Regulation of ROMK1 channel by protein kinase A via a phosphatidylinositol 4,5-bisphosphate-dependent mechanism

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ABSTRACT ROMK inward-rectifier K⁺ channels control renal K⁺ secretion. The activity of ROMK is regulated by protein kinase A (PKA), but the molecular mechanism for regulation is unknown. Having found that direct interaction with membrane phosphatidylinositol 4,5-bisphosphate (PIP₂) is essential for channel activation, we investigate here the role of PIP₂ in regulation of ROMK1 by PKA. By using adenosine-5'-[γ-thio]triphosphate (ATP[γS]) as the substrate, we found that PKA does not directly activate ROMK1 channels in membranes that are devoid of PIP₂. Rather, phosphorylation by PKA + ATP[γS] lowers the concentration of PIP₂ necessary for activation of the channels. In solution-binding assays, anti-PIP₂ antibodies bind PIP₂ and prevent PIP₂–channel interaction. In inside-out membrane patches, antibodies inhibit the activity of the channels. PKA treatment then decreases the sensitivity of ROMK1 for inhibition by the antibodies, indicating an enhanced interaction between PIP₂ and the phosphorylated channels. Conversely, mutation of the PKA phosphorylation sites in ROMK1 decreases PIP₂ interaction with the channels. Thus, PKA activates ROMK1 channels by enhancing PIP₂–channel interaction.

Inward-rectifier K⁺ channels more readily conduct current inward than outward. They are widely present and regulate many important cellular processes, including resting membrane potential, cell and synaptic excitability, pancreatic insulin secretion, and renal K⁺ transport (1). Many cDNAs for the inward-rectifier K⁺ channel family have been isolated, including the rat kidney ROMK1, the strongly rectifying IRK1, the G protein-gated GIRK1, and the pancreatic beta cell inward rectifier BIR (2). These cDNAs encode polypeptides of ≈300–500 aa, which share ≈40% or more amino acid identity and have the common structure of a cytoplasmic N terminus, two hydrophobic segments (M1 and M2) that span the membrane as α-helices, one pore-forming partial membrane-spanning region (H5), and a long cytoplasmic C terminus.

Opening of the G protein-gated GIRK1/4 channels requires G protein βγ subunits (3, 4). Other inward-rectifier K⁺ channels, such as ROMK1 and IRK1, are constitutively open. Inward-rectifier K⁺ channels run down when inside-out membrane patches are excised into ATP-free, Mg²⁺-containing solution. Recent evidence implicates PIP₂ as a regulator of inward-rectifier channels. We and others (5–8) have reported that depletion of membrane PIP₂ causes channel run-down. Direct application of PIP₂-containing liposomes to the membrane patches reactivates run-down channels, and application of Mg-ATP to membrane patches reproduces the effect by activating membrane-associated lipid kinases (which phosphorylate phosphatidylinositol and phosphatidylinositol 4-phosphate) to generate PIP₂ *in situ* (9).

Phosphorylation by cAMP-dependent protein kinase (PKA) controls the activity of ion channels in many tissues by a variety

of mechanisms (10). For example, PKA phosphorylation on the voltage-gated delayed-rectifier K⁺ channels in squid axons markedly alters the voltage-dependent activation by addition of negative charges on the cytoplasmic side of the channels (11). In epithelia, activation of the cystic fibrosis transmembrane conductance regulator Cl[−] channel requires PKA phosphorylation as well as binding and hydrolysis of ATP (12). The phosphorylation of serine residues in the regulatory domain increases the affinity of the nucleotide-binding domain for ATP and thus facilitates channel gating by ATP (13). Phosphorylation of the skeletal muscle L-type voltage-sensitive Ca²⁺ channels by PKA increases voltage-dependent potentiation of Ca²⁺ current by shifting the voltage dependence of activation to more negative membrane potentials (14, 15). PKA phosphorylation of the L-type Ca²⁺ channels in cardiac cells underlies the increase in contractility by β-adrenergic stimulation (16, 17). Another effect of PKA phosphorylation for the cardiac L-type Ca²⁺ channels is to regulate run-down of the channel (18).

Several lines of evidence suggest that run-down of the ROMK channels also is prevented by PKA phosphorylation: First, run-down of the inward-rectifier K⁺ can be prevented, at least partially, by specific protein phosphatase inhibitors (19, 20). Second, application of PKA catalytic subunit and Mg-ATP reactivates the run-down channels by a direct phosphorylation (20, 21). Third, the importance of direct phosphorylation for channel function is further supported by the finding that one of the genetic defects in Bartter's syndrome is caused by a mutation in a PKA phosphorylation site in the ROMK channel (22). Moreover, PKA phosphorylation is important for regulation of the renal K⁺ channels by arginine vasopressin (23, 24). However, it is not known how phosphorylation of ROMK leads to an increase in the activity of the channels. As experiments with PKA catalytic subunit were performed in the presence of Mg-ATP (20, 25) and Mg-ATP can generate PIP₂ via lipid kinases, we test the hypothesis that PKA phosphorylation regulates the ROMK channels by modulating PIP₂ activation of the channel.

MATERIALS AND METHODS

Molecular Biology. Site-directed mutagenesis was performed and confirmed by nucleotide sequencing as described (7). mCAP RNAs of the wild-type and mutant channels were *in vitro*-transcribed by using T7 RNA polymerase (7, 27).

This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: PKA, cAMP-dependent protein kinase; PIP₂, phosphatidylinositol 4,5-bisphosphate; ATP[γS], adenosine-5'-[γ-thio]triphosphate; GST-RKC, glutathione S-transferase fusion protein of the carboxyl terminus of ROMK1; FVPP, fluoride, vanadate, and pyrophosphate.

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Patch-Clamp Recording. *Xenopus* oocytes were injected with ≈ 5 ng of cRNA for the wild-type or mutant ROMK1 and giant patch-clamp recording (at $\approx 23^\circ\text{C}$) was performed as described (7, 27, 28). The pipette (extracellular) solution contains (in mM) 100 KCl, 2 CaCl_2 , and 5 Hepes (pH 7.4). Bath (cytoplasmic) solution contains either 100 KCl, 5 Hepes (pH 7.4), 5 EGTA, and 1 MgCl_2 (Mg^{2+} solution) or 100 KCl, 5 Hepes, 5 EDTA, 4 NaF, 3 Na_3VO_4 , and 10 $\text{Na}_4\text{P}_2\text{O}_7$ (FVPP solution) as indicated for each experiment. Inward K^+ currents (at -30 mV holding potential) were recorded in a chart recorder by using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Chart recording strips were scanned and analyzed in a computer for presentations. Each vial of anti-PIP₂ mAb stock (PerSeptive Biosystems, Framingham, MA) is reconstituted in 0.5 ml of distilled water and diluted 40:1 into experimental solutions (to yield a final concentration of 40 nM). PIP₂ (Boehringer Mannheim) was diluted in water (1 mg/ml) and sonicated to form liposomes (7). Stocks of PKA catalytic subunit (Sigma, 2,000 units/ml dissolved in water containing 40 mM DTT) were diluted 20:1 into experimental solutions to yield a final concentration of 100 units/ml with 2 mM DTT.

³H-PIP₂ Binding Assay. ³H-PIP₂ (American Radiolabeled Chemicals, St. Louis) (at 0.05, 0.1, 0.3, 0.5, 1, 3, and 5 μM) was incubated with purified glutathione S-transferase fusion protein of the carboxyl terminus of ROMK1 (GST-RKC) (100 nM) in 50 μl of PBS as described (7). When indicated, ³H-PIP₂ (at all concentrations) was preincubated with 1 or 10 μM of anti-PIP₂ antibodies before addition of GST-RKC. The complexes of GST-RKC and ³H-PIP₂ were then precipitated by 10

μl of glutathione 4B-Sepharose beads (1:1 PBS–beads suspension). After one wash in PBS, the precipitates were dissolved in SDS-gel loading buffer and counted in a β -scintillation counter by using a window for ³H. Nonspecific binding was determined by incubating ³H-PIP₂ and GST-RKC in the presence of 100 μM unlabeled PIP₂ (without anti-PIP₂ antibodies). Specific binding was determined by subtracting nonspecific binding from the total binding performed in the presence of 1 μM , 10 μM , or no anti-PIP₂ antibodies. To determine the binding of PIP₂ by anti-PIP₂ antibody, the ³H-PIP₂ and anti-PIP₂ antibody complexes (with or without unlabeled lipids at indicated concentrations) were precipitated by 10 μl of protein A-Sepharose beads. Background binding of ³H-PIP₂ to protein A-Sepharose beads (determined in a parallel experiment without addition of anti-PIP₂ antibodies) was subtracted from the total binding.

RESULTS

Role of PIP₂ in the Activation of the ROMK1 Channel by Mg-ATP. Current-voltage (*I-V*) relationships (from -80 to $+80$ mV, Fig. 1A) and inward K^+ currents (holding at -30 mV; Fig. 1B) through ROMK1 channels were measured by using giant patch-clamp recording in *Xenopus* oocytes, first in “on-cell” configuration and subsequently in excised inside-out configuration (labeled “ex”). The *I-V* relationship for all currents measured shows the characteristic weak inward-rectification for ROMK1 channels (Fig. 1A). As reported previously (7, 20, 25), the activity of ROMK1 channels ran down in Mg^{2+} -containing solution, and was restored to the

FIG. 1. Inhibition of ATP-dependent channel activation by anti-PIP₂ antibody. (A) Pulse protocol and *I-V* curves for channels on-cell, after run-down, after reactivation by Mg-ATP, and after inhibition by anti-PIP₂ antibody as in panel B. The horizontal time bar is 120 sec. (B) Run-down channels were activated by addition of 0.5 mM Mg-ATP (labeled ATP) to the Mg^{2+} solution. Inward currents (labeled I (nA) in B) was measured and normalized to the on-cell level ($I_K = 1$, for on-cell current; $I_K = 0$, for residual leak current after channels ran down completely). After reaching maximal current, bath solution was changed to FVPP. Anti-PIP₂ antibodies (40 nM final concentration in FVPP, labeled PIP₂ Ab) inhibited the channels. After washing with Mg^{2+} -containing solution, DTT (2 mM) partially reversed the antibody-induced inhibition. A complete reversal by 2 mM DTT occurred if the membrane patches were washed with FVPP solution (see Fig. 2B), demonstrating that Mg^{2+} washing activated lipid phosphatases and reduced PIP₂ in the membrane. DTT at 0.1–2 mM reversed the inhibition dose-dependently (data not shown). (C) Binding of ³H-PIP₂ (10 nM) to anti-PIP₂ antibodies (100 nM) in the presence of nonradioactive phosphatidylcholine (PC, ■), phosphatidylinositol 4-phosphate (PIP, ▲) or PIP₂ (●). (D) Binding of ³H-PIP₂ to GST-RKC (100 nM) in the absence of antibody (control, ●), in the presence of 1 μM (◆) or 10 μM (◇) antibody (labeled Ab). Curve fittings were performed assuming one PIP₂ binding site for each molecule of GST-RKC or anti-PIP₂ antibody. Because ³H-PIP₂ is multimeric in the form of liposomes, this analysis may overestimate affinity.

on-cell level by Mg-ATP ("ATP") with a $t_{1/2} = 60 \pm 18$ sec (mean $\pm$ SEM, $n = 4$) (Fig. 1*A* and *B*). Channels were then stabilized in FVPP solution (which contains the phosphatase inhibitors fluoride, vanadate, and pyrophosphate). Addition of anti-PIP₂ mAbs (40 nM) inhibited the channels completely ($t_{1/2} = 45 \pm 10$ sec, $n = 5$), demonstrating the role of PIP₂ in channel activation by ATP. Heat-inactivated anti-PIP₂ antibodies and control mouse IgG did not inhibit the channels (data not shown). PKA inhibitor H89 (5–25 μ M) did not affect the activation of the channels by Mg-ATP (data not shown).

The sensitivity of the channels to the anti-PIP₂ antibodies correlates with the *in vitro* biochemical interaction between channels and PIP₂, and also with the rate of activation of the channels by PIP₂ (7), suggesting that anti-PIP₂ antibodies probably inhibit channel activity by binding to PIP₂ in the membrane and uncoupling its interaction with the channels. Although preapplication of excess PIP₂ to membrane patches prevents the inhibition by the antibodies, addition of more PIP₂ after channels are inhibited by the antibodies does not reverse the inhibition (7). Clustering and formation of acidic lipid microdomains by basic residues of protein is well described (29, 30). If the ROMK1 channels and PIP₂ also are clustered in microdomains, the complexes of PIP₂ and anti-PIP₂ antibodies may remain localized around the channels and thus prevent reactivation of the channels by the exogenous PIP₂. Consistent with this notion, application of DTT after anti-PIP₂ antibodies [probably by reducing the interchain and/or intrachain disulfides of the Ig molecules (31) and dissociating the PIP₂-antibody complexes] reversed the inhibition (Fig. 1*B*). Another antioxidant, glutathione, also reversed the effect of the antibodies (data not shown). After reversal by DTT or glutathione, channels can be once again inhibited by anti-PIP₂ antibodies (Fig. 1*B*; see also Fig. 2*B*). DTT alone did not reverse the Mg²⁺-induced channel run-down, and anti-PIP₂ antibodies failed to inhibit channels when they were preincubated with DTT (data not shown).

Anti-PIP₂ Antibody Binds PIP₂ and Inhibits PIP₂-Channel Interaction. Monoclonal anti-PIP₂ antibody binds PIP₂ and inhibits many PIP₂-dependent cellular processes (32, 33). Binding of PIP₂ by anti-PIP₂ antibody was examined. Preliminary results showed that 1–10 nM [³H]-PIP₂ bound to 100 nM anti-PIP₂ antibodies dose-dependently (data not shown). We then found that 0.1–1,000 nM unlabeled PIP₂ (but not PIP or PC) blocked by competition the binding of 10 nM [³H]-PIP₂ to 100 nM anti-PIP₂ antibodies dose-dependently (Fig. 1*C*). The apparent K_d for PIP₂ and anti-PIP₂ antibody binding is estimated to be ≈ 8 nM.

³H-PIP₂ (0.05 to 5 μ M) bound to GST-RKC (100 nM) dose-dependently (Fig. 1*D*, $\bullet$). Anti-PIP₂ antibodies ($\blacklozenge$, 1 μ M; $\diamond$, 10 μ M) reduced binding of ³H-PIP₂ to GST-RKC dose-dependently (Fig. 1*D*). Scatchard analysis revealed K_d to be ≈ 0.58 μ M and >5 μ M for binding of ³H-PIP₂ to GST-RKC without and with 1 μ M antibody, respectively. Because the free ³H-PIP₂ (total ³H-PIP₂ added minus the amount bound to GST-RKC) includes both antibody-bound and antibody-unbound ³H-PIP₂ molecules, the increase in the K_d for binding of ³H-PIP₂ to GST-RKC by antibody presumably represents sequestering of ³H-PIP₂ by anti-PIP₂ antibody (see Fig. 1*C*).

The Relationship of PKA vs. ATP in Channel Activation. Application of PKA catalytic subunit and Mg-ATP (PKA + ATP) to run-down channels also restored them to the same maximal (on-cell) level as ATP (Fig. 2*A*, compare Fig. 1*A* and *B*). The maximal restorable current by PKA + ATP is $105 \pm 9\%$ of the on-cell level ($n = 14$). The rate of channel activation was apparently faster for PKA + ATP than for ATP alone ($t_{1/2} = 24 \pm 22$ vs. 60 ± 18 sec, $P \approx 0.1$). When activated by PKA + ATP, channels were only partially inhibited ($25 \pm 10\%$, $n = 6$) by anti-PIP₂ antibodies (40 nM) in 5 min (Fig. 2*A* and *C*), suggesting that PIP₂ was more tightly bound to the phosphorylated channels. When channels were already activated max-

FIG. 2. PKA activates run-down channels to the same maximal restorable level as ATP and reduces the sensitivity of the channels to anti-PIP₂ antibody. Membrane patches were excised and allowed to run down in Mg²⁺ solution as in Fig. 1. The horizontal time bar is 120 sec. (*A*) PKA catalytic subunit (100 units/ml) + Mg-ATP (0.5 mM) activated the channels to the same maximal level of on-cell current as ATP (see Fig. 1*A* and *B*). Anti-PIP₂ antibodies (40 nM) inhibited the channel only partially in 5 min. (*B*) PKA + ATP was not further stimulatory to the channels that were maximally activated by ATP. Increasing concentrations of anti-PIP₂ antibodies (5 $\times$ Ab, 200 nM, and 10 $\times$ Ab, 400 nM) inhibited the channels faster. (*C*) Dose-dependent inhibition of the channels by anti-PIP₂ antibody. Channels were allowed to run down in Mg²⁺ solutions and were reactivated by ATP (shaded bars) or PKA + ATP (open bars). Thereafter, anti-PIP₂ antibodies (8, 40, or 200 nM) were applied to the channels in FVPP solutions. Mean $\pm$ SEM, $n = 3$ –16 for each group.

imally by ATP, addition of PKA + ATP did not further stimulate the activity but decreased the sensitivity of the channels to inhibition by anti-PIP₂ antibodies (Fig. 2*B*). Addition of 5- to 10-fold (200 to 400 nM) antibodies then inhibited the channels completely. In some of the patches, ATP alone activated the channels only partially from run-down, probably because of a lower endogenous lipid kinase activity. Addition of PKA + ATP to these channels after ATP then stimulated the activity of the channels further to the on-cell level (data not shown). Fig. 2*C* shows the dose-dependent relationship for inhibition of the channels by anti-PIP₂ antibody. Anti-PIP₂ antibody at ≥ 40 nM inhibited the ATP-activated channels completely in 5 min (Fig. 2*C*, shaded bar). When run-down channels were activated by PKA + ATP, however, it required ≥ 200 nM anti-PIP₂ antibodies to inhibit the channels (Fig. 2*C*, open bar). Taken together, these results support the idea that PKA modulates ATP activation of the channels via enhancing PIP₂-channel interaction. The role of PKA in relationship to ATP was further examined by using ATP[γ S] as the substrate for PKA (see below).

Phosphorylation by PKA Enhances PIP₂-Channel Interaction. Although the phosphorothioate derivative of ATP,

ATP[γ S], can serve as a substrate for PKA (34), ATP[γ S] could not activate lipid kinases to produce PIP₂ (Fig. 3A). Addition of ATP thereafter activated the channels by generating PIP₂. When ATP[γ S] was substituted for ATP, addition of PKA did not reactivate the run-down channels (Fig. 3B), indicating that PKA phosphorylation alone is not sufficient for channel activation. This pretreatment with PKA + ATP[γ S], however, apparently rendered the channels to be activated by ATP faster ($t_{1/2}$ for channel activation 60 ± 18 secs for ATP alone, $n = 4$, vs. 22 ± 16 secs for ATP after PKA + ATP[γ S], $n = 3$; $P < 0.1$) and reduced the sensitivity of the channels to anti-PIP₂ antibodies (Fig. 3B). The decrease in the sensitivity to the anti-PIP₂ antibody confirms that channel phosphorylation by PKA indeed occurs by using ATP[γ S] as a substrate. Other PIP₂ binding agents, such as heptalysine (29), also inhibits the activity of the ROMK channels when applied to the excised membrane patches (7). We found that PKA phosphorylation also reduced the sensitivity of the channels to inhibition by heptalysine (5 μ M) ($103 \pm 14\%$ inhibition in 5 min for control vs. $34 \pm 12\%$ inhibition for PKA-treated; mean $\pm$ SEM, $P < 0.05$).

Further evidence for modulation of PIP₂ activation of the channels by PKA phosphorylation is provided from the following experiments by direct application of PIP₂ (Fig. 3C) as described (7). Channels were allowed to run down in the Mg²⁺ solution, then either pretreated by PKA + ATP[γ S] or without pretreatment before addition of PIP₂ liposomes. Without PKA + ATP[γ S], it required >10 μ M PIP₂ to fully activated ROMK1 channels from Mg²⁺-induced run-down over 3 min (Fig. 3C). When channels were phosphorylated by pretreatment with PKA + ATP[γ S], the concentrations of PIP₂ required to activate the channels were reduced by $\approx 90\%$, such that 1 μ M PIP₂ could fully activate the channels. The *I-V* relationship for currents activated by PIP₂ was characteristic of that for ROMK1, and was not altered by pretreatment with PKA + ATP[γ S] (data not shown).

When phosphorylated by using ATP[γ S] as a substrate, proteins are resistant to dephosphorylation by protein phosphatases (34). We further examined the role of Mg²⁺-dependent protein phosphatases in channel run-down by using the thio-phosphorylated channels. Channels were allowed to run down completely in Mg²⁺-containing solution and then reactivated either by PKA + ATP or by PKA + ATP[γ S]. After PKA + ATP or PKA + ATP[γ S] was washed off, channels were reexposed to Mg²⁺ solutions for run-down. For those channels activated by PKA + ATP, reexposure to Mg²⁺ caused a complete run-down, as expected ($97 \pm 11\%$ run-

down, $n = 4$). In contrast, reexposure to Mg²⁺ caused a very slow run-down for those channels reactivated by PKA + ATP[γ S], reaching only $38 \pm 8\%$ ($n = 5$) run-down over 30 min. As a time-control, exposure of the thio-phosphorylated channels to FVPP solution did not lead to a significant run-down in 30 min ($8 \pm 4\%$, $n = 4$; $P < 0.05$ for comparing Mg²⁺ vs. FVPP solution). As the thio-phosphorylated channels are resistant to protein phosphatases, the slow run-down for these channels by Mg²⁺ is likely caused by a gradual loss of PIP₂ as a result of activation of the lipid phosphatases. These results support that, under physiological conditions, Mg²⁺ causes channel run-down by activating both protein and lipid phosphatases, leading to dephosphorylation of the channels as well as depletion of PIP₂ in the membrane, and further support that phosphorylation of the channels maintains PIP₂-channel interaction.

Mutation of PKA Phosphorylation Sites Decreases PIP₂ Interaction with the Channels. Phosphopeptide mapping reveals three serine residues (equivalent to Ser-45, Ser-219, and Ser-313 of ROMK1, Fig. 4A) are phosphorylated by PKA (21). Single mutation of Ser-219 or Ser-313 (but not Ser-45) to alanine reduced the single channel open probability by $\approx 50\%$ (35). These effects from mutation of Ser-219 and Ser-313 are similar to the effect of mutation on PIP₂-binding site in Kir6.2 (6), suggesting that phosphorylation of these two residues by PKA may be responsible for enhancing channel affinity for PIP₂. Indeed, the sensitivity to antibody for the S219A and the S313A mutants of ROMK1 was increased compared with the wild-type (Fig. 4, B and C). Similarly, the sensitivity of the channels to inhibition by heptalysine was also increased by S219A or S313A mutation ($t_{1/2}$ for inhibition of the channels in FVPP = 100 ± 7 sec, 42 ± 4 sec, and 55 ± 6 for the wild-type, S219A, and S313A, respectively; $P < 0.05$ for wild-type vs. S219A and wild-type vs. S313A). Mutation of control serine residues to alanine (S45A or S195A) did not alter the sensitivity of the channels to anti-PIP₂ antibodies (Fig. 4C) or to heptalysine (data not shown) as compared with the wild-type ROMK1.

Because the electrostatic interaction between PIP₂ and the multiple basic residues of proteins is likely cooperative (36), mutation of a single residue in the PIP₂-binding region as in R188Q resulted in $\approx 90\%$ reduction in PIP₂ affinity (Fig. 4B and C; see also ref. 7). Substitution of Arg-188 by another basic residue lysine (R188K) did not alter the rate of channel inhibition by anti-PIP₂ antibodies as compared with the wild-type ROMK1 channels (Fig. 4B and C), confirming that the ionic interaction between Arg-188 and PIP₂ is important for

FIG. 3. Enhancement of PIP₂ activation of the channels by PKA phosphorylation. In each experiment, channels were excised and allowed to run down in Mg²⁺ solution. The horizontal time bar is 120 sec. (A) Mg-ATP[γ S] (0.5 mM in Mg²⁺ solution) did not activate channels after run-down in Mg²⁺ solution. Subsequent addition of Mg-ATP (0.5 mM) activated the channels. ($n = 3$.) (B) Although PKA catalytic subunit (100 units/ml) + Mg-ATP[γ S] (0.5 mM) did not activate the channels, it apparently increased the rate of channel activation by ATP and reduced the sensitivity of the ATP-activated channels to inhibition by anti-PIP₂ antibodies (40 nM). ($n = 3$.) (C) Dose-response curves (assuming one binding site) for PIP₂ (0.03–50 μ M) activation of the channels with or without pretreatment by PKA + ATP[γ S]. Currents activated by PIP₂ (at -30 mV holding potential) are shown as percentage of on-cell currents. As the PIP₂ concentration in the membrane is likely different from that calculated based on the amount of PIP₂ dispersed in aqueous solution (as shown here), it is difficult to compare the half-maximal concentration for PIP₂ activation of the channels to the K_d determined from the *in vitro* binding of ³H-PIP₂ to GST-RKC (see Fig. 1 C and D).

FIG. 4. Serine → alanine mutation at PKA phosphorylation sites reduces channel's interaction with PIP₂. (A) Membrane topology of ROMK1 in relationship to PIP₂ in the inner leaflet of the membrane. Like all inward-rectifier K⁺ channels, it consists of a short N-terminal cytoplasmic domain, two transmembrane domains, one partial membrane domain, and a long C-terminal cytoplasmic tail. The proximal C-terminal region of channel (amino acid 180–223 of ROMK1) contains many conserved basic residues that form the putative PIP₂-binding region. A critical role for Arg-188 (R188) in forming an electrostatic interaction with PIP₂ is described in the text. Ser-219 (S219) and Ser-313 (S313) are PKA sites that are involved in enhancing PIP₂ affinity (see text for details). (B) Membrane patches were excised and stabilized in FVPP solution. Anti-PIP₂ antibodies (40 nM in FVPP) were applied to inhibit the channels. The on-cell currents for wild-type (WT) and mutant channels were normalized and superimposed. Time bar is 120 sec. (C) Half-time ($t_{1/2}$) for maximal inhibition by anti-PIP₂ antibody (40 nM) for the wild-type (WT) and the mutant channels of ROMK1. Mean ± SEM, $n = 4$ –16 for each group. * indicates $P < 0.01$ by unpaired t test, S219D vs. S219A. ** indicates statistically insignificant, S313D vs. S313A.

channel activation by PIP₂. This result also confirms the specificity of anti-PIP₂ antibodies in assessing channel interaction with PIP₂. The intermediate sensitivity to antibody for S219A and S313A compared with the wild-type and the R188Q mutant channels ($t_{1/2}$ for inhibition by anti-PIP₂ antibodies 39 ± 4 , 32 ± 3 , 100 ± 7 , and 14 ± 3 sec respectively, Fig. 4 B and C) indicates that both serine residues are available for PKA and phosphorylation of one serine gives a partial effect. The requirement of both serine residues for maximal enhancement of PIP₂ affinity was further examined by using channels phosphorylated by exogenous PKA. S219A or S313A mutant channels were allowed to run down and were reactivated by PKA + ATP. In contrast to a partial inhibition for the wild-type channels ($25 \pm 10\%$ inhibition, $n = 6$; Fig. 2C), 40 nM anti-PIP₂ antibody completely inhibited the activity of the S219A ($98 \pm 3\%$ inhibition, $n = 3$) and the S313A mutants ($99 \pm 2\%$ inhibition, $n = 3$) that were reactivated by exogenous PKA + ATP.

PKA phosphorylation may modulate function of the proteins by inducing a conformational change (10) and/or addition of negative charges (37). We found that mutation of either Ser-219 or Ser-313 to aspartate (S219D or S313D) could not substitute for the effect of PKA in enhancing PIP₂-channel interaction ($t_{1/2}$ for inhibition by anti-PIP₂ antibody: 8 ± 5 sec and 44 ± 6 sec for S219D and S313D, respectively vs. 100 ± 7 sec for the wild-type channels; Fig. 4C). These results suggest that the enhancement of PIP₂-ROMK interaction by PKA phosphorylation requires conformational changes of the channel proteins. Of interest, S219D mutants were more sensitive to the antibody than S219A ($t_{1/2}$ 8 ± 5 sec, $n = 4$ vs. 39 ± 4 sec, $n = 16$; $P < 0.05$). The region between amino acid 180 and 223 of the C terminus of ROMK1 contains at least four conserved basic residues (including Arg-188) that are critical for PIP₂-channel interaction (H.-H.L. and C.-L.H., unpublished results, see also legend to Fig. 4A). Thus, the higher anti-PIP₂ antibody sensitivity for S219D (compared with S219A) further suggests that the electrostatic PIP₂-channel interaction is weakened if negative charges are introduced into the PIP₂-binding region without a concomitant change in the protein conformation induced by phosphorylation.

Double mutant S219A/S313A did not express detectable current on-cell (data not shown; see also ref. 21). Unlike the channels dephosphorylated by Mg²⁺, the double mutant S219A/S313A could not be activated by exogenous PIP₂ (up to 50 μ M) in inside-out patches (data not shown). It is possible that channels are not completely dephosphorylated by Mg²⁺

and the residual phosphorylation is essential for PIP₂ activation of the channels. Alternatively, PKA may have other effect(s) on the channels besides enhancing the interaction with PIP₂. These additional stabilizing effects may be irreversibly lost for channels with mutation of both PKA target sites, but not for channels transiently dephosphorylated in Mg²⁺ solution.

DISCUSSION

PKA Activates ROMK1 by Enhancing PIP₂ Activation of the Channels. In the present study, we show that whereas PIP₂ can fully activate the channels after their run-down in the Mg²⁺ solution, PKA does not activate the channels in the absence of PIP₂. PKA phosphorylation, however, increases the sensitivity of the channels to activation by PIP₂. The dose-response curve for PIP₂ activation of the channels is shifted ≈ 10 -fold to the left, allowing channels to be activated by lower PIP₂ concentrations. Furthermore, phosphorylation by PKA decreases the sensitivity of the channels to inhibition by anti-PIP₂ antibody, and mutation of the PKA phosphorylation sites increases the sensitivity to the antibody. These results suggest that phosphorylation of ROMK1 by PKA strengthens its interaction with PIP₂ and thereby increases the activity of the channels.

The C-terminal domain of ROMK1 binds PIP₂ (7) and contains PKA phosphorylation sites (21). In multiple experiments, we could not detect significant increases in the binding of ³H-PIP₂ (0.5 μ M) to GST-RKC (100 nM) by PKA phosphorylation (not shown). Phosphorylation modulates protein function by altering its conformation and/or by addition of negative charges (10, 11, 37). For ROMK1 channels, mutation of the PKA target site Ser-219 or Ser-313 to aspartate does not substitute for the effects of PKA phosphorylation in enhancing interaction with PIP₂, suggesting that the PKA-induced increase in the affinity of the C-terminal domain of ROMK1 for PIP₂ requires conformational changes of the protein. The truncated C terminus of ROMK1 in solution (as in GST-RKC) may not be able to adopt the conformation in the absence of the remaining part of the channel proteins. Because of the dominant hydrophobic interaction between lipids and the membrane-spanning regions, it is not feasible to use the full-length proteins to study the weaker electrostatic interaction between the anionic PIP₂ and the cationic amino acids in the cytoplasmic PIP₂-binding region *in vitro*.

Validity of Anti-PIP₂ Antibody. Anti-PIP₂ antibody binds PIP₂ (with $K_d \approx 8$ nM) and prevents it from binding to the

C-terminal fragment of the channels *in vitro*. This binding K_d can fit with the observed concentration of antibodies (40–200 nM) required to inhibit the activity of the channels. The theoretical concentration of PIP₂ at the inner leaflet of the plasma membrane is ≈ 5 mM in human neutrophils (38). If PIP₂ concentration in oocyte membrane is the same, a 20- μ m giant excised membrane patch will contain approximately 10⁵ molecules of PIP₂ and 10³ molecules of the channels. A subpopulation of PIP₂ molecules may be clustered with the channels in microdomains, and the channels may be regulated only by this small fraction of PIP₂ molecules. If the affinity of the antibody for these PIP₂ molecules is equivalent to that in the solution, 40–200 nM antibodies in 50 μ l of bath solution (containing $1.2\text{--}6 \times 10^{12}$ molecules of antibodies) will be sufficient to bind 80–96% of the channel-associated PIP₂ to cause inactivation of the channels. As a further support for the specificity of anti-PIP₂ antibody, the sensitivity to anti-PIP₂ antibody for R188K is not different from that for the wild-type channels. Finally, the sensitivity of the channels to heptalysine (another PIP₂-binding agent) also is modulated by PKA phosphorylation and by mutation of PKA sites. Collectively, these results strongly support the validity of by using the sensitivity to anti-PIP₂ antibody as an index of the channel's affinity for PIP₂.

PIP₂ as the Essential "Gating Molecule" for Inward Rectifier K⁺ Channels. PIP₂ directly interacts with the proximal C terminus of the inward-rectifier K⁺ channels, including ROMK1, IRK1, and GIRK1 (7). This direct interaction between PIP₂ and the channel is important for the constitutive opening of ROMK1 and IRK1 channels. Channels close (run down) when PIP₂ is depleted from the membrane. Because of their lower affinity for PIP₂, the GIRK channels are relatively inactive in the absence of G $\beta\gamma$. G $\beta\gamma$ activates GIRK channels by enhancing PIP₂-channel interaction (7, 8). The activation of the GIRK channels by intracellular Na⁺ also depends on PIP₂ (8). Our present results that PKA regulates channel activity via PIP₂ further strengthen the idea that PIP₂ is fundamental for gating of inward-rectifier K⁺ channels, probably by stabilizing the open channel pore. Thus, modulation of the interaction between channel and the gating molecule PIP₂ regulates the activity of the inward-rectifier K⁺ channels. This mechanism for regulation of the inward-rectifier K⁺ channels is similar to that for cystic fibrosis transmembrane conductance regulator Cl[−] channel, in which phosphorylation in the R domain increases the activity of the channel by a mechanism consistent with increased binding of ATP (13). Together, these two studies provide examples for a new mechanism for PKA regulation of ion channels by enhancing the interaction with agonists.

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