Protein Kinase A Is Central for Forward Transport of Two-pore Domain Potassium Channels K_{2p}3.1 and K_{2p}9.1*

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Acid-sensitive two-pore domain potassium channels (K_{2p}3.1 and K_{2p}9.1) play key roles in both physiological and pathophysiological mechanisms, the most fundamental of which is control of resting membrane potential of cells in which they are expressed. These background “leak” channels are constitutively active once expressed at the plasma membrane, and hence tight control of their targeting and surface expression is fundamental to the regulation of K^{+} flux and cell excitability. The chaperone protein, 14-3-3, binds to a critical phosphorylated serine in the channel C termini of K_{2p}3.1 and K_{2p}9.1 (Ser^{393} and Ser^{373}, respectively) and overrides retention in the endoplasmic reticulum by βCOP. We sought to identify the kinase responsible for phosphorylation of the terminal serine in human and rat variants of K_{2p}3.1 and K_{2p}9.1. Adopting a bioinformatic approach, three candidate protein kinases were identified: cAMP-dependent protein kinase, ribosomal S6 kinase, and protein kinase C. In vitro phosphorylation assays were utilized to determine the ability of the candidate kinases to phosphorylate the channel C termini. Electrophysiological measurements of human K_{2p}3.1 transiently expressed in HEK293 cells and cell surface assays of GFP-tagged K_{2p}3.1 and K_{2p}9.1 enabled the determination of the functional implications of phosphorylation by specific kinases. All of our findings support the conclusion that cAMP-dependent protein kinase is responsible for the phosphorylation of the terminal serine in both K_{2p}3.1 and K_{2p}9.1.

Two-pore domain (K_{2p}) potassium channels are widely expressed and play a vital role in stabilizing cellular membrane potential. The acid-sensitive K_{2p} subgroup (TASK) K_{2p}3.1 (also known as TASK1) and K_{2p}9.1 (TASK3) are inactivated by low external pH (1). TASK channels are implicated in playing regulatory roles in cell proliferation (and oncogenesis), chemoreception, activation of T-cells, and have neuroprotective roles in response to ischemia and inflammation (1). These channels are sensitive to an array of physiological and pharmacological modulators, including extracellular acidification, hypoxia, local anesthetics, and endocannabinoids (2–10). K_{2p} channels are constitutively active once expressed on the cell surface and therefore “leak” potassium from the cell via highly regulated pathways. Consequently, these channels are key contributors to the resting membrane potential of cells and are therefore vital components in determining the characteristics of cell excitability. The control of channel surface expression and targeting to the cell membrane are of critical importance because any change in the channel number at the plasma membrane influences the electrical properties of the cell in which they are expressed. K_{2p} channel activity is regulated via quality control pathways, which control the targeting, cell surface delivery, and recovery of these channels.

We and others have demonstrated that the export of newly synthesized K_{2p}3.1 channels from the endoplasmic reticulum to the cell surface is subject to tight quality control mechanisms (11–15). Although the exact roles and binding domains of regulators are disputed, there is general agreement that regulation of forward transport of K_{2p}3.1 and K_{2p}9.1 is dependent on their interaction with either 14–3–3, a ubiquitously expressed chaperone protein, or βCOP, a component of the multiprotein coatamer-coated vesicle COPI. Dibasic endoplasmic reticulum retrieval motifs bind βCOP and inhibit forward transport of the channel to the membrane, whereas recruitment of 14–3–3 to a non-canonical 14–3–3 binding motif (MKRRESSV-cooh) at the C terminus of TASK channels on the COPI comprises the βCOP binding and enables forward transport of the channels to the membrane (11, 12, 14). We previously observed 14–3–3 recruitment to the K_{2p}3.1 C terminus to be dependent on the phosphorylation of the penultimate C-terminal residue (−RRSPSV, where pS denotes phosphorylated serine). Disruption of this C-terminal motif eliminated 14–3–3β binding to the channel and trafficking of the channel to the plasma membrane. Replacing the terminal five residues with a canonical 14–3–3 motif, as found in Raf1 kinase (−RSApSEP), restored both 14–3–3β binding and cell surface expression (11).

14–3–3 proteins are an abundant, highly conserved family of ubiquitously expressed adapter proteins that modulate a multitude of functionally diverse proteins, including functional expression of an expanding number of ion channels (16, 17). 14–3–3 binds to phosphoserine-containing motifs in a sequence-specific manner; our previous studies have shown that, although there are two potential phosphorylation sites within the C terminus of human K_{2p}3.1, 14–3–3 binding is dependent on phosphorylation of the terminal serine, Ser^{393} (11, 12, 14). A hitherto undetermined kinase is responsible for this phosphorylation. Here we investigate the phosphorylation

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| Channel | Experimental system | Sequence of C terminus | Mutation |
|---------|---------------------|------------------------|----------|
| K<sub>2P</sub>3.1 human | Electrophysiology (full-length); in vitro kinase assays (C terminus fused to GST) | SLSTFRGGLMKRRSSV | Wild type |
| GFP-K<sub>2P</sub>3.1-HA rat | Immunofluorescence; flow cytometry | SFTDHQRLMKRRSSV | Wild type |
| K<sub>2P</sub>9.1 human | Electrophysiology (full-length); in vitro kinase assays (C terminus fused to GST) | TCGENHRHHRKSSV | Wild type |
| K<sub>2P</sub>9.1 rat | In vitro kinase assays (C terminus fused to GST) | S373A |
| GFP-K<sub>2P</sub>9.1-His rat | Flow cytometry | S395A |

of the human (h) and rat (r) K<sub>2P</sub>3.1 and K<sub>2P</sub>9.1 channels and present in vitro and in vivo evidence to demonstrate that the cAMP-dependent protein kinase (protein kinase A; PKA)<sup>2</sup> is responsible for phosphorylation of the terminal serine of K<sub>2P</sub>3.1 and K<sub>2P</sub>9.1, thus enabling forward transport of these channels.

**EXPERIMENTAL PROCEDURES**

Molecular Biology—Table 1 summarizes the constructs used and shows the sequence of the C-terminal 15 amino acids of each. WT hK<sub>2P</sub>3.1 DNA in the vector pRAT was produced as described previously (11). Mutations to create alanine or aspartate substitutions in the C terminus of the full-length channel were made with an XL QuikChange kit (Stratagene, La Jolla, CA) and confirmed by DNA sequencing. HA-(YPYDVPDYA)-tagged rK<sub>2P</sub>3.1 was created by QuikChange PCR mutagenesis of full-length rK<sub>2P</sub>3.1 fused to N-terminal eGFP (a gift from Dr. R. Preisig-Müller, Philipps Universität, Marburg, Germany) in pcDNA3.1 (Invitrogen). The tag was introduced to an external loop of the second pore-forming domain, between alanine 213 and leucine 214. Similarly, a His (HHHHHH) tag was introduced into GFP-rK<sub>2P</sub>3.1 between Ala<sup>213</sup> and Leu<sup>214</sup>.

The glutathione S-transferase (GST)-conjugated C terminus of hK<sub>2P</sub>3.1 was created by annealing phosphorylated oligonucleotides encoding the final 15 amino acids of WT hK<sub>2P</sub>3.1 (5’-AAT TCC ACA GAT TAT CCA GAT TTC GTC TCA TGA AGC GAA GAA GCT CAG TGT AAT CTA GAG GC-3’ and 5’-GGC CGC TCG ATT ACA ATG ATG TCT GTC TCA TGA GAC CTC GTA AGT TCG ATA AAC TGT GG-3’) and ligating them into the pGEX-6P-1 plasmid (GE Healthcare) between the EcoRI and NotI sites in the multiple cloning site. Corresponding hK<sub>2P</sub>9.1 and rK<sub>2P</sub>9.1 constructs were made in a similar manner (hK<sub>2P</sub>9.1, 5’-AAT TCA GCT TTA CGC ACC ACC AGA GGC TGA TGA ACG GAC AGT CCG TTT AAA CTC CGA GC-3’ and 5’-GGC CGC TCG AGG TTT AAA CGG ACT TCC GCC GGT TTA TCA TCA GCC TTT GGT CGG TAA AGC TG-3’; rK<sub>2P</sub>9.1, 5’-AAT TCA TTA CGG GGG AAA ACC ACA GGC TGC ACA TCC GTC GCA AGT CCA TTA AAA CCA CGA GC-3’ and 5’-GGC CGC CG TCG AGG TTT AAA TGG ACT TGC GAG GCA TGT GCA GCC TGT GGT TTC CCC CGC AGG TG-3’). Mutations were introduced by PCR, using the Stratagene QuikChange kit (Agilent Technologies UK Ltd., Stockport, UK), and all cDNAs and mutants were confirmed by DNA sequencing.

Recombinant Fusion Protein Expression— Cultures of Escherichia coli strain BL21 (DE3), containing constructs of hK<sub>2P</sub>3.1 and hK<sub>2P</sub>9.1 C termini fused to GST or GST alone, were grown at 37 °C, 200 rpm in 100 ml of LB medium plus 100 μg/ml ampicillin to OD 0.5 and then induced with 0.5 mM IPTG for 3 h. Bacteria were harvested by centrifugation and resuspended in 2 ml of PBS, 1 mM EDTA containing 2× Complete Protease Inhibitor Mixture (Roche Applied Science). Triton X-100 was added to 1% (v/v). Bacterial suspensions were sonicated in an ice water bath by a Misonix 3000 Sonicator (Misonix Inc., Farmingdale, NY) with cup horn probe for 3 × 1 min at power setting 1.5 and then centrifuged at 17,000 × g for 20 min at 4 °C. The supernatant was used as a substrate for in vitro phosphorylation assays.

In Vitro Phosphorylation Assays—The DNA sequence of the catalytic subunit of human PKA (PKAc) was amplified by PCR and cloned into the vector pET30 Ek/LIC (Novagen, Nottingham, UK). Recombinant His-tagged PKA was purified from the soluble supernatant of 180-mld electroeluted in imidazole, and dialyzed into kinase buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 0.1% 2-mercaptoethanol, 10 mM MgCl2, 0.5 mM ATP in a 180-ml total volume. RSK2 assays contained 45 μl of sonicate supernatant (typically 160 μg of total protein), 1.5 μg of PKA, 50 μM Tris, pH 7.4, 100 mM NaCl, 0.1% 2-mercaptoethanol, 10 mM MgCl2, and 100 μM ATP in a 180-μl total volume. RSK2 assays contained 45 μl of sonicate supernatant, 1.5 μg of RSK2, 50 mM Tris, pH 7.4, 10 mM MgCl2, 2 mM DTT, 1 mM EGTA, 0.01% Triton X-100, and 100 μM ATP in a 180-μl total volume. PKCα, MAPK I, and casein kinase II were purchased from Invitrogen. PKA assays contained 45 μl of sonicate supernatant (typically 160 μg of total protein), 1.5 μg of PKA, 50 μM Tris, pH 7.4, 100 mM NaCl, 0.1% 2-mercaptoethanol, 10 mM MgCl2, and 100 μM ATP in a 180-μl total volume. PKC assays contained 45 μl of sonicate supernatant, 0.7 μg of PKCα, 50 mM Tris, pH 7.4, 100 mM NaCl, 0.1% 2-mercaptoethanol, 10 mM MgCl2, 100 μM ATP, and 39 μl of lipid activator mix (Millipore, Watford, UK) in a 180-μl total volume. Samples were incubated for 30 min at 30 °C, and then the fusion proteins were purified using affinity resin. Glutathione-Sepharose was prepared according to the manufacturer’s instructions (GE Healthcare) and 100 μl of slurry was added per sample. Tubes

2 The abbreviations used are: PKA, protein kinase A; eGFP, enhanced green fluorescent protein; RSK, ribosomal protein S6 kinase; 8-Br-cAMP, 8-bromo-cyclic AMP.
were rotated end-on-end for 1 h at room temperature. Samples were centrifuged at 500 × g for 5 min at room temperature, and the Sepharose pellet was washed three times with 10 volumes of PBS, 1% (v/v) Triton X-100 and two times with 10 volumes of 50 mM Tris, pH 8.0. Fusion proteins were eluted twice from the resin, each time with 1 volume of 10 mM reduced glutathione, 50 mM Tris, pH 8.0, for 10 min at room temperature, and then the eluates were pooled and frozen at −20 °C. Positive control substrates for PKCa, MAPK, and casein kinase II were histone H1 and myelin basic protein (Millipore) and the casein kinase II substrate peptide RRRDDDSDDD fused to GST as described above.

**Phos-tag Electrophoresis**—Detection of phosphorylated proteins was achieved by SDS-PAGE incorporating Phos-tag acrylamide (Phos-tag Consortium, Hiroshima, Japan). 10% polyacrylamide gels (29:1 acrylamide/bisacrylamide) containing 50 μM Phos-tag ligand and 50 μM MnCl₂ were prepared according to the manufacturer’s instructions. Eluted proteins were mixed with sample buffer, heated for 5 min at 95 °C, and electrophoresed at 30 mA/gel at room temperature. Gels were fixed and stained using InstantBlue (Expedeon, Cambridgeshire, UK).

**Two-electrode Voltage Clamp Recordings**—Oocytes were isolated from Xenopus laevis toads (Nasco, Atkinson, WI), treated with collagenase to ease removal of the follicular layer, and injected with 0.1–1 ng of K₂p.3.1 WT or mutant channel cRNA in 46 nl of sterile water. Currents were measured 48 h after injection by two-electrode voltage clamp (Warner Instruments Corp., Hamden, CT). Data were filtered at 1 kHz and sampled at 4 kHz. Electrodes of 1.5-mm borosilicate glass tubes (Garner Glass Co., Claremount, CA) contained 3 m KCl and had resistances of 0.3–1 millionohm. Recordings were performed at room temperature with perfusion of 0.4–1.0 ml/min ND96 (93 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 0.3 mM CaCl₂, 5 mM HEPES, pH 7.5 or 6.5, with NaOH or HCl). Holding potential was −80 mV. Currents were evoked by step depolarization from −135 to +60 mV in 15-mV increments. Acid sensitivity was routinely determined (data not shown) by exchanging external bath solutions during repeated step depolarization from holding potential to +30 mV at 10-s intervals.

**Whole Cell Patch Clamping Recordings**—HEK293 cells were plated in 10-cm dishes, 2 × 10⁶ cells/dish, and transfected transiently with 10 μg of GFP-rK₂p.3.1-HA, GFP-rK₂p.9.1-His, eGFP alone, or empty pcDNA3.1 (Invitrogen), as described above. After overnight incubation, cells were harvested using trypsin, pooled, and replated to obtain a single starting population with the same efficiency of transfection. Cells were then grown in the presence or absence of 8-Br-cAMP (concentration indicated in the figure legend) for 16 h, before harvesting by scraping, staining on ice with mouse monoclonal anti-HA tag antibody (clone 16B12, Covance (Leeds, UK)) at 1:400 dilution, mouse monoclonal anti-His tag (catalog no. 70796-3, Novagen, VWR International, Leicestershire, UK) at 1:400 dilution, mouse monoclonal anti-His (catalog no. 70796-3, Novagen, VWR International, Leicestershire, UK) at 1:400 dilution, an isotype control, or without antibody. Cell lysates were centrifuged at 5000 × g for 5 min, and the supernatant was examined by Western blotting using a FACS-Canto (BD Biosciences), collecting 10⁵ transfected cells or 10⁵ cells in total for empty vector samples.

**Microscopy**—HEK293 cells were transfected transiently with plasmids encoding epitope-tagged PKA, PKC, MAPK, and casein kinase II. Green fluorescent proteins (GFP) were used for visualization. After 16 h, cells were fixed and stained with primary antibodies (1:500 dilution) and Alexa Fluor 647 (Invitrogen) (1:1000 dilution). Cells were then analyzed by flow cytometry using a FACSCanto (BD Biosciences), collecting 10⁵ transfected cells or 10⁵ cells in total for empty vector samples.
PKA Regulates Forward Transport of K_{2p}3.1 and K_{2p}9.1

Expression of Functional hK_{2p}3.1 in Xenopus Oocytes—Phosphorylation of the terminal serine in the hK_{2p}3.1 C terminus is critical for 14-3-3 interaction with the channel (11). Similarly, in rK_{2p}3.1, substitution of this serine with an alanine (S410A) causes loss of current and membrane expression in Xenopus oocytes (12). We tested the effect of substituting amino acids at the channel C terminus on the function of hK_{2p}3.1 expressed in Xenopus oocytes (Fig. 1A). Substituting the terminal serine for alanine (S393A) resulted in a total loss of current, as did either the double substitution S392A/S393A or the removal of the terminal valine residue (ΔV394). These mutations are known to result in the abrogation of 14-3-3 binding (11, 12, 15) and thus prevent the channel from being correctly targeted to the plasma membrane. Of interest, no significant difference in current expression of the channel was observed when S392A substitution was performed (data not shown); this is in agreement with previous reports, which propose that this residue is not critical to 14-3-3 binding (11). Substitution of the terminal serine for aspartate (S393D), in an attempt to mimic the negative charge of a phosphoserine residue, restored some current (0.81 μA at 60 mV, S.E. = 0.28, n = 6 versus the control (2.97 μA, S.E. = 0.37, n = 4)). Fig. 1B shows the surface current expressed in Xenopus oocytes at 60 mV for each mutant channel relative to wild type hK_{2p}3.1. The reduction in current expression for each of the mutants is significant (p ≤ 0.01 by Student’s t test or Mann-Whitney rank sum test), confirming the critical nature of the terminal serine of hK_{2p}3.1 for functional channel expression. Currently, the kinase responsible for phosphorylating this residue is unknown.

Phosphorylation of hK_{2p}3.1 and hK_{2p}9.1—We analyzed the sequences of human and rat K_{2p}3.1 and K_{2p}9.1 using the NetPhosK 1.0 server (Danish Technical University) (18) to predict candidate kinases for Ser^{392} (hK_{2p}3.1), Ser^{410} (rK_{2p}3.1), Ser^{373} (hK_{2p}9.1), and Ser^{395} (rK_{2p}9.1) (Table 2). For all channels, PKA was predicted as the most likely candidate to phosphorylate the terminal serine (predicted score range 0.87–0.89; maximum score = 1.0); RSK and PKC were predicted with much lower probabilities (RSK predicted score range = 0.61–0.70; PKC predicted score range 0.68–0.74; Table 2). The C-terminal 15 residues of K_{2p}3.1 contain an additional serine residue, Ser^{392} (hK_{2p}3.1) or Ser^{409} (rK_{2p}3.1), and this also conforms to the known site specificity for PKA. However, Ser^{409} is not required for surface expression of rK_{2p}3.1 (12) (data not shown), as demonstrated by substitution of Ser^{392} by alanine causing no significant inhibition of channel current when expressed in Xenopus oocytes. Aside from the terminal serine residues, C-terminal residues Ser^{382} and Thr^{383} in hK_{2p}3.1 are predicted to be phosphorylated by PKC and cyclin-dependent kinase 2, respectively, and Thr^{400} of rK_{2p}3.1 is predicted to be phosphorylated by PKC (Table 2).

To determine if the predicted kinase could phosphorylate the terminal serine of these channels, the C-terminal 15 amino acids of each channel fused to GST were expressed in E. coli. We conducted in vitro phosphorylation assays using crude lysate supernatant as a substrate and recombinant purified PKA, RSK2, and PKCα. RSK2 was chosen because it exhibits the tissue distribution pattern most similar to those of K_{2p}3.1 and K_{2p}9.1 (14, 19–22). We also selected two serine/threonine-specific kinases, MAPK I (ERK1) and casein kinase II, which were not predicted to phosphorylate the C-terminal motifs. Fig. 2 shows the purified fusion proteins after separation by standard (A and C) and Phos-tag (B and D) SDS-PAGE, a non-radioactive technique that reduces the electrophoretic mobility of phosphorylated proteins relative to their non-phosphorylated counterparts (23).

PKA phosphorylated GST-hK_{2p}3.1 (Fig. 2D), as shown by the upward bandshift relative to the control incubations of the fusion proteins without kinase (Fig. 2D, Con lane) and relative to GST alone (Fig. 2B). For hK_{2p}3.1, there are three distinct bands (Fig. 2D, PKA lane). The first (marked 0P) migrates at a position corresponding to the fusion protein incubated without...
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PKA and RSK phosphorylate $K_{2p}.3.1$ at Ser393 in vitro. Lysates of E. coli expressing fusion proteins were added to ATP and purified kinases, and then the fusion proteins were purified using glutathione-Sepharose. Purified proteins were subjected to either standard or Phos-tag SDS-PAGE, as indicated to the left, and then stained with Coomassie Blue. Con, control (no kinase); CKII, casein kinase II; MK, MAPK I. As a control, GST alone was incubated with the purified kinases (A and B). C and D, wild type $K_{2p}.3.1$ C terminus. Bands marked 1P and 2P represent single- and double-phosphorylated proteins, respectively. The band marked with an asterisk is most likely to be co-purifying MAPK I. E, $K_{2p}.3.1$ wild type (SSV) compared with mutant S393A (SAV). Panel F, $K_{2p}.3.1$ wild type (SSV) compared with S392A/S393A (AAV).

### TABLE 2
Phosphorylation sites in the C-terminal 15 amino acids of $K_{2p}.3.1$ and $K_{2p}.9.1$, as predicted by the NetPhosK 1.0 server

| Channel     | Sequence of C terminus | Residue | Kinase     | Score (cut-off = 0.5, maximum = 1.0) |
|-------------|------------------------|---------|------------|----------------------------------|
| $K_{2p}.3.1$ human | 380SLATFRGLMKRRS^SV|^Ser^ | PKA      | 0.88 |
|             |                        |         | PKC      | 0.74 |
|             |                        |         | RSK      | 0.67 |
|             |                        |         | CKI      | 0.54 |
| $K_{2p}.3.1$ rat | 397SLATFRGLMKRD^SV|       | PKA      | 0.75 |
|             |                        |         | PKC      | 0.90 |
|             |                        |         | RSK      | 0.70 |
|             |                        |         | CKI      | 0.54 |
| $K_{2p}.9.1$ human | 360SFTDHQLRKRRS^SV|^Ser^ | PKA      | 0.88 |
|             |                        |         | PKC      | 0.88 |
|             |                        |         | RSK      | 0.68 |
|             |                        |         | CKI      | 0.68 |
| $K_{2p}.9.1$ rat | 382TCHENHRLHR^S|^Ser^ | PKA      | 0.87 |
|             |                        |         | RSK      | 0.61 |

kinase (Con lane), which represents a population of unphosphorylated fusion proteins. The second band (marked 1P) corresponds to a population of molecules stoichiometrically phosphorylated at a single serine, whereas the third population (2P) is phosphorylated at both serines. That the respective band shifts are due to phosphorylation of GST-hK2P3.1 at Ser392 and Ser393 is confirmed by the loss of the uppermost band in the mutant S393A, where the terminal serine has been mutated to the non-phosphorylatable alanine (Fig. 2E, S393A, PKA lane) and both bands in S392A/S393A (Fig. 2F, S392A/S393A, PKA lane). RSK2 phosphorylated the majority of GST-hK2P3.1 at a single serine; this was abolished in the mutant S393A. By contrast, any phosphorylation of GST-hK2P3.1 by PKC is at the limit of detection, although the enzyme does phosphorylate a control substrate histone H1 (supplemental Fig. 1). Thus, in vitro, PKCa does not appreciably phosphorylate either Ser393 or indeed Ser392, the second residue in the C-terminal 15 amino acids of hK2P3.1 predicted to be a target for PKC. Negative control kinases MAPK and casein kinase II also failed to phosphorylate the fusion proteins but were active when presented with an appropriate substrate (supplemental Fig. 1).

We performed similar assays with both GST-hK2P9.1 and GST-rK2P9.1 (Fig. 3, A and B). In the presence of PKA, all of the fusion protein migrates more slowly than the control, indicative of phosphorylation-dependent decreased motility through the Phos-tag gel. By contrast, RSK2 phosphorylated only a minor proportion of the human and rat fusion proteins, and PKC phosphorylated almost none at all. When the terminal serine residues were mutated to alanine (C, S373A (hK2P9.1); D, S395A (rK2P9.1)), no phosphorylation by PKA, RSK2, or PKC was detected (a corollary of which is that any phosphorylation by PKC is not at residue Thr400). Thus, in vitro assays support the NetPhosK prediction that PKA is a good candidate to phosphorylate the terminal serine of both hK2P3.1 and human and rat K2P9.1. However, of the small panel of kinases tested, RSK2 is also able to phosphorylate the terminal serine of hK2P3.1 effectively in vitro.
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**Sensitivity of Membrane Expression of hK$_{2p3.1}$ to Kinase-modulating Drugs**—To test whether phosphorylation by PKA and RSK2 regulates plasma membrane targeting of hK$_{2p3.1}$, the channel was transiently expressed in HEK293 cells, in the presence and absence of the PKA activator, 8-Br-cAMP; two PKA inhibitors, myristoylated PKI (a specific inhibitor of PKA catalytic subunit) and the small molecule KT5720; or a highly specific RSK inhibitor, SL0101, which binds to an adenosine-interacting loop that is unique to RSK and differs markedly from other kinases, including PKA (24). Transfected cells were identified by co-transfecting GFP in a separate plasmid and selecting green fluorescent cells. K$_{2p3.1}$ is constitutively active at the cell surface, so measured K$^+$ flux is directly proportional to the number of channels inserted in the plasma membrane. We determined the current passed in transfected cells at different applied potentials, using whole-cell patch clamp (Fig. 4). Cells transfected with WT-K$_{2p3.1}$ (plus pEGFP-C1) show a negative shift in resting membrane potential from $-32.6$ mV ($S.E. = 1.16, n = 6$) in non- or pEGFP-C1 transfected cells to $-54.6$ mV ($S.E. = 3.12, n = 14$) in cells transfected with the channel. Currents evoked by step depolarization from $-100$ to $+90$ mV showed instantaneous outward currents, with a small time-dependent component, non-inactivating outward current, with a reversal potential negative of $-50$ mV typical of K$_{2p3.1}$. Currents also showed characteristic sensitivity to external acidosis (data not shown). For cells transfected with hK$_{2p3.1}$ and cultured in the presence of $0.4$ mM 8-Br-cAMP (Fig. 4A), increased current was observed at test potentials between $-50$ mV and $+90$ mV. At $60$ mV, evoked current increased from $0.59$ nA ($S.E. = 0.05, n = 7$) to $1.09$ nA ($S.E. = 0.14, n = 9$) in 8-Br-cAMP treated cells, with an associated modest negative shift in the resting membrane potential to $-58.6$ mV ($S.E. = 2.45, n = 7$). The effect of PKA enhancement by 8-Br-cAMP on serine-substituted channels S393A and S393D was also examined. HEK293 cells transfected with either hK$_{2p3.1}$ S393A or S393D failed to express whole-cell currents significantly different from that of non-transfected cells; furthermore, treatment with $0.4$ mM 8-Br-cAMP failed to enhance whole cell currents of non-transfected HEK293 cells or HEK293 cells transfected with either of the two phosphorylation mutant channels (Fig. 4B).

Unexpectedly, the RSK inhibitor, SL0101, causes an increase in current ($1.01$ nA, $S.E. = 0.12$, at $60$ mV, $n = 8$; Fig. 4A) although without a concomitant decrease in membrane potential ($V_m$ for RSK-treated cells was $-37.9$ mV; $S.E. = 2.05$ mV; $n = 5$) compared with non-treated matched controls ($-47.4$ mV, $S.E. = 4.47, n = 5$), suggesting that RSK inhibition may have either nonspecific effects on other channels expressed in HEK293 or additional (direct or indirect) regulatory effects on K$_{2p3.1}$. In addition, a nonspecific target of SL0101 might explain these data.

In a separate experiment (Fig. 4C), the effects of two different PKA inhibitors on current expression in HEK293 cells transfected with hK$_{2p3.1}$ were tested. Transfected cells were cultured in the presence of either $1$ mM KT5720 or $20$ mM PKI for $16$ h prior to whole-cell voltage clamp analysis. Both PKA inhibitors resulted in a decrease in current at all test potentials when compared with non-treated cells. Non-treated hK$_{2p3.1}$-transfected cells passed $1.15$ nA ($S.E. = 0.08, n = 8$) of current at $60$ mV, whereas cells treated with $1$ mM KT5720 or $20$ mM PKI showed reduced current of $0.81$ nA ($S.E. = 0.09, n = 6$) and $0.67$ nA ($S.E. = 0.06, n = 9$) at the same test potential. Statistical analysis indicates that all drug treatments are significantly different from the untreated control ($p = 0.05$; Fig. 4D). Similarly, hK$_{2p3.1}$ and hK$_{2p9.1}$ channels expressed in *Xenopus* oocytes demonstrated inhibition of their current by PKI comparable with that seen for these channels in HEK293 cells, with statistical analysis indicating that PKI treatment was significantly different from untreated controls ($p = 0.014$ for hK$_{2p3.1}$ and $p = 0.002$ for hK$_{2p9.1}$; supplemental Fig. 2). Taken together, our data provide strong evidence that functional PKA is necessary for plasma membrane expression of hK$_{2p3.1}$.

**Immunofluorescence of Tagged K$_{2p3.1}$**—We next sought to corroborate our cellular observations using confocal microscopy. HEK293 cells were transfected with rK$_{2p3.1}$ fused at the N terminus to eGFP and incorporating a non-interfering HA tag in an external loop of the second pore-forming domain (GFP-rK$_{2p3.1}$-HA). The external tag made it possible to compare the relative amounts of channel expressed at the cell surface under different conditions by staining with anti-tag antibodies. Transfected cells were incubated with 8-Br-cAMP or PKA inhibitors and then fixed (but not permeabilized) and stained with anti-HA tag antibody (Fig. 5). In control cells (Fig. 5, A–C), anti-HA staining (Fig. 5B, red) can be seen around the periphery of a subset of transfected cells; not all green fluorescent cells display detectable tagged channel on the plasma membrane, suggesting that aberrant folding and/or targeting of the
tagged channel may take place when expressed in HEK293 cells. In the presence of 8-Br-cAMP (Fig. 5, D–F), more HA-positive cells could be observed over multiple fields of view, and these cells stained more brightly than control cells. By contrast, when cells were incubated with PKA inhibitors KT5720 (Fig. 5, G–I) or PKI (J–L), anti-HA staining was weaker. In the presence of the RSK inhibitor SL0101 (Fig. 5, M–O), anti-HA staining was comparable or slightly brighter than the control. Cells transfected with eGFP-C1 alone were not markedly affected by the drugs (supplemental Fig. 3). Therefore, immunofluorescence evidence supports both the in vitro and functional evidence that PKA is the central kinase that regulates forward transport of K2P3.1.

**Effect of PKA Activation on Surface Expression of Tagged K2P3.1 and K2P9.1 Channels**—To obtain a quantitative measure of the effect of activating PKA on the surface expression of both K2P3.1 and K2P9.1, we used the rat variants of these channels with GFP fused to the channel N terminus and either an HA tag (K2P3.1) or His tag (K2P9.1) in the external loop of the second pore-forming domain (GFP-rK2P3.1-HA and GFP-rK2P9.1-His, respectively). HEK293 cells were transiently transfected with either one of the tagged channel constructs, eGFP-C1 alone or empty vector, and surface expression was analyzed by flow cytometry (Fig. 6). After transfection, cells were incubated overnight with or without 0.4 mM 8-Br-cAMP to activate PKA. Fig. 6A shows the distribution of GFP fluorescence (i.e. total expression of GFP-hK2P3.1-HA fusion protein within the cell population). The peaks under the horizontal marker represent transfected cells, as judged by analysis of cells transfected with vector alone and eGFP-C1 alone (supplemental Fig. 4). Activation of PKA with 8-Br-cAMP results in a relative increase in the number of brightly fluorescent cells (Fig. 6A, black line).

To determine the level of GFP-rK2P3.1-HA channel expressed at the plasma membrane, 10⁵ transfected cells from each treatment (green fluorescent cells, defined by the marker in Fig. 6A; see also supplemental Fig. 4) were compared for binding of anti-HA antibody (B, far red fluorescence). In agreement with the immunofluorescence data above, not all transfected cells stain positive for the externally facing HA tag. In this experiment, activation of PKA by 8-Br-cAMP treatment results in a 27% increase in the number of HA-positive cells relative to the control. There is an accompanying slight increase in the mean fluorescence intensity from 2036 (control) to 2206 arbitrary fluorescence units compared with the control (an 8% increase in mean fluorescence intensity). 8-Br-cAMP treatment did not increase the expression of GFP alone; nor did it alter the background fluorescence of cells transfected with empty vector (supplemental Fig. 4).

A similar pattern emerged for GFP-rK2P9.1-His surface expression in response to 8-Br-cAMP treatment. A 53% increase in the proportion of transfected cells that express detectable channel at the plasma membrane was detected as well as a 20% increase in mean fluorescence intensity from 740 to 890 arbitrary fluorescence units (Fig. 6D). From these observations, we conclude that activating PKA promotes forward transport of both K2P3.1 and K2P9.1 channels.
Precise control of cell surface expression of the acid-sensitive K$_{2p}$3.1 and K$_{2p}$9.1 is critically important in the control of membrane potential in both excitable and non-excitable cells. Here, we used bioinformatic analysis, in vitro assays, and recombinant functional assays to elucidate the kinase responsible for control of forward transport of these channels.

**DISCUSSION**

**FIGURE 5. Immunostaining shows PKA activation results in increased surface expression of K$_{2p}$3.1.** Cells transfected with GFP-K$_{2p}$3.1-HA were grown in the presence of DMSO (control; A–C), 50 μM 8Br-cAMP (D–F), 1 μM KT5720 (G–I), 20 μM PKI (J–L), or 50 μM SL0101 (M–O) and then fixed and stained with anti-HA tag antibody and an Alexa Fluor 647-conjugated secondary antibody. The cells were not permeabilized, so only surface-exposed HA tag in correctly targeted channel was accessible to the antibody. Images are a confocal z-stack. GFP, total expression of the fusion protein; HA, surface-exposed HA tag; Merge, composite image. Scale bar (A), 10 μm.
The C termini of both K_{2P}3.1 and K_{2P}9.1 contain a number of residues that are predicted targets for modulation by phosphorylation. Among these, some residues are already identified as being phosphorylated and playing a role in channel modulation, including PKC activation resulting in a 40% decrease in murine K_{2P}3.1 current over a 30-min time course (4). Also, hK_{2P}9.1 contains a C-terminal threonine (Thr341), which is a target for phosphorylation by PKC, with a resultant reduction in current amplitude (25). In 2002, we and others identified a phosphorylation-dependent forward transport pathway for K_{2P}3.1, which has subsequently been shown to be utilized by K_{2P}9.1 and a number of other proteins. Phosphorylation of the terminal serine of the acid-sensitive K_{2P} channels was shown to result in recruitment of 14-3-3, which we demonstrated to be critical for channel forward transport (11, 12, 14). 14-3-3 has also been shown to play a role in the intracellular transport of a number of other membrane proteins, including the KATP channel Kir6.2, invariant chain protein, nicotinic acetylcholine receptor (nAChR/H9251), kainate receptor KA2, and G-protein-coupled receptor 15 (11, 26). Furthermore, another K_{2P} channel member (K_{2P}18.1/TRESK) has recently been shown to be negatively regulated by 14-3-3 in a phosphorylation-dependent manner (27).

Serine substitution and targeted phosphorylation of peptides analogous to the terminal residues of K_{2P}3.1 demonstrated the critical nature of the terminal serine (and its phosphorylation) to 14-3-3 recruitment (11, 12). Here, we build upon the previously reported biochemical evidence by providing functional confirmation of the importance of the terminal serine (Ser^{393}) in hK_{2P}3.1. Mutation of the Ser^{393} to alanine, which can neither be phosphorylated nor mimic a phosphorylated residue, resulted in the channel failing to pass current when expressed in Xenopus oocytes. This correlates well with reports from Rajan et al. (12) for an equivalent residue (Ser^{410}) in the rat homolog.

To understand the physiological mechanism and signaling pathways that enable regulation of cell surface expression of K_{2P}3.1, it is crucial to identify the kinase responsible for phosphorylation of the serine critical to 14-3-3 recruitment (Ser^{393} in hK_{2P}3.1). Prior to this study, the identity of this kinase was unknown. Here we use algorithms to predict the kinases most likely to phosphorylate the C-terminal forward transport motif of the acid-sensitive channels (hK_{2P}3.1, rK_{2P}3.1, hK_{2P}9.1, and rK_{2P}9.1) and then exploit a combination of approaches to directly test these predictions: kinase assays to determine the ability of each kinase to phosphorylate the site in vitro, patch clamp analysis of HEK293 cells expressing hK_{2P}3.1 to determine K^+ current in the presence of kinase-modulating drugs, and immunofluorescence and flow cytometry to measure and quantify changes in the surface expression of channel in response to activating or inhibiting specific kinases.

**Figure 6.** Activation of PKA increases the number of K_{2P}3.1 and K_{2P}9.1 on the cell surface. Cells transfected with GFP-K_{2P}3.1-HA or GFP-K_{2P}9.1-His were grown in the presence or absence of the PKA activator 8Br-cAMP (0.4 mM) and then harvested and stained with anti-tag antibodies and an Alexa Fluor 647-conjugated secondary antibody before analysis by flow cytometry. A, GFP-K_{2P}3.1-HA, total expression as measured by GFP fluorescence. Shaded area, control; black line, 8-Br-cAMP. The horizontal marker indicates GFP-positive, transfected cells. B, 10^5 transfected cells from A were analyzed for surface expression of the channel, as detected by binding of antibody to the external HA tag. The numbers denote the proportion of transfected cells that stain positive for the HA tag. C, GFP-K_{2P}9.1-His, total expression as measured by GFP fluorescence. D, 10^5 transfected cells from C were analyzed for surface expression of the channel, as detected by binding of antibody to the external His tag. The numbers denote the proportion of transfected cells that stain positive for the His tag.
PKA Regulates Forward Transport of K_{2p}3.1 and K_{2p}9.1

For human and rat K_{2p}3.1 and K_{2p}9.1, PKA was predicted and subsequently demonstrated to be the most likely kinase to phosphorylate the terminal serine. Through serine substitution and mutation analysis, we further demonstrated that phosphorylation of the C-terminal fusion proteins was lost upon substitution of the terminal serine (Figs. 2 and 3). Two interesting observations were borne out by the in vitro kinase assays. (i) We observed that the ribosomal S6 kinase, RSK2, phosphorylated the hK_{2p}3.1 C-terminal fusion protein as effectively as PKA in vitro (Fig. 2). (ii) In vitro phosphorylation analysis also detected phosphorylation of Ser^{992} of hK_{2p}3.1 by PKA. Ser^{992} has previously been shown not to be required for 14-3-3 recruitment and hence forward transport of the channel (11, 12). Patch clamp analysis, flow cytometry, and immunocytochemistry studies of HEK293 transfected with WT hK_{2p}3.1 and cultured in the presence of PKA activators or inhibitors all confirm that activation of PKA resulted in an increase in hK_{2p}3.1 current expression (Figs. 4–6) and demonstrate the dynamic regulatory effect of PKA activity on K_{2p}3.1 channel expression.

RSK2 phosphorylated hK_{2p}3.1 at the critical serine (Ser^{993}) in our in vitro studies, and we hypothesized that if RSK2 phosphorylation was critical to Ser^{993} phosphorylation and hence forward transport of the channel, a reduction in hK_{2p}3.1 current is predicted in response to RSK2 inhibition. In contrast, we observed that current expression in cells transfected with hK_{2p}3.1 was increased when they were treated with the RSK inhibitor SL0101. Although this finding was unexpected, it does demonstrate that RSK phosphorylation of the channel is not critical for the channel to achieve cell surface expression. A number of potential explanations for the observed increase in K_{2p}3.1 current expression exist, including a role of RSK in acute channel modulation following cell surface expression of the channel. In this study, we focused on a single phosphorylation site known to be important in cell surface expression of K_{2p}3.1. However, a second non-related serine (Ser^{344} in hK_{2p}3.1) is also predicted to be phosphorylated by RSK, and this site could have additional modulatory roles on channel function. Furthermore, RSKs modulate the activity of K^{+} currents in both mouse ventricular myocytes and HEK293 cells. In both cases, currents were inhibited by co-expression of RSK (28). Consequently, it is possible that the increased whole-cell currents observed in response to RSK inhibition in this study are due to altered modulation of endogenous current. Moreover, evidence suggests that RSK may have an inhibitory effect on PKA (29). Hence, inhibition of RSK may simply alleviate constitutive inhibition of PKA, resulting in an apparent increase in PKA activity and an increase in channel expression. Immunofluorescence in HEK293 cells transfected with GFP-RK_{2p}3.1-HA, however, did not reveal an appreciable increase in the levels of this fusion protein at the plasma membrane after SL0101 treatment. Further experiments are necessary to determine the nature of the K^{+} flux in HEK293 cells in response to RSK inhibition.

Therefore, from the data presented in this work, we conclude that in vivo expression of hK_{2p}3.1 can be modulated by drugs and kinases that activate or inhibit PKA; that activation of PKA leads to phosphorylation of the 14–3–3 C-terminal binding site; and that phosphorylation of a single critical serine is responsible for an increased current. Several hormones and growth factors induce the activation of adenylyl cyclase in vivo, which, via generation of cAMP, liberates the PKA catalytic subunit from its inactive tetrameric form. Further studies are now required to uncover the signal transduction mechanisms involved in PKA and hence channel activation. It is likely that these will demonstrate some level of tissue specificity, but further studies will provide valuable information on the biology of these channels, whose exact function remains largely unknown, despite their widespread distribution and their implication in numerous physiological and pathophysiological roles.

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