Phosphatidylinositol 3-Phosphate Indirectly Activates KCa3.1 via 14 Amino Acids in the Carboxy Terminus of KCa3.1

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KCa3.1 is an intermediate conductance Ca²⁺-activated K⁺ channel that is expressed predominantly in hematopoietic cells, smooth muscle cells, and epithelia where it functions to regulate membrane potential, Ca²⁺ influx, cell volume, and chloride secretion. We recently found that the KCa3.1 channel also specifically requires phosphatidylinositol-3 phosphate [PI(3)P] for channel activity and is inhibited by myotubularin-related protein 6 (MTMR6), a PI(3)P phosphatase. We now show that PI(3)P indirectly activates KCa3.1. Unlike KCa3.1 channels, the related KCa2.1, KCa2.2, or KCa2.3 channels do not require PI(3)P for activity, suggesting that the KCa3.1 channel has evolved a unique means of regulation that is critical for its biological function. By making chimeric channels between KCa3.1 and KCa2.3, we identified a stretch of 14 amino acids in the carboxy-terminal calmodulin binding domain of KCa3.1 that is sufficient to confer regulation of KCa2.3 by PI(3)P. However, mutation of a single potential phosphorylation site in these 14 amino acids did not affect channel activity. These data together suggest that PI(3)P and these 14 amino acids regulate KCa3.1 channel activity by recruiting an as yet to be defined regulatory subunit that is required for Ca²⁺ gating of KCa3.1.

INTRODUCTION

KCa3.1 subunits (also known as IKCa and KCNN4) are components of an intermediate conductance Ca²⁺-activated K⁺ channel that is found predominantly in peripheral tissues such as blood cells, epithelia, and smooth muscle cells where it functions to couple alterations in cytosolic Ca²⁺ to K⁺ flux (Bond *et al.*, 1999; Jensen *et al.*, 2001; Wulff *et al.*, 2003). KCa3.1 channels thereby play an important physiological role to set the membrane potential at negative values close to the K⁺ equilibrium potential (Bond *et al.*, 1999; Jensen *et al.*, 2001; Wulff *et al.*, 2003; Stocker, 2004). This effect on membrane potential can have diverse physiological responses in a variety of cell types, including water movement and volume regulation in red blood cells, mitogen activation of T-lymphocytes, Cl[−] secretion of exocrine epithelial cells, and control of proliferation by a variety of cells such as T- and B-lymphocytes, vascular smooth muscle cells, keratinocytes, and some cancer cell lines (Khanna *et al.*, 1999; Ghan-shani *et al.*, 2000; Fanger *et al.*, 2001; Koegel and Alzheimer, 2001; Kohler *et al.*, 2003; Maher and Kuchel, 2003; Ouadid-Ahidouch *et al.*, 2004; Wulff *et al.*, 2004). Based on these activities, pharmacological modulation of KCa3.1 channels has been proposed to treat proliferative diseases such as restenosis after angioplasty and cancer, transplant rejection

as well as secretory diarrheas, sickle cell anemia, and cystic fibrosis (Rufo *et al.*, 1997; Jensen *et al.*, 2001; Maher and Kuchel, 2003; Wulff *et al.*, 2003).

Over the past several years, the mechanism of activation of KCa3.1 and the three related small conductance Ca²⁺-activated K⁺ channels, KCa2.1, KCa2.2, and KCa2.3 channels (also known as SK1, SK2, and SK3), has been determined. These channels are all structurally similar and possess six transmembrane domains with cytosolic N and C termini. Functional channels, however, are heteromeric complexes with constitutively bound calmodulin (CaM), and channel opening occurs only after Ca²⁺ binds to CaM. The C termini of all of the Ca²⁺-activated K⁺ channels contain a proximal 100-amino acid calmodulin binding domain (CBD) (Xia *et al.*, 1998; Keen *et al.*, 1999; Maylie *et al.*, 2004). The model proposed for Ca²⁺-dependent gating is that when Ca²⁺ binds to calmodulin, cross-linking of two adjacent C-terminal tails occurs, which results in channel opening (Schumacher *et al.*, 2001; Schumacher and Adelman, 2002; Maylie *et al.*, 2004).

We recently found that, in addition to requiring Ca²⁺ for activation, the KCa3.1 channel uniquely also depends on the lipid phosphatidylinositol-3 phosphate [PI(3)P] for activation (Srivastava *et al.*, 2005). Interestingly, the PI(3)P dependence does not seem to exist for the related KCa2.2 channel. Our initial insight that KCa3.1 channels are regulated by PI(3)P followed the finding that the CT coil-coil (CC) domain of KCa3.1 bound the CC domain in myotubularin-related protein 6 (MTMR6), a PI(3)P-specific phosphatase (Laporte *et al.*, 2003; Taylor and Dixon, 2003; Srivastava *et al.*, 2005). These studies demonstrated that KCa3.1 channel activity was inhibited >90% in cells overexpressing MTMR6 or in cells treated with the PI3 kinase inhibitors wortmannin and

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2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002). Moreover, the finding that addition of PI(3)P, but not other phosphoinositides, to the pipette solution restored KCa3.1 channel activity indicated that channel inhibition by both MTMR6 and wortmannin is mediated via decreased PI(3)P levels.

Fourteen different MTMs have been identified in mammalian cells (Laporte *et al.*, 2003; Taylor and Dixon, 2003). The founding member of this family of phosphatases, MTM1, was identified as the gene mutated in a congenital muscular dystrophy. MTMR2 and MTMR13 were identified as genes mutated in a subset of patients with Charcot Marie Tooth Syndrome type 4B (Laporte *et al.*, 1996; Bolino *et al.*, 2000). The finding that MTMR6 inhibits KCa3.1 suggests that MTMR6 plays an important and unexpected role in negatively regulating biological responses mediated by KCa3.1 by dephosphorylating PI(3)P in the membrane adjacent to KCa3.1. It is unlikely that the congenital diseases that result from loss of MTM1 or MTMR2 are due to abnormal regulation of KCa3.1 because inhibition of KCa3.1 is specific to MTMR6; neither MTM1 nor MTMR2 inhibits KCa3.1 (Srivastava *et al.*, 2005). In this study, we sought to determine the mechanism whereby PI(3)P regulates KCa3.1.

MATERIALS AND METHODS

Constructs

CHO-KCa3.1 cells are a stable cell line that overexpressed KCa3.1 and have been described previously (Srivastava *et al.*, 2005). Human KCa3.1 and human KCa2.3 were amplified by PCR and cloned in frame with green fluorescent protein (GFP) into the vector pEGFP (BD Biosciences Clontech, Palo Alto, CA). KCa3.1-KCa2.3 chimeric channels were made by overlapping PCR and cloned into pEGFP. The following chimeric channels were generated: KCa3.1-KCa2.3/CT: AA 1–273 KCa3.1, AA 536–736 KCa2.3; KCa2.3-KCa3.1/CT: AA 1–535 KCa2.3, AA 274–427 KCa3.1; KCa2.3-KCa3.1/CC, AA 1–642 KCa2.3, AA 376–427 KCa3.1; KCa2.3-KCa3.1/355–427; AA 1–616 KCa2.3, AA 355–427 KCa3.1; and KCa2.3-KCa3.1/369–427: AA 1–631 KCa2.3, AA 369–427 KCa3.1. Accession numbers for the various constructs are: human KCa3.1, NP_002241; rat SK2, NP_062187; and human KCa2.3, NP_00240.

Cell Culture, Transfection, and Patch Clamping

The GFP-tagged constructs were transfected into Chinese hamster ovary (CHO) cells using FuGENE (Roche Diagnostics, Indianapolis, IN) and whole cell-patch clamping was performed 48 h after transfection on GFP-positive cells as described previously (Srivastava *et al.*, 2005). For electrophysiological studies, CHO cells transfected with GFP-tagged constructs were plated on 18-mm coverslips at the time of transfection. After 48 h, a coverslip was positioned in a recording chamber, and patch clamping was performed at room temperature using a pipette solution containing 100 mM K⁺-gluconate, 30 mM KCl, 10 mM HEPES, 1.15 mM MgCl₂, 5 mM EGTA, and 4.27 mM CaCl₂, pH 7.2, with 1 N KOH (calculated free Ca²⁺, 1 μ M) and a bath solution containing 140 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, and 10 mM glucose. Patch-clamp pipettes had resistances ranging between 2.2 and 3.5 M Ω . Current-voltage (I-V) relationships were measured using ramp voltage-clamp protocols (at 15-s intervals) from a holding potential of -70 to -120 mV, followed by ramp depolarization to $+60$ mV (symmetrical ramp rate of 0.18 mV ms⁻¹). The current-voltage relationship was obtained by plotting the current during the depolarizing ramp phase as a function of the corresponding voltage. Membrane currents were filtered (-3 dB at 1 kHz) and digitized at 10 kHz (pClamp 9.2 with Digidata 1200 ADC interface; Molecular Devices, Sunnyvale, CA). Cell capacitance and pipette series resistances were compensated (usually $>80\%$), and these were obtained using the "membrane test" function of Clampex. Whole cell current density was expressed as picoamperes per picrofarad.

The identity of the currents were verified by 1) their sensitivity to cytosolic Ca²⁺; 2) the dependence of the reversal potential on the extracellular K⁺ concentration; 3) their absence in parental CHO cells; 4) voltage-independent gating; and 4) their sensitivity to either apamin, a specific inhibitor of KCa2.3 or Tram-34, a specific inhibitor of KCa3.1.

Inhibition of PI3K and Treatment with PI(3)P

To determine whether PI3-kinase inhibition affects channel activity, transfected cells were treated with the PI3K inhibitor wortmannin (1 μ M) 45 min before patch clamping, and I-V relationship was performed as de-

Figure 1. KCa3.1 channels require membrane-bound PI(3)P for activity and are not directly activated by P(3)P in isolated, I/O membrane patches. (A) CHO cells overexpressing KCa3.1 (CHO-KCa3.1) were treated with wortmannin, and whole cell patch clamping was performed in the absence (control) or presence of wortmannin. To assess whether soluble PI(3)P was sufficient for KCa3.1 channel activity, current activity was assessed in wortmannin-treated cells containing either C₁₇H₂₉Na₃O₁₆P₂ [diC4-PI(3)P, soluble] or C₄₁H₄₅Na₃O₁₆P₂ [diC16-PI(3)P, membrane bound] in the pipette solution. Bar graph summary of results at -120 and $+60$ mV are shown. (B and C) Control recording of I/O patches recorded in a bath solution containing 170 nM Ca²⁺ without (top tracing) and after the addition of 10 μ M PI(3)P (bottom tracing). PI(3)P did not affect single channel activity ($n = 3$). (C) The recording conditions were the same as in B but the Ca²⁺ activity was 33 nM. When switching to a higher Ca²⁺ (100 μ M), channels were activated which verifies the presence of functional channels in the patch (top left). Addition of PI(3)P (10 μ M) to the low Ca²⁺ bath (33 nM) did not produce single channel activity (bottom tracing). Similar results were observed in three other patches. All recordings in B and C were at -80 mV.

scribed above. Channels that were inhibited by wortmannin were determined to be due to inhibition of PI(3)P by showing that channel activity could be rescued by the addition of 100 nM PI(3)P into the pipette solution during patch clamping. PI(3)P [C₄₁H₄₅Na₃O₁₆P₂ (C₆) or C₁₇H₂₉Na₃O₁₆P₂ (C₂)] were purchased from Echelon Biosciences (Salt Lake City, UT) and used according to specifications. PI(3)P was resuspended in water and flash frozen in liquid nitrogen and used at a concentration of 100 nM in the pipette solution as described previously.

Figure 2. Wortmannin inhibits KCa_{3.1} but not KCa_{2.3} channels. CHO cells were transfected with GFP-KCa_{3.1} or GFP-KCa_{2.3}, and patch clamping in the whole cell configuration was performed on GFP-positive cells in the absence or presence of wortmannin (1 μ M). Shown are the I-V plots. (A) I-V plot of KCa_{3.1} cells before and after treatment with Tram-34 (1 μ M for 3–5 min), demonstrating the identity of the current as being formed by KCa_{3.1} channels. The K⁺ current displays the reversal potential of -73 mV (not corrected for the liquid junction potential, calculated to be ~ 13 mV), which is similar to the calculated reversal potential of -84 mV at an extracellular K⁺ of 5 mM. (B) Currents recorded from the same cells treated with 1 μ M wortmannin. (C) I-V plots of KCa_{2.3} cells before and after treatment with apamin, demonstrating that the current measured is KCa_{2.3}. (D) Currents from same cells treated with 1 μ M wortmannin ($n = 8$).

Inside-Out Patch Clamping to Record KCa_{3.1} Channels

CHO cells stably transfected with KCa_{3.1} were used for single-channel recordings. These were performed using the inside-out (I/O) patch-clamp mode, and data were obtained using standard patch-clamp techniques with an Axopatch-200B amplifier (Molecular Devices). Currents were recorded at room temperature (21–23°C), low-pass filtered (~ 3 -dB cut-off frequency at 1 kHz), and recorded on computer disk at a sampling frequency of 5 kHz (Clampex; Molecular Devices). The pipette resistance was 6–8 M Ω when filled with a solution containing 145 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, and 5 mM HEPES, pH 7.4. The bath solution contained 141 mM KCl, 5 mM HEPES, 1 mM EGTA, 1.01 mM MgCl₂, 0.244 or 0.62 mM CaCl₂, and 10 mM glucose, pH 7.3. The Ca²⁺ activity was calculated to be 33 or 170 nM (WinMAX Chelator software) (Bers *et al.*, 1994). PI(3)P (0.2–10 μ M) was added to the bath solution as needed. Data were analyzed using pClamp9.0 (Molecular Devices) and Origin 7.0 (OriginLab, Northampton, MA) software.

RESULTS

KCa_{3.1} Is Activated Indirectly by Membrane-bound PI(3)P

We previously found that KCa_{3.1} channels are inhibited by PI(3)P depletion and that channel activity in these cells could be rescued by including PI(3)P in the pipette solution (Srivastava *et al.*, 2005). To determine whether activation of KCa_{3.1} channels requires membrane-localized PI(3)P, we compared C₁₇H₂₉Na₃O₁₆P₂ [diC4-PI(3)P, soluble] with C₄₁H₄₅Na₃O₁₆P₂ [diC16-PI(3)P, membrane bound] in their ability to restore KCa_{3.1} channel activity in cells treated with the PI3 kinase inhibitor wortmannin. These studies showed that addition of C₄₁H₄₅Na₃O₁₆P₂, but not C₁₇H₂₉Na₃O₁₆P₂, in the pipette solution rescued KCa_{3.1} channel activity in cells treated with wortmannin, indicating that membrane-bound PI(3)P is required for KCa_{3.1} channel activity (Figure 1A).

To determine whether membrane bound PI(3)P directly or indirectly regulates KCa_{3.1} channel activity, we next performed single-channel recording using I/O membrane patches and examined whether application of PI(3)P to the cytosolic face of the channel increased KCa_{3.1} channel activity. KCa_{3.1} channels had an open probability that depended on the Ca²⁺ activity of the bath solution. Addition of PI(3)P (up to 10 μ M) did not increase channel activity (Fig-

ure 1B). It is possible that PI(3)P functions to increase Ca²⁺ sensitivity of the KCa_{3.1} channel. Therefore, we also measured single-channel activity in excised patches at lower cytosolic Ca²⁺ activities. In these experiments, I/O patches were initially exposed to high Ca²⁺ (100 μ M) to verify the presence of KCa_{3.1} channels in the patch. The bath solution was then switched to a solution with lower Ca²⁺ activity (33 nM), and phosphatidylinositol phosphates (PIPs) were then added to the low Ca²⁺ solution. PI(3)P also failed to activate KCa_{3.1} channels in the low Ca²⁺ solution (Figure 1C). Experiments were also performed by exposing excised patches to various solutions, respectively, containing (10 μ M each) PI(3)P ($n=4$), phosphatidylinositol-3,4-bisphosphate [PI(3,4)P₂] ($n=4$), or phosphatidylinositol trisphosphate [PI(3,4,5)P₃] ($n=3$) (our unpublished data). Similar to the results in Figure 1, no activation of KCa_{3.1} channels was observed by addition of any of these PIPs (our unpublished data). Thus, these data suggest that PI(3)P does not directly activate the KCa_{3.1} channel.

KCa_{2.1}, KCa_{2.2}, and KCa_{2.3} Channels Are Not Inhibited by PI(3)P Depletion

In contrast to KCa_{3.1} channels, we previously found that PI(3)P depletion with wortmannin had no effect on the related KCa_{2.2} channel (Srivastava *et al.*, 2005). We now find that PI(3)P depletion with wortmannin similarly has no effect on KCa_{2.3} channels (Figure 2) or KCa_{2.1} channels (our unpublished data). To determine the PI(3)P sensitivity of channels, CHO cells were transfected with GFP-tagged KCa_{3.1} or KCa_{2.3}, and patch clamp in the whole cell configuration was performed on GFP-positive cells that were untreated or treated with wortmannin (Srivastava *et al.*, 2005). The KCa_{3.1} channel current was identified as the current blocked by Tram-34, and this current component was significantly reduced in cells pretreated with wortmannin (1 μ M for 45 min) (Figure 2, A and B). In contrast, GFP-tagged KCa_{2.3} channel current (identified as the current component blocked by apamin) was not inhibited by wortmannin pretreatment (Figure 2, C and D; $n = 8$). Thus,

Figure 3. The CT cytosolic domain of KCa3.1 confers regulation by PI(3)P. Chimeric KCa2.3/KCa3.1 channels were generated as described in *Materials and Methods* in which the CT cytosolic portion of KCa2.3 and KCa3.1 was replaced with similar regions of the other channel to generate GFP-KCa3.1-KCa2.3(CT) and GFP-KCa2.3-KCa3.1(CT). CHO cells were transfected with each construct, and whole cell patch clamping was performed on GFP-positive cells as described in Figure 2. I-V plots of KCa2.3-KCa3.1(CT) before (A) or after (B) treatment with 1 μ M wortmannin. I-V plots of KCa3.1-KCa2.3(CT) before (C) or after (D) treatment with 1 μ M wortmannin. Although both channels are constitutively active in the presence of PI(3)P, only KCa2.3-KCa3.1(CT), which contains the CT of KCa3.1, is inhibited by wortmannin. Bar graph summary is shown in E.

of the KCa family members, KCa3.1 is uniquely dependent upon PI(3)P for channel activation.

The C Terminus of KCa3.1 Is Necessary and Sufficient for Channel Activation by PI(3)P

Chimeric channels between KCa2.2 and KCa3.1 and between KCa2.3 and KCa3.1 have previously provided important insights into regulation of this family of channels (Gerlach *et al.*, 2000, 2001; Wulff *et al.*, 2001). The finding that KCa3.1, but not KCa2.3, is inhibited by wortmannin and MTMR6 (the latter data are unpublished) provides a unique opportunity to determine the region(s) of KCa3.1 responsible for conferring regulation by PI(3)P and MTMR6 by generating a series of KCa2.3/KCa3.1 chimeric channels.

We first assessed whether the C terminus of KCa3.1 mediates channel regulation by PI(3)P. Chimeric GFP-KCa2.3/KCa3.1 channels were generated in which the C-terminal cytosolic region of KCa3.1 and KCa2.3 were swapped to generate KCa2.3-KCa3.1(CT) and KCa3.1-KCa2.3(CT). Chimeric constructs were transfected into CHO cells, and whole cell patch clamping was performed on GFP-positive cells that were untreated or treated with wortmannin. Currents

produced by the KCa2.3 chimera containing the KCa3.1 C terminus were inhibited by wortmannin (Figure 3, A and B). These studies demonstrated that the C terminus of KCa3.1 was necessary and sufficient for channel regulation by PI(3)P. In contrast, we found that the chimeric KCa3.1 channel containing the KCa2.3 C terminus [KCa3.1-KCa2.3(CT)] was constitutively active, but it was not inhibited by wortmannin (Figure 3, C–E). The ability of wortmannin to inhibit KCa2.3-KCa3.1/CT was due to loss of PI(3)P because wortmannin-inhibited channel activity could be rescued by including PI(3)P in the pipette solution (our unpublished data).

A Stretch of 14 Amino Acids in the KCa3.1 C Terminus Mediates Activation of KCa3.1 by PI(3)P

Alignment of the C termini of KCa2.1, KCa2.2, KCa2.3, and KCa3.1 is shown in Figure 4A. Each of the C termini contains a membrane-proximal CBD that mediates activation of these channels by calcium and a more carboxy terminus CC domain. To narrow down the region in KCa3.1 that mediates activation by PI(3)P, a further series of chimeric KCa2.3/KCa3.1 constructs were generated in which various regions

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Figure 5. Fourteen amino acids in the CT of KCa3.1 is sufficient to confer regulation by PI(3)P. GFP-KCa2.3 containing the substitution of 14 amino acids in CT portion of the CBD of KCa3.1 [KCa2.3-KCa3.1/14aa] was transfected into CHO cells and analyzed as described in Figures 1 and 2. I-V plots of KCa2.3-KCa3.1/14aa before (A) or after (B) treatment with 1 μ M wortmannin. (C) Whole cell patch clamping in CHO cells cotransfected with GFP-KCa2.3/KCa3.1/14aa and MTMR6. (D) Bar graph summary of results. In all experiments, the current was inhibited by apamine, indicating that the current measured was KCa2.3.

in the CT of KCa2.3 were replaced with corresponding regions from KCa3.1. The boundaries between chimeric channels tested are shown in Figure 4A. As before, PI(3)P sensitivity of the chimeric channels was assessed by its ability to be inhibited by wortmannin pretreatment as well as whether wortmannin-inhibited current could be rescued by including PI(3)P in the pipette solution. We found that the CC domain of KCa3.1 did not mediate its unique regulation by PI(3)P because a KCa3.1 chimera containing the KCa2.3 CC domain was still sensitive to wortmannin (Figure 4B). Using a number of different chimeras, we narrowed down the region of KCa3.1 that mediates regulation by PI(3)P to a 14-amino acid stretch within the C-terminal CBD of KCa3.1 (Figure 4, A and B). To determine whether these 14 amino acids are sufficient to confer regulation by PI(3)P, we replaced the corresponding residues of KCa2.3 with these 14 amino acids [KCa2.3-KCa3.1/14aa]. This mutant channel was active under baseline conditions, but unlike wild-type KCa2.3, it was inhibited by wortmannin treatment (Figure 5, A and B). In addition, KCa2.3-KCa3.1/14aa was inhibited by MTMR6 overexpression (Figure 5C). Thus, substitution of only 14 amino acids from KCa3.1 onto KCa2.3 was sufficient to change KCa2.3 into a channel that resembled KCa3.1 with respect to its sensitivity to PI(3)P.

PI(3)P Activation of KCa2.3-KCa3.1/14aa Is Not Mediated by Direct Phosphorylation of the Channel

PI(3)P could indirectly regulate KCa3.1, for example, by recruiting a kinase that contains a PX domain to the plasma membrane, which is then able to phosphorylate and activate KCa3.1. Several serine/threonine kinases with PX domains that bind PI(3)P have been identified (Liu *et al.*, 2000; Xing *et al.*, 2004). KCa3.1 contains a single potential phosphorylation site in these 14 amino acids, serine 367, which is a threonine in all the other mammalian SK channels (Figure 4A). To determine whether serine 367 is important for KCa3.1 channel activity, serine 367 was mutated to alanine [KCa3.1(S367A)], and channel activity was determined in the presence and absence of wortmannin. Our prediction

was that if PI(3)P was required for the constitutive phosphorylation of serine 367, KCa3.1(S367A) would be constitutively inactive and resemble the KCa3.1 channel activity in CHO cells expressing wild-type KCa3.1 [KCa3.1(WT)] and treated with wortmannin. We found that in the absence of wortmannin, channel activity of KCa3.1(S367A) was similar to channel activity of KCa3.1(WT) (Figure 6A). These findings indicate that phosphorylation of S367 is not required for KCa3.1 channel activity.

In contrast to KCa3.1(WT), the S367A mutant was insensitive to wortmannin (Figure 6B). The finding that KCa3.1(S367A) is no longer inhibited by wortmannin raised the possibility that inhibition of KCa3.1 is mediated by a phosphorylation event at residue S367 and that PI(3)P is required for constitutive KCa3.1 channel activity by stimulating perpetual dephosphorylation of S367, perhaps by recruiting a phosphatase to the plasma membrane. Previous studies have indicated that substitution of a serine for a negatively charged amino acid such as glutamic acid can mimic the effect of phosphorylation under some circumstances. To test this possibility, we mutated S367 to glutamic acid (S367E). If phosphorylation of S367 was inhibitory, KCa3.1(S367E) may be constitutively inactive if 367E mimicked phosphoserine at this site. However, KCa3.1(S367E) channel activity resembled KCa3.1(WT) in that it was active in the absence of wortmannin, and unlike KCa3.1(S367A), was inhibited by wortmannin (Figure 6, C and D). Thus, these data together suggest that phosphorylation of S367 does not play a role in the regulation of KCa3.1 channel activity. We can, however, not rule out the possibility that phosphorylation plays a role in negatively regulating the KCa3.1 channel because glutamic acid does not always mimic phosphoserine.

DISCUSSION

We found that, unlike KCa3.1, the three related Ca^{2+} -activated K^{+} channels, KCa2.1, KCa2.2, and KCa2.3, do not require PI(3)P for channel activity. Several other factors also

Figure 6. Mutation of the potential phosphorylation site at serine 367 of KCa_{2.3} did not inhibit channel activity. Serine 367 in KCa₁ was mutated to alanine and glutamic acid by site-directed mutagenesis to generate GFP-KCa_{3.1}(S367A) and GFP-KCa_{3.1}(S367Q). Both constructs were transfected into CHO cells, and whole cell patch clamping was performed as described in text. I-V plots are shown. (A) GFP-KCa_{3.1}(S367A). (B) GFP-KCa_{3.1}(S367A) treated with 1 μM wortmannin. (C) GFP-KCa_{3.1}(S367E). (D) GFP-KCa_{3.1}(S367E) treated with 1 μM wortmannin. Bar graph summary is shown in E.

distinguish KCa_{3.1} from KCa_{2.1}, KCa_{2.2}, and KCa_{2.3} channels. For example, KCa_{3.1} is expressed predominantly in T- and B-cells, epithelial cells, and smooth muscle cells where they function to stimulate cell proliferation and activation by regulating membrane potential and Ca²⁺ influx, whereas KCa_{2.1}, KCa_{2.2}, and KCa_{2.3} channels are expressed predominantly in the nervous system where they function to regulate membrane potential, neuronal firing, and Ca²⁺ influx (Ghanshani *et al.*, 2000; Jensen *et al.*, 2001; Kohler *et al.*, 2003; Maylie *et al.*, 2004; Stocker, 2004; Wulff *et al.*, 2004). In addition, only KCa_{2.1}, KCa_{2.2}, and KCa_{2.3} channels are found in invertebrates. These data would indicate that KCa_{3.1} channels have evolved unique functions in higher organisms and with these different functions have evolved unique means for regulation. We propose that the requirement of KCa_{3.1} for PI(3)P provides a means to rapidly turn on or turn off KCa_{3.1} channel activity, which may play important roles in the function of KCa_{3.1} channels in these cells. This would enable KCa_{3.1} channel activity to be rapidly activated by increasing PI3 kinase activity leading to PI(3)P generation adjacent to KCa_{3.1} or to be rapidly inhibited via the recruitment of MTMs (MTMR6 subfamily members) via their CC domains to KCa_{3.1}, leading to dephosphorylation of PI(3)P and KCa_{3.1} channel inhibition.

One other difference between KCa_{3.1} and KCa_{2.1}, KCa_{2.2}, and KCa_{2.3} channels is their regulation by kinases (Khanna *et al.*, 1999; Gerlach *et al.*, 2000; Gerlach *et al.*, 2001). Although KCa_{3.1} channels are activated by serine/threonine kinases, KCa_{2.1}, KCa_{2.2}, and KCa_{2.3} channels are not. Interestingly, many of the requirements for KCa_{3.1} channel activation by kinases are remarkably similar to our findings for PI(3)P activation of KCa_{3.1}. Gerlach *et al.* found that the same 14 amino acids that we found are required for PI(3)P activation of KCa_{3.1} are also required for kinase activation of KCa_{3.1} (Gerlach *et al.*, 2000, 2001). In fact, we used these data to help guide the construction of some of the chimeric channels studied herein. Based on these findings, we tested whether PI(3)P functions to activate KCa_{3.1} by recruiting a kinase with a PX domain that binds PI(3)P to the plasma membrane where it can then phosphorylate and activate KCa_{3.1} (Liu *et al.*, 2000; Xing *et al.*, 2004). However, in agreement with a previous study (Gerlach *et al.*, 2001), KCa_{3.1} is not activated by direct phosphorylation because mutation of the only serine (serine 367) in these 14 amino acids did not inhibit channel activity. Surprisingly, mutation of 367 to alanine resulted in a channel that was constitutively active, but no longer inhibited by wortmannin. This finding suggests that S367 plays a role in negatively regulating KCa_{3.1} channel

activity, and one function of PI(3)P may be to antagonize this inhibition. The mechanism whereby S367 functions to negatively regulate KCa3.1 is currently unknown. Our data cannot exclude the possibility that constitutive phosphorylation of S367 negatively regulates KCa3.1.

Our current working model is that PI(3)P together with (at least some of) these 14 amino acids in the C-terminal CBD of KCa3.1 is required to recruit another protein that functions as a subunit for the active KCa3.1 channel. We can in theory explain all of the above-mentioned results if direct phosphorylation of this putative regulatory subunit increases KCa3.1 channel activity. This hypothesis is consistent with PI(3)P indirectly activating KCa3.1 as well as the same 14 amino acids mediating regulation by PI(3)P and kinases; PI(3)P and these 14 amino acids would be required to recruit the putative regulatory subunit to the active channel that would then be the target for phosphorylation. It should be pointed out that no other subunit that associates with KCa3.1 has been identified to date. However, the requirement for PI(3)P would suggest that this subunit likely contains a PI(3)P binding domain (such as a FYVE or PX domain) that is required for membrane targeting (Stenmark and Aasland, 1999; Gillooly *et al.*, 2001; Ellson *et al.*, 2002).

In addition to requiring PI(3)P, KCa3.1 channel activity also requires Ca^{2+} (Srivastava *et al.*, 2005). Thus, any proposed model for PI(3)P regulation of KCa3.1 channels must also take into account a concomitant requirement for Ca^{2+} . Important insight into the role for Ca^{2+} in the activation of channels in this family has come from crystallographic studies of KCa2.2 and calmodulin (Schumacher *et al.*, 2001). These studies demonstrated that in the absence of Ca^{2+} , the C-terminal lobe of calmodulin is constitutively bound to the amino-terminal region of the CBD of one KCa2.2 channel, resulting in a calmodulin/CBD monomer. Binding of Ca^{2+} to the N-terminal lobe of calmodulin then stimulates its binding to the C-terminal region of an adjacent KCa2.2 CBD, resulting in a calmodulin/CBD dimer that has then been proposed to transmit a rotary force to the S6 pore region leading to opening of the channel (Schumacher *et al.*, 2001; Schumacher and Adelman, 2002; Maylie *et al.*, 2004). Because the functional KCa2.2 channel is tetrameric, these data would suggest that in the presence of Ca^{2+} the active channel is composed of two calmodulin/CBD dimers. It is intriguing that the region of KCa3.1 that mediates its activation by PI(3)P is also within the same region of the CBD that interacts with N-terminal lobe of calmodulin. Thus, it is possible that in the absence of PI(3)P, Ca^{2+} is unable to stimulate binding of the N-terminal lobe of calmodulin to an adjacent KCa3.1 CBD and therefore calmodulin/CBD dimers do not form. PI(3)P, possibly by recruiting a regulatory subunit, may then function to relieve this inhibition by enabling Ca^{2+} to now stimulate binding of the N-terminal lobe of calmodulin to an adjacent KCa3.1 CBD. Validation of this hypothesis will ultimately require the identification and characterization of this putative regulatory subunit.

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