Phosphatidylinositol 4,5-Bisphosphate (PIP<sub>2</sub>) Stimulates Epithelial Sodium Channel Activity in A6 Cells*

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Phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) is a membrane lipid found in all eukaryotic cells, which regulates many important cellular processes, including ion channel activity. In this study, we used inside-out patch clamp technique, immunoprecipitation, and Western blot analysis to investigate the effect of PIP<sub>2</sub> on epithelial sodium channel activity in A6 cells. A6 cells were cultured in media supplemented with 1.5 μM aldosterone. Single sodium channel activity in excised, inside-out patches was increased by perfusion of the bath solution with 30 μM PIP<sub>2</sub> plus 100 μM GTP (NP<sub>α</sub> = 1.34 ± 0.14) compared with the paired control (NP<sub>α</sub> = 0.09 ± 0.02). However, neither 30 μM PIP<sub>2</sub> (NP<sub>α</sub> = 0.11 ± 0.02) nor 100 μM GTP (NP<sub>α</sub> = 0.10 ± 0.02) alone stimulated the sodium channels. The PIP<sub>2</sub>-stimulated channel activity was abolished by application of 10 nM G protein βγ subunits (NP<sub>α</sub> = 0.14 ± 0.05). However, 10 nM Go<sub>α1a</sub> plus 30 μM PIP<sub>2</sub> increased both NP<sub>α</sub> and NP<sub>γ</sub>. The stimulating effect of 10 nM Go<sub>α1a</sub> plus 30 μM PIP<sub>2</sub> is similar to that of 30 μM PIP<sub>2</sub> plus 100 μM GTP. Immunoprecipitation and Western blot analysis show that both Go<sub>α1a</sub> and PIP<sub>2</sub> bind β and γ epithelial Na<sup>+</sup> channels (ENaC), but not α ENaC. These results indicate that PIP<sub>2</sub> increases ENaC activity by direct interaction with β or γ xENaC in the presence of Go<sub>α1a</sub>.

However, the direct regulation of ENaC activity by membrane lipid has, to our knowledge, never before been demonstrated.

Phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) is a trace, but ubiquitous, component of eukaryotic membrane phospholipid. In the traditional pathway of phosphoinositide metabolism, phosphatidylinositol is phosphorylated to produce phosphatidylinositol 4,5-bisphosphate by phosphatidylinositol 4-kinase and phosphatidylinositol 5-kinase. PIP<sub>2</sub> can be further phosphorylated to produce phosphatidylinositol 3,4,5-trisphosphate (PIP<sub>3</sub>) by phosphatidylinositol (PI) 3-kinase (3). Alternatively, PIP<sub>2</sub> can be hydrolyzed by phospholipase C to produce 1,4,5-inositol triphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). For a long time, it was assumed that the sole purpose of PIP<sub>2</sub> was to act as precursor of the two intracellular second messengers, IP<sub>3</sub> and DAG, in response to phospholipase C activation. IP<sub>3</sub> and DAG are known to mobilize calcium from the endoplasmic reticulum and to activate protein kinase C, respectively. However, recent evidence has suggested that this lipid has additional functions including regulation of ion channels. For example, PIP<sub>2</sub> can modify inwardly rectified K<sup>+</sup> channels, G protein-gated inwardly rectified K<sup>+</sup> channels (4, and ATP-sensitive K<sup>+</sup> channels (K<sub>ATP</sub>) (5–7). Besides various types of K<sup>+</sup> channels, a Na<sup>+</sup>-gated nonselective cation channel in lobster olfactory receptor neurons is also activated by PIP<sub>2</sub> (8). Since a growing number of ion channels appear to be regulated by PIP<sub>2</sub>, it is possible that PIP<sub>2</sub> is generally important in maintaining ion channel activity (8).

Recent studies using short circuit measurements demonstrated that phosphatidylinositol 3-kinase (PI 3-kinase) is present in A6 cells and that this enzyme is required for regulation of ENaC by insulin (6), aldosterone, and vasopressin (9–12). The products of PI 3-kinase include phosphatidylinositol 3-phosphate, phosphatidylinositol 3,4-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate (13). These studies indicate that phosphoinositides are involved in the regulation of ENaC activity, and these membrane lipids may be common effector molecules for hormonal regulation. Nevertheless, the short circuit current measurements in these studies could not answer the following questions. 1) Do phosphoinositides regulate ENaC or other membrane transporters? 2) If phosphoinositides regulate ENaC, do they directly interact with ENaC or interact through some other closely associated intermediate signaling molecule? 3) Which of the phosphoinositides alter ENaC activity? 4) How do the lipids modify single channel activity?

In the present work, we examined the PIP<sub>2</sub> regulation of single ENaC activity in A6 cells with the patch clamp technique and identified PIP<sub>2</sub> binding to ENaC subunits by co-immunoprecipitation and Western blot analysis.

MATERIALS AND METHODS

A6 Cells Preparation—A6 cells were purchased from American type Culture collection (Manassas, VA) in the 68th passage. All experiments...
were performed on passages 71–80 with no discernable variation among different passages. The cells were maintained in plastic tissue flasks (Corning, Corning, NY) at 27 °C in a humidified incubator with 4% CO₂ in air. The culture medium was a mixture of Coon’s medium F-12 (3 parts) and Leibovitz’s medium I-15 (7 parts) supplemented with 10% fetal bovine serum (Invitrogen) and 1.5 μM aldosterone for amphibian cells with 103 mM NaCl, 25 mM NaHCO₃, pH 7.4. For patch clamp experiments, the cells were subcultured on permeable, collagen-coated aluminosilicate supports (Nunc Corp.) attached to the bottoms of plastic rings.

**Patch Recording**—A plastic ring containing an A6 cell monolayer was mounted in a recording chamber on an inverted microscope (Nikon, Tokyo, Japan). Both the apical side and basolateral side of the monolayer were bathed in amphibian saline solution containing (in mM): 96 NaCl, 3.4 KCl, 0.8 CaCl₂, 0.8 MgCl₂, and 10 HEPES at pH 7.4 (titrated with 1 N NaOH). A perfusion setup was used to exchange the apical bath solution from amphibian saline to high potassium solution containing (in mM): 3 NaCl, 85.4 KCl, 4 CaCl₂, 1 Mgl₂, 10 HEPES, and 5 EGTA at pH 7.4 (titrated with 1 N KOH). Experiments were performed at room temperature (22–23 °C). Single channel events from inside-out patches were measured with a Micropatch 620 amplifier, low pass filtered at 5 kHz, recorded on a digital video recorder (Sony, Tokyo, Japan), and then digitized at 500 Hz using a scientific Solutions A/D converter and pentium computer equipped with Axoscope software (Axon Inc., Foster City, CA). The convention for applied voltage to the apical membrane patch (Vpipette) digitized at 500 Hz using a scientific Solutions A/D converter and pentium computer was measured with an Axopatch 200 amplifier, low pass filtered at 5 kHz. The assumptions made are that the channels within a patch are identical and open and close independently.

**Immunoprecipitation and Western Blot**—Lysates of A6 cells are incubated with PIP₂ (Roche Molecular Biochemicals), GTP (Sigma), G protein α subunit (Gαi) (Calbiochem), or G protein βγ subunit (a generous gift from Dr. David Clapham, Harvard University). For these experiments, we used inside-out patch clamp methods following standard procedure. Patch pipettes were fabricated from TW 150 glass (World Precision, New Haven, CT) and fire-polished to produce tip resistance of 5–10 MΩ when filled with amphibian saline solution. Inside-out patches were obtained from cell-attached patches following exchange of the apical bath solution from amphibian saline to high potassium solution containing (in mM): 96 NaCl, 3.4 KCl, 0.8 CaCl₂, 0.8 MgCl₂, and 10 HEPES at pH 7.4. Bath contained 3 NaCl, 85.4 KCl, 4 CaCl₂, 1 Mgl₂, 10 HEPES, and 5 EGTA at pH 7.4. Untreated patches have characteristically low activity, and addition of either GTP or PIP₂ by themselves to the cytosolic surface of the patches does not significantly alter the activity. Addition of PIP₂ + GTP dramatically increased channel activity. The lower panel shows the effects of PIP₂ and GTP on single channel activity from five patches expressed as Np (left) and Po (right). PIP₂ or GTP alone did not change Np or Po, while PIP₂ + GTP increased both number of active channels and open probability, *p < 0.05 compared with control by repeated ANOVA and Dunnett’s post test. Mean ± S.D. values for Np for different treatments were: untreated = 0.0900 ± 0.0406, PIP₂ = 0.108 ± 0.0502, GTP = 0.104 ± 0.0483, PIP₂ + GTP = 1.34 ± 0.322. Mean ± S.D. values for Po were: untreated = 0.0900 ± 0.0406, PIP₂ = 0.108 ± 0.0502, GTP = 0.104 ± 0.0483, PIP₂ + GTP = 0.346 ± 0.106.

**RESULTS**

Fig. 1 (upper panel) illustrates representative single channel records from a cell-free, inside-out patch. The control group has a low level of activity which resulted from the general loss of activity after the switch from cell-attached patch mode to inside-out, excised patch mode. Neither PIP₂ nor GTP alone altered the channel activity, but the combination of these two agents dramatically increased the number of active channels and open probability. Po. This figure shows that PIP₂ or GTP alone did not change Np or Po, while PIP₂ + GTP increased both the apparent number of active channels (Np) and open probability (Po). The requirement for GTP suggests that a G protein is involved in the PIP₂-mediated regulation of Na⁺ channel activity. This idea is supported by the observation that stimulation of ENaC activity by PIP₂ + GTP was abolished by addition of G protein βγ subunit protein in the bath solution (cytoplasmic side of membrane) (Table 1). The G protein, which has been previously described as associated with the apical membranes of A6 cells,
is G$_{i-3}$ (21). Application of the activated a subunit of G$_{i-3}$ protein (G$_{i-3}$a) by itself to the cytosolic surface of excised patches does not increase single Na$^+$ channel activity expressed as $N_P$, or $P_o$; however, application of G$_{i-3}$a + PIP$_2$ strongly activated Na$^+$ channels (Table II). The stimulating effect of G$_{i-3}$a + PIP$_2$ is comparable with that of PIP$_2$ + GTP (as shown in Fig. 1). The results in Tables I and II imply that the presence of G protein alpha subunit (G$_{i-3}$a), but not beta subunit, is required for the effect of PIP$_2$ on Na$^+$ channel activity. On the other hand, the presence of beta subunit may prevent the effect of PIP$_2$ by binding the endogenous alpha subunit in the excised patches. Unlike PIP$_2$, PIP$_3$ + GTP did not change Na$^+$ channel activity (data not shown), suggesting that PIP$_2$-induced Na$^+$ channel activity is not mediated by one of its metabolic products, PIP$_3$. In addition, application of the PIP$_2$ isomer, phosphatidylinositol 3,4 bisphosphate, also had no effect on channel activity (data not shown).

Three homologous subunits of ENaC have been isolated from A6 cells (alpha ENaC, beta ENaC, and gamma ENaC). We examined the PIP$_2$ binding subunits using immunoprecipitation and Western blot analysis. Fig. 2 shows that anti-PIP$_2$ antibody was unable to precipitate alpha ENaC, but was able to precipitate beta and gamma subunits. This result suggested that beta xENaC, and gamma ENaC, but not alpha xENaC, are the PIP$_2$ binding ENaC subunits in A6 cells. Since PIP$_2$ is only about 1100 daltons and does not stay associated with ENaC under the high detergent conditions of the gels, we could not perform the reverse experiment in which we immunoprecipitated with anti-ENaC antibodies and detected PIP$_2$.

The Western blots in Fig. 2 suggest that beta xENaC and gamma xENaC, but not alpha xENaC, are responsible for PIP$_2$ binding. Yu et al. (22) compared the putative PIP$_2$ binding sites in gelsolin with that of other PIP$_2$ binding proteins. They found 2 consensus sequences associated with PIP$_2$ binding in gelsolin. Each of the two contains a stretch of basic amino acid sequence. One of the consensus sequences, K R X X X X X K K, is also found in the N-terminal domain of gamma xENaC and is highly conserved among gamma xENaC, gamma hENaC, and gamma mENaC (Fig. 3, lower panel). The other consensus sequence is K R X X X X X X X X X X K R. A similar sequence, K R X X X X X X X X X X K R is present in all the N-terminal regions of beta xENaC, beta hENaC, beta mENaC, and gamma mENaC (Fig. 3, upper panel). Interestingly, a similar consensus sequence, K R X X X X X X K K, was identified in the PIP$_2$ binding domain of several types of inwardly rectified K$^+$ channels (4). Since both beta and gamma subunits contain potential PIP$_2$ binding sequences, while alpha ENaC does not, it is not surprising that PIP$_2$ precipitated beta xENaC and gamma xENaC. Therefore, PIP$_2$ may bind the N-terminal domains of beta xENaC and gamma xENaC and, thereby, modulate the Na$^+$ channel activity in A6 cells.

**DISCUSSION**

Our single channel recordings using inside-out patch clamp methods demonstrated that PIP$_2$, a trace membrane lipid, could dramatically alter the activity of ENaC in A6 cells. PIP$_2$ activated ENaC by increasing the single channel open probability and the apparent number of active channels. However, since ENaC open probability is extremely low under untreated conditions, we might have been unable to observe two or more channels opening simultaneously, a necessary criterion to determine the number of channels within a patch. That we failed to observe simultaneous openings does not mean that there was only one active channel under untreated conditions, since when open probability is very low, different open events may be due to different channel proteins with a very low probability of simultaneous openings. Therefore, it is hard to determine the true number of active channels under untreated conditions. Therefore, we cannot be sure PIP$_2$ increased the true number of active channels, although it certainly increased the single channel open probability.

The metabolic products of 4,5-PIP$_2$ include IP$_3$, DAG, and IP$_3$. IP$_3$ stimulates calcium release from endoplasmic reticu-
lum, but it is apparently not responsible for PIP2 stimulation of ENaC activity, since endoplasmic reticulum is not present in inside-out patches. DAG also cannot be responsible for the activation, since DAG activates protein kinase C that inhibits ENaC (25, 26). 3,4,5-PIP3 is also not responsible, since direct addition of PIP3 did not alter ENaC activity in A6 cells. Moreover, phosphatidylinositol 3,4-bisphosphate, a membrane lipid structurally similar to 4,5-PIP2, did not activate ENaC activity (data not shown). These results suggest that only 4,5-PIP2 and not its metabolic products, stimulate ENaC activity. This conclusion is different from the reports that activation of PI 3-kinase and production of PIP3 can stimulate Na+/H+ transport in Ussing chambers (9, 11). There are three possible explanations for this apparent discrepancy. First, PIP3 stimulates membrane transporters other than Na+ channels, since short circuit current could reflect not only Na+ transport but also K+ or Cl− transport. Second, PIP3 might stimulate Na+ channel activity through an intermediate intracellular signaling molecule, which would be missing in inside-out patch clamp experiments. Third, PIP3 is increased, but is broken down by a lipid 3-phosphatase to 4,5-PIP2 at the apical membrane where its increase is difficult to measure against a large cellular background of other inositol lipids.

Western blot analysis suggests that β xENaC and γ xENaC, but not α xENaC, are responsible for PIP2 binding (Fig. 2). In fact, both β and γ subunits contain consensus PIP2 binding sequences that are conserved across subunits from several species (Fig. 3). Therefore, PIP2 may bind the N-terminal domains of β xENaC and γ xENaC and thereby modulate the Na+ channel activity in A6 cells. Further investigation with mutagenesis and PIP2 binding assay will identify the PIP2 sites in ENaC subunits. Since the crystal structure of ENaC is still unknown, the molecular interaction between PIP2 and ENaC and subsequent regulation of ENaC activity is not clear, but allosteric regulation is always a possible explanation.

Despite the questionable quality of the commercial antibody, Fig. 4 shows that, besides β and γ ENaC, anti-PIP2 antibody can also co-immunoprecipitate Gαi3. However, it is unlikely that PIP2 directly binds Gαi3, because unlike β and γ ENaC, Gαi3 has no obvious PIP2 binding sites. A reasonable explanation for the result in Fig. 4 is that both PIP2 and Gαi3 bind β or γ ENaC (and are consequently all immunoprecipitated together). This is consistent with the previous finding that Gαi3 is associated with the ENaC channel protein complex (23, 27) and with the observation that PIP2 directly binds the K+ channel protein in a G protein-coupled inward rectifier K+ channel (4).
Since our patch clamp studies indicate that the presence of PIP2 regulated ENaC activity possibly by interaction between PIP2 and N-terminal domains of β xENaC and γ xENaC.

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**Fig. 4. Anti-PIP2 co-immunoprecipitates Gαi,3.** A commercially available antibody (Calbiochem) was used to detect Gαi,3 in the anti-PIP2 immunoprecipitate. Since the antibody stained several bands, we used the antigenic peptide to compete for specific binding. Two bands were reduced after treatment with peptide. The band slightly above 40 KDα is the correct molecular mass for Gαi,3. The higher molecular mass band is likely Nedd4, since Nedd4 has an amino acid sequence that has 7 of 10 amino acids in common with the antigenic peptide (and which, other than G proteins, is the only protein with such identity to the antigenic peptide).

**Fig. 5. An anti-PIP5 kinase antibody (kindly provided by Helen Liu, University of Texas, Southwestern) detected a band of the appropriate size in A6 cells confirming the existence of the enzyme responsible for production of 4,5-PIP2.**

Since our patch clamp studies indicate that the presence of Gαi,3 is indispensable for PIP2 activation of ENaC activity, PIP2 and Gαi,3 must cooperate to interact and regulate ENaC. Recent reports regarding K+ channels show that PIP2 is responsible for the gating of the channels (4, 28), while βγ subunits of G protein activate G protein-gated inwardly rectified K+ channels by stabilizing the interaction of PIP2 and C-terminal region (4). We will examine the roles of PIP2 and Gαi,3 in regulation of ENaC activity.

PIP 5-kinase is a key enzyme for production of PIP2. The presence of PIP 5-kinase in A6 cells (Fig. 5) suggests that PIP2 is produced in A6 cells. While the present excised patch clamp study has shown that the exogenously applied PIP2 activated ENaC activity, further investigation will clarify whether PIP 5-kinase is responsible for the endogenous PIP2 production and subsequent ENaC activation in intact A6 cells.

In summary, the present patch clamp study suggested that PIP2 increases Na+ channel activity in A6 cells by direct interaction with ENaC protein in the presence of G protein. The PIP2-induced Na+ channel activity is not mediated by one of its metabolic products, PIP3, or other intracellular lipid metabolites. PIP2 regulated ENaC activity possibly by interaction between PIP2 and N-terminal domains of β xENaC and γ xENaC.