeds by a pathway that is distinct from, but analogous to, ubiquitin conjugation. Thus, SUMOs are first activated and linked to Aos1/Uba2 and then transferred to the cysteine side chain of the conjugating enzyme Ubc-9 (Gong et al., 1999; Bernier-Villamor et al., 2002). Ubc-9 then binds to the acceptor protein and transfers its thioester-linked SUMO to the acceptor-protein lysine. The SUMO conjugation machinery is present en toto in extracts from *Xenopus laevis* oocytes (Saitoh et al., 1998).

As interaction of Ubc-9 and the acceptor is required to accomplish sumoylation, we sought evidence for interaction of Ubc-9 and K2P1. First, human Ubc-9 was cloned and confirmed to interact with the C-terminal portion of K2P1 that carries lysine 274 using a yeast two-hybrid assay (Figure 2A). To next determine if complete K2P1 and Ubc-9 proteins interact in oocytes, a GFP tag was introduced at the N terminus of Ubc-9 (GFP-Ubc-9) for studies by confocal microscopy. Expressed on its own, GFP-Ubc-9 was observed at the oocyte plasma membrane in a uniform, nonpolarized fashion (Figure 2B). In contrast, coexpression of wild-type K2P1 (no tag) and GFP-Ubc-9 led to restricted localization of the conjugating enzyme at the animal pole (Figure 2C), the surface pattern observed for K2P1 channels (Figure 1B), arguing for assembly of the two proteins.

Native Ubc-9 endogenous to oocytes was found to interact with K2P1 in the same fashion as overexpressed human Ubc-9. The human protein is identical to the *Xenopus* product (accession number BC046273), allowing use of a commercial antibody to human Ubc-9. Native Ubc-9 shows a uniform signal at the plasma membrane in untreated cells (Figure 2D). When K2P1 is expressed, the native Ubc-9 signal is polarized to the animal pole (Figure 2E). This suggested that surface distribution of native Ubc-9 was also restricted by assembly with

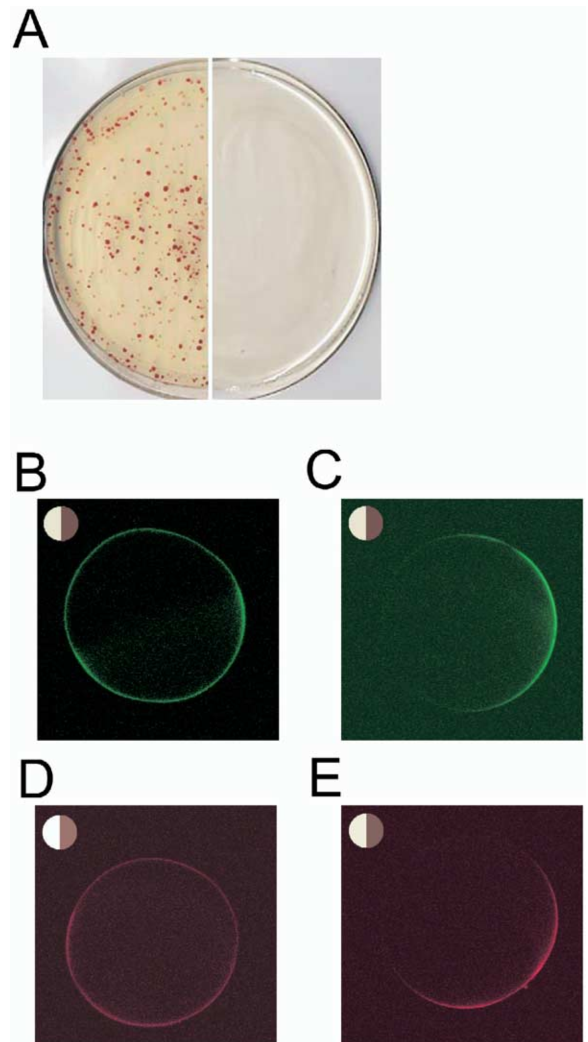

Figure 2. K2P1 Assembles with SUMO-Conjugating Enzyme in the Plasma Membrane

(A) Yeast two-hybrid analysis shows strong interaction of the K2P1 C terminus and SUMO-conjugating enzyme (Ubc-9). (Left panel) Yeast with the K2P1 C terminus (in pGBT-9) and Ubc-9 (in pGAD-424) show growth on histidine-deficient plates whereas (right panel) yeast with the K2P1 C terminus (in pGBT-9) and empty pGAD 424 vector do not.

(B) A representative oocyte expressing GFP-Ubc-9 shows uniform surface staining in confocal images.

(C) A representative oocyte expressing GFP-Ubc-9 and K2P1 shows that surface staining for the conjugating enzyme becomes polarized to the animal pole.

(D) Native Ubc-9 visualized in a naive oocyte stained with anti-Ubc-9 antibody and a Texas red-conjugated secondary antibody shows uniform surface staining.

(E) Native Ubc-9 visualized in an oocyte expressing wild-type K2P1 and stained with anti-Ubc-9 antibody as in (D) shows that the native conjugating enzyme also becomes polarized to the animal pole.

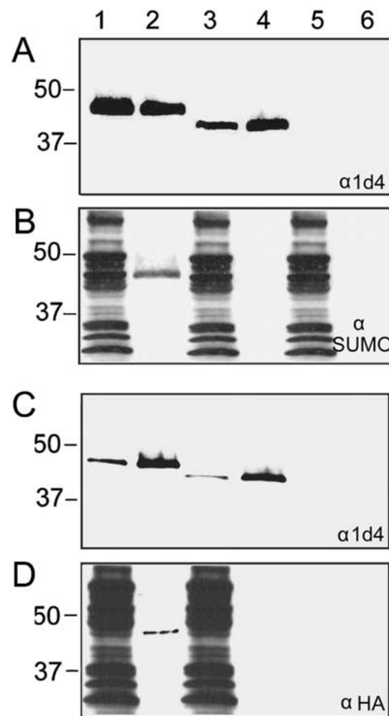

Figure 3. K2P1 Is Modified by Native or Overexpressed SUMO on Lysine 274

Oocytes were injected with cRNA for the indicated subunits, incubated for 48 hr, and proteins purified by immunoprecipitation (IP) with 1d4 antibodies for separation by SDS-PAGE and Western blotting.

(A) Cells expressing K2P1-1d4 or K274E-K2P1-1d4 blotted with anti-1d4 antibodies. Lane 1: K2P1-1d4 total extract. Lane 2: IP of lane 1 with 1d4 antibody. Lane 3: K274E-K2P1-1d4 total extract. Lane 4: IP of lane 3 with 1d4 antibody; the point mutant migrates with a lower apparent kDa than wild-type. Lane 5: total extract from mock injected cells. Lane 6: IP of lane 5 with 1d4 antibody.

(B) Materials as in (A) visualized with an antibody to SUMO-1 showing that many native proteins are sumoylated (lanes 1 and 3), as is K2P1-1d4 (lane 2), whereas K274E-K2P1-1d4 does not carry SUMO (lane 4).

(C) Cells expressing K2P1-1d4 or K274E-K2P1-1d4 and human HA-SUMO blotted with anti-1d4 antibodies. Lanes as in (A).

(D) Materials as in (C) visualized with an antibody to HA showing that many native proteins are modified with HA-SUMO (lanes 1 and 3), as is K2P1-1d4 (lane 2), whereas K274E-K2P1-1d4 does not bear HA-SUMO (lane 4).

K2P1. Direct evidence for sumoylation of K2P1 was thus sought.

K2P1 Is Modified by SUMO at Lysine 274

Human K2P1 was shown to be coupled to native SUMO by enzymes endogenous to the oocyte as follows. Wild-type K2P1 and mutant subunits with lysine 274 changed to glutamate (K274E-K2P1) were produced with C-terminal 1d4 tags to allow immunopurification. Channels were expressed in oocytes, solubilized in detergent, affinity isolated via the 1d4 tag, subjected to separation by SDS-PAGE, and analyzed by Western blotting with anti-1d4 (Figure 3A) or anti-SUMO-1 antibodies (Figure 3B). Consistent with SUMO modification of wild-type K2P1-1d4 on lysine 274 and failure to sumoylate K274E-K2P1-1d4 channels, the latter subunits migrate at a lower apparent kDa (Figure 3A). While many native proteins in oocytes are modified by SUMO (Figure 3B, lane 1), immunoprecipitation isolated a single sumoylated band that migrated at the same position as K2P1-1d4 (Figure 3B, lane 2). Furthermore, no sumoylated band was isolated from cells expressing K274E-K2P1-1d4 (Figure 3B, lane 4), despite successful isolation of the subunit (Figure 3A, lane 4).

Evidence that lysine 274 was the single essential residue in K2P1 required for SUMO linkage and activity was also obtained by cloning and study of human SUMO-1. First, SUMO was produced with a nine-residue tag from hemagglutinin at the N terminus (HA-SUMO). HA-SUMO was overexpressed with K2P1-1d4 or K274E-K2P1-1d4, and after purification, anti-1d4 and anti-HA antibodies were used to visualize channel subunits or human SUMO-1, respectively. Whereas both K2P1-1d4 and K274E-K2P1-1d4 subunits were purified (Figure 3C), HA-SUMO was observed only with wild-type subunits bearing lysine 274 (Figure 3D, lane 2). In addition, mutation or deletion of the other potential SUMO modification sites in K2P1 produced no current (data not shown; examined constructs included K275Q and R, K283Q, K286Q and E, and K312Q and E).

SENP-1 Protease Removes SUMO from K274 to Activate Wild-Type K2P1 Channels

If sumoylation silences K2P1, desumoylation is expected to activate the channels. This was found to be the case. Recently, proteases were identified that specifically cleave SUMO from target proteins (Gong et al., 2000). One such enzyme, SENP-1, was cloned from a human brain library and expressed with wild-type human K2P1 in oocytes. Coexpression resulted in macroscopic, potassium-selective currents with attributes like those of K274E-K2P1 channels (Figures 4A and 4B). Wild-type K2P1 channels activated by SENP-1 were open at rest and potassium selective, showing 51 ± 3 mV change in reversal per 10-fold change in bath potassium (data not shown). Specificity of the SENP-1 effect was confirmed by study of a point mutant where the active-site cysteine at position 603 was altered to serine to produce C603S-SENP-1, a change that ablates enzymatic activity (Bailey and O'Hare, 2004); expression of C603S-SENP-1 with K2P1 failed to induce currents (Figure 4C). Neither expression of SENP-1 nor C603S-SENP-1 alone yielded significant currents (Figures 4D and 4E).

To demonstrate that the effect of wild-type SENP-1 on K2P1 was to remove SUMO from the channel subunit, fusion proteins of glutathione S-transferase and SENP-1 (GST-SENP-1) and C603S-SENP-1 (GST-C603S-SENP-1) were synthesized in bacteria, purified, and applied to K2P1-1d4 channels immunopurified from oocytes also expressing HA-SUMO. Figures 4F and 4G show that the isolated K2P1-1d4 subunits traveled as expected for sumoylated K2P1 (Figure 3) and were modified by HA-SUMO, and that GST-SENP-1 treatment removed the HA-SUMO adduct from the channel (lane 3), whereas inactive GST-C603S-SENP-1 did not (lane 4).

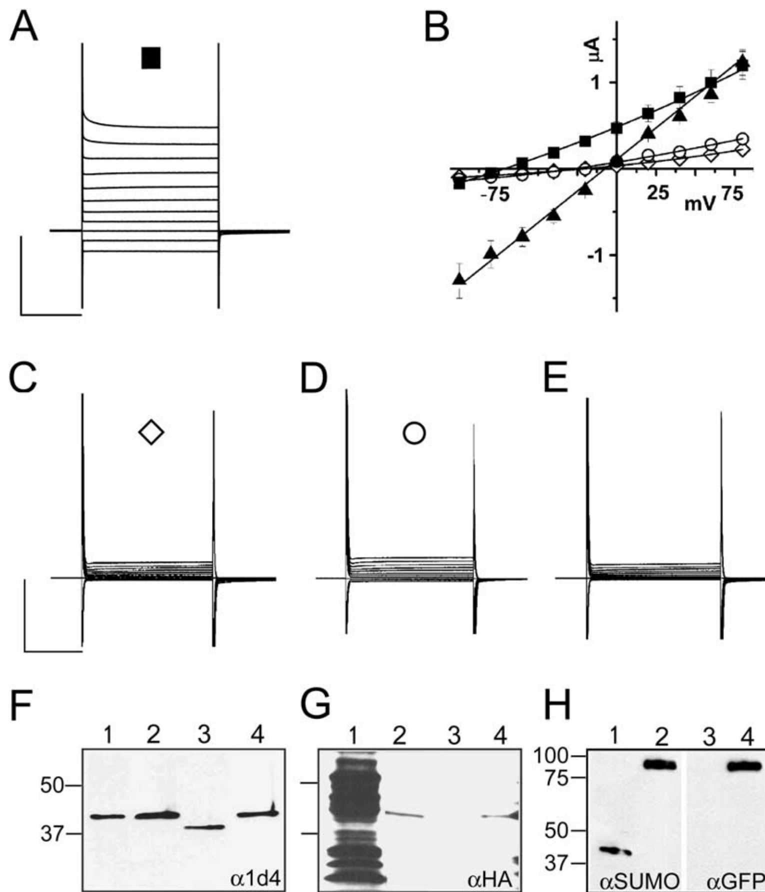

Figure 4. SENP-1, a SUMO Protease, Awakens Wild-Type K2P1

The indicated subunits were expressed in *Xenopus* oocytes for 48 hr before surface currents were recorded by two-electrode voltage clamp and proteins purified for Western blot analysis.

(A) Wild-type K2P1 and SENP-1. Current trace from a representative oocyte studied in 4 mM potassium. Protocol as in Figure 1A. Scale bars, 1 μA and 200 ms.

(B) Average current-voltage relationships for groups of six cells (mean ± SEM) studied as in (A) (■, K2P1 + SENP-1; ◇, K2P1 + C603S-SENP-1; ○, SENP-1) or in 100 mM potassium (▲, K2P1 + SENP-1).

(C) Wild-type K2P1 and the point mutant C603S-SENP-1 fail to induce currents on expression when studied as in (A). Current trace from a representative oocyte. Current-voltage relationship for six cells (mean ± SEM) in (B) (◇).

(D) Wild-type SENP-1 alone yields no new currents when studied as in (A). Current trace from a representative oocyte. Current-voltage relationship for six cells (mean ± SEM) in (C) (○).

(E) C603S-SENP-1 alone yields no new currents when studied as in (A). Current trace from a representative oocyte.

(F) K2P1-1d4 immunopurified (IP) from oocytes expressing HA-SUMO (as in Figure 3C) visualized with anti-1d4 antibodies. Lane 1: total extract. Lane 2: IP of lane 1 with 1d4 antibody. Lane 3: lane 2 treated with GST-SENP-1. Lane 4: lane 2 treated with GST-C603S-SENP-1. The active enzyme (lane 3) decreases the apparent kDa of the channel subunit consistent with removal of the SUMO adduct.

(G) Samples in (F) stained with anti-HA antibody to visualize HA-SUMO. This shows that active enzyme (lane 3) removes the SUMO adduct from K2P1-1d4.

(H) K2P1-1d4 purified from oocytes with anti-1d4 antibodies is modified by endogenous SUMO (lanes 1 and 3) or coexpressed GFP-SUMO (lanes 2 and 4). Lanes 1 and 2 with anti-SUMO antibodies; lanes 3 and 4 with anti-GFP antibodies. Not shown: K274E-K2P1-1d4 is not modified; the upper band seen with anti-GFP and anti-SUMO antibodies in lanes 2 and 4 also stains with anti-1d4 antibodies identifying it as K2P1-1d4.

Of note, the increase in apparent mass on modification of K2P1 with SUMO or HA-SUMO is less than expected (∼12 kDa). This is best explained by anomalous gel migration, because terminal epitopes are not lost from purified SUMO-modified channels. Indeed, isolation of K2P1-1d4 channels after coexpression with GFP-SUMO (∼35 kDa) shows incorporation of the larger adduct on gels visualized with antibodies to either SUMO or GFP (Figure 4H).

Like K2P3 and K2P9, a Histidine in K2P1 Mediates Block by External Proton

To support the conclusion that currents induced by SENP-1 were due to activation of K2P1 channels (rather than effects on other oocyte proteins), a predicted behavior of the channel based on its sequence was studied. A point mutation was introduced into K2P1 to change a natural histidine at position 122 in the first P loop to asparagine (H122N-K2P1). This residue is homologous to a histidine in K2P3 (Lopes et al., 2000; Lopes et al., 2001) and K2P9 (Rajan et al., 2000) that allows external protons to block in the physiological pH range (pKa ∼ 7.24 with 4 mM KCl in the bath for K2P3). As predicted, SENP-1-induced K2P1 currents were inhibited as external pH was lowered from 8.0 to 6.2 (pKa = 6.67 ± 0.04, Hill coefficient 0.99, n = 6 cells), while H122N-K2P1 channels were insensitive to the manipulation (Figure 5).

Mutation of K274 or SENP-1 Coexpression Activates K2P1 Channels in COS-7 Cells

The operation of the SUMO regulatory pathway in mammalian cells was confirmed by studies of COS-7, a fibroblast cell line from African green monkey kidney. Alone, K2P1 channel expression yields no current in the cells (Figure 6A), as previously reported (Orias et al., 1997; Goldstein et al., 1998). Conversely, as in oocytes (Figure 1), K274E-K2P1 and K274R-K2P1 channels are constitutively active (Figures 6B and 6C). Furthermore, expression of SENP-1 alone produces no significant flux, whereas coexpression of wild-type K2P1 and SENP-1 yields robust, potassium-selective currents (Figures 6D–6F).

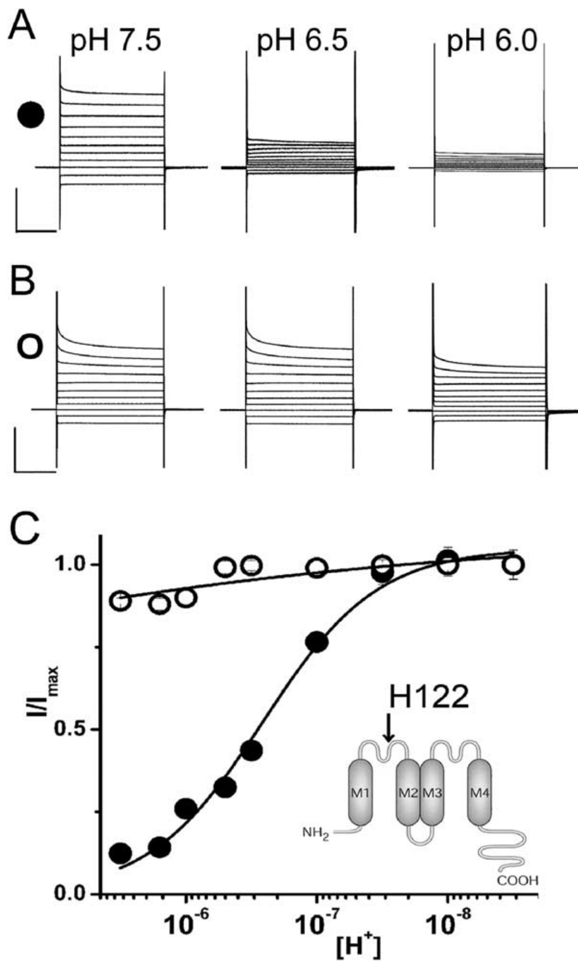

Figure 5. As Predicted, a Histidine in K2P1 Mediates Block by External Proton

The indicated subunits were expressed in oocytes for 48 hr before surface currents were recorded at various pH levels with 4 mM bath potassium by two-electrode voltage clamp.

(A) Wild-type K2P1 and SENP-1. Current trace from a representative oocyte studied at the indicated pH. Protocol as in Figure 1A. Scale bars, 1 μA and 200 ms.

(B) H122N-K2P1 and SENP-1. Current trace from a representative oocyte studied as in (A) shows that the point mutant is insensitive across the physiological range. Scale bars are 1 μA and 200 ms. Inset shows location of H122 in K2P1.

(C) Normalized current at +40 mV with changes in bath pH for groups of cells (n = 6) expressing wild-type K2P1 (●) or H122N-K2P1 (○) channels (mean ± SEM). The solid line represents a fit of the data to $(1+ [B]/K_i)^{-1}$, where B is the concentration of proton and K_i is the level required to achieve half-block (pKa).

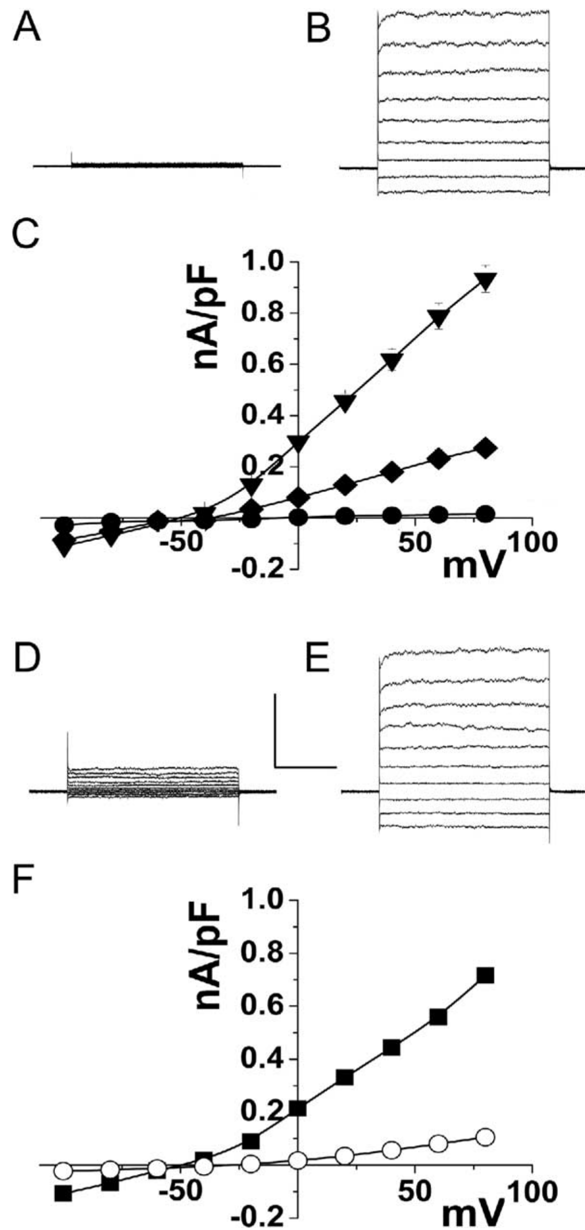

Figure 6. SUMO Regulates K2P1 in Mammalian Cells

The indicated subunits were expressed in COS-7 cells for 48 hr before currents were recorded. Whole-cell currents were evoked by 500 ms pulses to voltages between −100 mV and +80 mV from a holding potential of −50 mV with 20 mM and 140 mM potassium in the bath and pipette, respectively. Scale bars, 1 nA and 200 ms.

(A) Wild-type K2P1. Current trace from a representative cell.

(B) K274E-K2P1. Current trace from a representative cell.

(C) Average current-voltage relationships for groups of 10–20 cells (mean ± SEM) studied in (A) and (B) (●, K2P1; ▼, K274E-K2P1; ♦, K274R-K2P1).

(D) Wild-type SENP-1 alone yields no new currents. Current trace from a representative cell.

(E) SENP-1 and wild-type K2P1. Current trace from a representative cell.

(F) Average current-voltage relationships for groups of 10–15 cells (mean ± SEM) as in (E) (○, SENP-1; ■, K2P1 + SENP-1).

Single K2P1 Channels Are Silenced by Sumoylation and Activated by SENP-1

To verify mechanistic interpretations gleaned from macroscopic studies, individual K2P1 channels were studied. Whereas cell-attached patches on oocytes expressing wild-type K2P1 or SENP-1 alone were indistinguishable from naive cells from −100 to 100 mV (data not shown), a new channel was observed in cells expressing wild-type K2P1 and SENP-1 (Figure 7). Silencing and activation of single K2P1 channels were shown

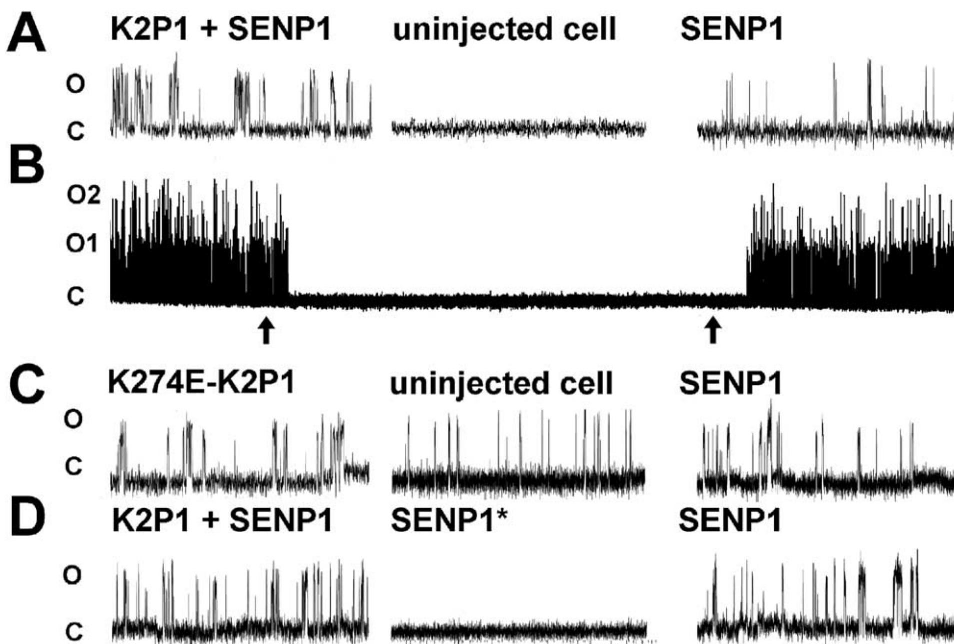

Figure 7. Single K2P1 Channels Are Silenced by Sumoylation and Activated by SENP-1

Individual K2P1 channels were studied in inside-out patches excised from oocytes expressing proteins as indicated. Eighteen of forty-two off-cell patches with a single channel remained intact on insertion into a cell (~36%); 15 of the 18 stable patches tolerated withdrawal and secondary insertion into another cell (85%). Panels show 1 of 5 patches studied under the condition.

(A) An active wild-type single K2P1 channel excised from a cell expressing K2P1 and SENP-1 (left panel) becomes silent on exposure to the interior of a naive oocyte (middle panel) and returns to full activity on insertion into a cell expressing SENP-1 (right panel).

(B) A continuous 4 min recording of two channels studied as in (A). Arrows indicate points of patch insertion into each oocyte.

(C) A single K274E-K2P1 channel shows that the point mutant is constitutively active when expressed alone (left panel) and retains full activity on exposure to the interior of a naive oocyte (middle panel), and also on insertion into a cell expressing SENP-1 (right panel).

(D) An active wild-type single K2P1 channel from a K2P1 and SENP-1 cell (left panel) is silenced on exposure to the interior of an oocyte expressing the inactive C603S-SENP-1 protease (SENP-1*, middle panel) but returns to full activity on insertion into a cell expressing SENP-1 (right panel).

to be reversible upon secondary exposure to native conjugating enzyme or SENP-1 as follows. Patches with active channels were excised from cells expressing K2P1 and SENP-1 in inside-out mode. The patches were first inserted into naive cells (or those expressing the inactive protease C603S-SENP-1) to expose the inner surface of the channels to the native SUMO conjugation machinery; next, the patches were withdrawn and inserted into cells expressing active SENP-1 alone. Figure 7A shows an active wild-type K2P1 channel from a K2P1- and SENP-1-expressing cell that was silenced on exposure to the interior of a naive oocyte and then returned to activity on subsequent insertion into a cell expressing SENP-1. On insertion into a naive cell, single wild-type channels became silent in 13 ± 7 s (n = 5); transfer into SENP-1-expressing cells restored activity in 18 ± 8 s (n = 5). Activity changes were stable, as demonstrated by a continuous recording of two active K2P1 channels exposed first to a naive oocyte and then a cell expressing SENP-1 (Figure 7B).

Arguing against the concern that suppressive effects of naive cells were nonspecific rather than activity of endogenous Ubc-9 were the following observations. First, suppression was not relieved by pulling patches back out of the cells, but only on subsequent entry into cells with SENP-1 (data not shown). Second, as shown in Figure 7C, constitutively active single K274E-K2P1 channels were insensitive to insertion into naive oocytes and retained activity on tertiary insertion into cells expressing SENP-1. Third, active channels from cells expressing K2P1 and SENP-1 were silenced on insertion into cells expressing the inactive desumoylase C603S-SENP-1 (due to unopposed native Ubc-9 activity) and returned to full activity on insertion into cells expressing active wild-type SENP-1 protease (Figure 7D). K2P1 channel activity was also induced by application of purified GST-SENP-1 to the inside of silent patches excised from cells expressing only wild-type K2P1 (data not shown).

Single Activated K2P1 Channels Show Open Rectification

Consistent with the behavior of single K2P0 (Zilberberg et al., 2000; Zilberberg et al., 2001; Ilan and Goldstein, 2001), K2P2 (Bockenhauer et al., 2001), and K2P3 channels (Lopes et al., 2000), single K2P1 channels are openly rectifying (Figure 8A). The K2P1 current-voltage relationship is nearly linear, with similar potassium levels across the membrane, and shows larger outward than inward currents under physiological conditions of high internal and low external potassium. With nearly symmetrical 140 mM potassium across the membrane,

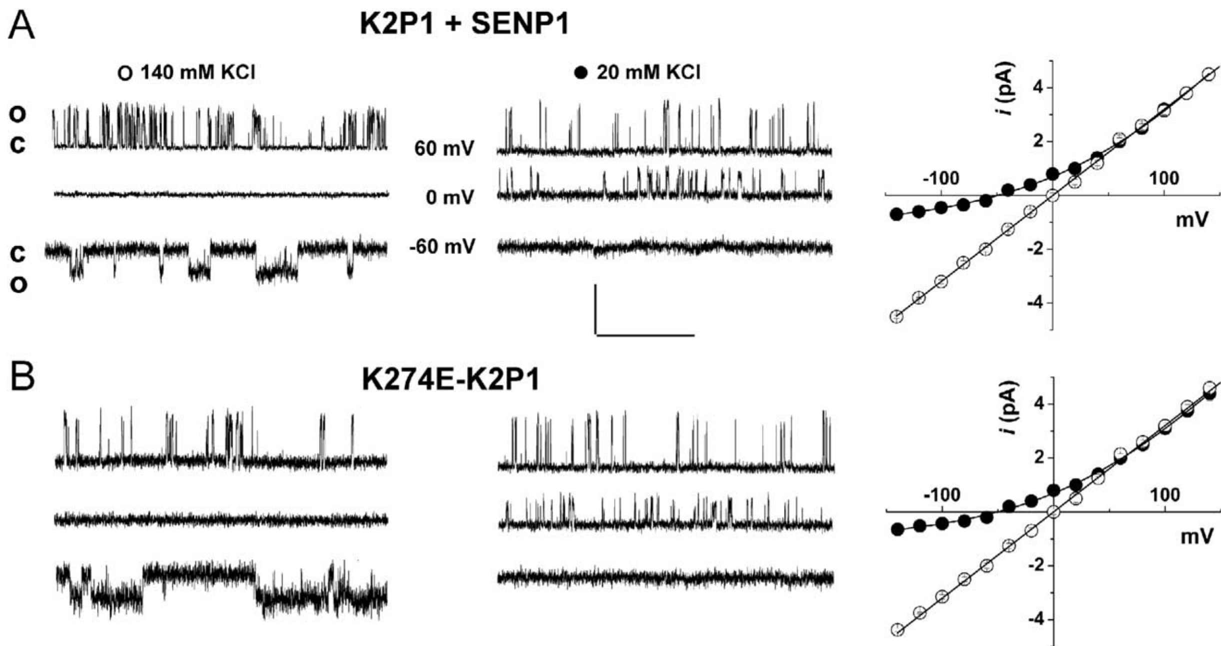

Figure 8. Single K2P1 Channels Are Potassium-Selective Leak Channels

Single wild-type K2P1 + SENP-1 or K274E-K2P1 channels studied in cell-attached patches.

(A) Sample traces of single K2P1 channels + SENP-1 with 140 or 20 mM potassium in the pipette and unitary current plotted against voltage; plot is mean ± SEM for 6–12 cells. The current-voltage relationships show open rectification. The open probability for 140 mM potassium at 60 and −60 mV were 0.08 ± 0.03 and 0.22 ± 0.09 (68 and 60 min with 12 and 8 patches, respectively). Scale bars, 2 pA, 500 ms.

(B) Single K274E-K2P1 channels at the voltages indicated in (A) operate like wild-type K2P1 channels expressed with SENP-1. Unitary currents were measured and plotted as above (n = 4–8 cells).

the unitary current slope conductance was 32 ± 2.2 pS (n = 34 channels) with a reversal potential of 0 mV. With asymmetrical potassium conditions (20 mM external, 140 mM internal), inward unitary currents are diminished in magnitude and the reversal potential shifts by 47 ± 2 mV (a 50 mV shift is predicted by the Nernst relation for an ideally potassium-selective pore). Like K2P0, openings of K2P1 are of shorter duration with greater depolarization; like K2P3, the open probability of K2P1 is 2- to 3-fold greater at negative potentials (Figure 8A). Nearly the same biophysical parameters are determined for single K274E-K2P1 channels, arguing against nonspecific effects from SENP-1 expression (Figure 8B).

Discussion

Posttranslational modification by SUMO has previously been shown to mediate import and export of target proteins from the nucleus, to regulate protein-DNA and protein-protein interactions, and to protect against ubiquitin-mediated degradation (Melchior et al., 2003). This report demonstrates the operation of the SUMO pathway at the plasma membrane and its role in regulating ion channel function. Vertebrates have three SUMO genes that are ubiquitously expressed (Hochstrasser, 2001). The first identified vertebrate target was RanGAP1, where sumoylation determines return from cytosol to the nuclear pore complex (Mahajan et al., 1998); additional substrates were described soon thereafter (Melchior, 2000). Although the majority of the ∼60 SUMO targets reported to date are nuclear proteins, it is perhaps sensible to discover the SUMO system active and resident in the plasma membrane. In *S. cerevisiae*, where SUMO-activating, -conjugating, and cleavage enzymes were first delineated, substrates include the septins, proteins essential for cell separation (Johnson and Blobel, 1999) that move near the plasma membrane at the neck between mother and daughter cells, the location of Siz1, an E3 factor that assembles with Ubc-9 to confer substrate specificity (Johnson and Gupta, 2001). Other suggestive findings are immunostaining of Ubc-9 in the cytosol of the mammalian cell lines HEK293, CHO, and NIH/3T3 (Machon et al., 2000); identification of Ubc-9 as a binding partner in yeast two-hybrid screens for the epithelial sodium channel ENaC (Malbert-Colas et al., 2003) and the intracellular glucose transporter GLUT4 (Giorgino et al., 2000); and influence of an E3 factor on voltage-gated channels in rat heart (Kuryshev et al., 2000). Significantly, Giorgino and colleagues (Giorgino et al., 2000) found Ubc-9 to alter GLUT4 abundance in L6 myoblasts on overexpression and to share distribution with the transporter as judged by membrane fractionation, showing high levels in intracellular membranes that cluster beneath the plasma membrane.

In this work, SUMO modification of lysine 274 is shown to silence K2P1 channels and removal of SUMO to allow channel activity. Mutation of lysine 274 yields K2P1 channels that are not subject to sumoylation and

are thus constitutively active. Single-channel studies reveal that sumoylation is readily reversible and proceeds in the plasma membrane. Native SUMO-conjugating enzyme (Ubc-9) is shown to be at the plasma membrane in a uniform manner in naive oocytes but to redistribute in polarized fashion with K2P1 on their coexpression. K2P1 is demonstrated to be decorated with native SUMO by endogenous conjugating enzyme under basal conditions and SUMO protease shown to remove the peptide adduct. Silence of surface K2P1 channels (in the absence of overexpressed SENP-1 protease) can therefore be understood to result from operation of the native SUMO conjugation machinery in oocytes (Saitoh et al., 1998) and mammalian cells (Machon et al., 2000). In contrast, other SUMO targets studied in experimental cells show a small fraction of their population to be sumoylated at baseline; this suggests existence of a pathway to regulate K2P1 desumoylation in cells where the channel is active in vivo.

Delineating the attributes of active K2P1 channels has resolved an outstanding controversy as to their nature. K2P1 channels are classical potassium-selective, openly rectifying background channels that show TASK-like acid sensitivity (that is, blockade via protonation of a pore-located histidine). Thus, K2P1 currents are greater in the outward direction under physiological conditions of high internal and low external potassium and of similar magnitude in both directions under symmetrical conditions. Active K2P1 single channels show nominal voltage dependence in their open probability (Figure 7A), a subject of ongoing study. The initial notion that K2P1 channels were weak inward rectifiers (TWIKs) was based on observation of outward currents that decreased at positive potentials when the gene was expressed in oocytes (Lesage et al., 1996); this behavior is not seen with active K2P1 channels (Figures 1F, 4B, and 7), suggesting that one or more native conductances contributed to the currents under study in that work.

The sumoylation site in K2P1, -LK^{274}KF-, is a variant of the classical -ψKxE/D- motif, where ψ is an aliphatic residue (Rodriguez et al., 2001; Melchior et al., 2003). This is not unexpected, as other variant sites have been demonstrated (i.e., -VK*YC- and -TK*ET- in the nuclear factors smad4 and PCNA, respectively). This suggests that motif variation will not differentiate acceptors sumoylated at the plasma membrane from other targets. While other potential SUMO modification sites are present in K2P1, our findings indicate that only K274 is subject to modification in oocytes. The motif and homologous lysine are present in all reported K2P1 clones (e.g., human, chimpanzee, fowl, guinea pig, mouse, rabbit, and rat).

A more complete biophysical characterization of K2P1 should help advance the process of determining the contribution of individual K2P channels to native currents. K2P1 is seen here to share sensitivity to protons via the same mechanism as K2P3 and K2P9, two channels thought to contribute to the standing outward current I_{Kso} that is sensitive to hypoxia and extracellular acidosis and key to activity of cerebellar granular neurons, CGN (Plant et al., 2002). Our findings support speculation that K2P1 may contribute to I_{Kso}, given that K2P1 mRNA is abundantly expressed in rat brain with highest levels in CGN (Talley et al., 2001). Moreover, Han and colleagues (Han et al., 2002) observed four channels in rat CGN that they attributed to K2P3, K2P9, K2P10, and a "type 4" current unlike any known K2P channel. The attributes of type 4 channels are quite similar to those we now can assign to active K2P1 channels (including a unitary conductance of 32 ± 2 pS under similar conditions, activity at all potentials, potassium selectivity, and block by external proton in the physiological pH range). Native currents in heart and kidney that correlate with K2P1 are more uncertain. K2P1 shares ~40% protein sequence identity with K2P6 and just ~20% with K2P3 and K2P9, although the latter carry the homologous histidine in the first P loop that confers sensitivity to lowered external pH.

Control of K2P1 via sumoylation adds reversible, covalent peptide linkage to the plethora of posttranslational modifications recognized to regulate ion channel function. The findings raise intriguing questions. Tight regulation of K2P channel activity is a hallmark of these portals that control cellular excitability (Goldstein et al., 2001). Thus, as-yet unknown pathways that control K2P1 sumoylation will modulate cellular excitability. SUMO influences some targets secondarily via suppression of ubiquitination or changes in binding to a receptor (Hochstrasser, 2001). Is this also the case for K2P1, or does SUMO directly alter channel function as do many other ion channel accessory subunits? Additional plasma membrane proteins are SUMO modified (data not shown); how this influences their operation is unknown. Indeed, sumoylation may prove to explain the silence of other ion channels that have yet to reveal their function.

Experimental Procedures

Molecular Biology

Human K2P1 (accession number NM_002245) was subcloned into pRAT, a dual-purpose expression vector (Bockenhauer et al., 2001) containing a CMV promoter for mammalian expression and a T7 promoter for cRNA synthesis. Human SUMO-1 (accession number BCOO5899), Ubc-9 (accession number BC004429), and SENP-1 (accession number BC045639) were amplified from a brain cDNA library (Clontech, Palo Alto, California) and inserted into pCR II TOPO (Invitrogen, Carlsbad, California) at XhoI and BamHI in the case of SUMO-1 and XbaI and HindIII in the case of Ubc-9 and SENP-1, and subcloned into pMAX, a version of pRAT with an altered MCS. pEGFP was amplified from pEGFPC3 (Clontech) and ligated to the N terminus of K2P1 with EcoRI and PstI. EGFP was inserted in SUMO-1 between XbaI and HindIII. Mutagenesis was carried out with a QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, California). 1d4 epitope (RVPDGDPDETSQVAPA) was introduced into K2P1 by mutating the stop codon to create an MluI site that was used to insert the epitope. cRNA was synthesized with an mMESSAGE mMACHINE kit after vector linearization (Ambion, Austin, Texas). HA-SUMO was produced by amplifying with a forward primer containing the code for the HA epitope (YPYDVPDYA) before the start codon. GST-SENP-1 was constructed by inserting SENP-1 between BamHI and SalI sites in pGEX6P1. GST-C603S-SENP-1 was produced by site-directed mutagenesis.

Yeast Two-Hybrid Analysis

Yeast strain HF7C was transformed with K2P1 C terminus (residues 260–336) in the vector pGBT-9 and Ubc-9 in pGAD-424. Transformants were plated on plates lacking tryptophan, leucine, and histidine. Red colonies appeared after 4 days with a positive interaction. The interaction was confirmed by a qualitative β-galactosidase assay (Clontech).

Biochemistry

cRNA encoding study proteins was injected in 250 *Xenopus laevis* oocytes. Extracts were obtained by grinding cells in lysis 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 Applied Science, Indianapolis, Indiana). The SUMO adduct is sensitive to proteolysis in the absence of NEM. 1d4 antibody (NCCC, Minneapolis) was used as before (Kim et al., 2004). Oocyte extract was incubated with 1d4-coated Sepharose beads for 2 hr at 4°C. Unbound material was removed with washing buffer containing (in mM) 300 NaCl, 40 KCl, 1 EDTA, 20 HEPES-KOH (pH 7.4), 0.5% Triton X-100, and protease inhibitor tablets. Bound proteins were eluted with 1d4 peptide (1 mg/ml). Eluates were subjected to SDS-PAGE analysis and Western blotting with anti-1d4 mAb (1:2000, NCCC), anti-SUMO mAb (1:200, 18-2306, Zymed Laboratories, San Francisco), anti-HA mAb (1:1000, 1867423, Roche Applied Science) or anti-GFP pAb (1:3000, R970-01, Invitrogen). The antibody to human SUMO-1 recognizes *Xenopus* SUMO-1, as the two are 92% identical. GST-SENP-1 was produced in bacterial strain BL21(DE3). SUMO cleavage from K2P1 was carried out by incubation with GST-SENP-1 by incubation for 60 min at 30°C in (in mM) 150 NaCl, 1 DTT, and 10 Tris-HCl (pH 7.4), as by others (Nishida et al., 2001).

Immunofluorescence

Oocytes were fixed in 1% paraformaldehyde in ND-91 (in mM: 91 NaCl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES [pH 7.5] with NaOH) for 1 hr. Oocytes were rinsed in PBS and blocked with 10% goat serum, 0.5% BSA, 0.1% Triton X-100 in PBS for 45 min followed by incubation with primary antibody in the blocking solution for 2 hr (1d4 antibody 1:200 or Ubc-9 antibody, 610749, BD Pharmingen, San Diego, California, 1:20). After thorough washing with PBS containing 0.1% Triton X-100, oocytes were incubated with secondary antibody for 30 min (1:100, anti-mouse FITC or Texas red, Jackson Labs, Bar Harbor, Maine) and then washed for study.

Electrophysiology

Two-Electrode Voltage Clamp

Defolliculated oocytes were injected with 15 ng *K2P1* cRNA with or without 5 ng *SENP-1* cRNA. Whole-cell currents were measured (Geneclamp 500, Axon Instruments, Union City, California) 48–72 hr after injection. Electrodes were filled with 3 M KCl and had resistances of 0.1–1 MΩ. Data were sampled at 5 kHz and recorded using Clampex software. Data analysis was performed using Clampfit 9.0, Excel, and Origin 6.0. Standard bath solution was (in mM) 96 NaCl, 4 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES (pH 7.5) with NaOH. Potassium levels were altered by isotonic substitution of KCl for NaCl.

Single-Channel Recording

Single channels were assessed in cell-attached or excised inside-out patches from oocytes after removal of vitelline by hypotonic stripping. Bath and pipette solutions were (in mM) 140 KCl, 0.3 $CaCl_2$, 1 $MgCl_2$, 5 HEPES (pH 7.4) with KOH. Potassium levels were altered by isotonic substitution of KCl for NaCl. Data were recorded with an Axopatch 200B amplifier using pCLAMP software (Axon Instruments, Claremont, California) at filter and sampling frequencies of 2 kHz and 10 kHz, respectively. Data were filtered at 1 kHz for off-line analysis using Clampfit 9.0. Open probabilities were determined in patches judged to contain only one channel. Events were detected using a half-amplitude threshold technique (Colquhoun and Sigworth, 1995). All experiments were performed at 20°C–22°C.

Whole-Cell Recording

COS-7 (ATCC, Manassas, Virginia) were transfected with plasmids carrying study genes and EGFP with lipofectamine (Invitrogen) as before (Bockenhauer et al., 2001). After 24 to 48 hr, currents were assessed by whole-cell patch-clamp recording using an Axopatch 200B amplifier at filter and sampling frequencies of 2 kHz and 10 kHz, respectively. Borosilicate glass pipettes were filled with a solution of (in mM) 140 KCl, 0.5 $CaCl_2$, 5 EGTA, 10 HEPES (pH 7.4) with KOH and had resistance of 3–5 MΩ. Cells were bathed in (in mM) 120 NaCl, 20 KCl, 0.3 $CaCl_2$, 1 $MgCl_2$, 10 HEPES (pH 7.4) with NaOH. Whole-cell capacitance did not differ between groups. Currents are normalized to capacitance for each cell and expressed as mean ± SEM.

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