Anionic Phospholipids Regulate Native and Expressed Epithelial Sodium Channel (ENaC)*

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Using patch clamp techniques, we found that the epithelial sodium channel (ENaC) activity in the apical membrane of A6 distal nephron cells showed a sudden rundown beginning at 4 min after forming the inside-out configuration. This sudden rundown was prevented by addition of anionic phospholipids such as phosphatidylinositol 4,5-bisphosphate (PIP2), phosphatidylinositol 3,4,5-trisphosphate (PIP3), and phosphatidylserine (PS) to the “cytoplasmic” bath. Conversely, chelation of en-}

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The phospholipid compositions of the two lipid bilayer leaflets of the plasma membrane are strikingly different. Anionic phospholipids are normally located in the inner leaflet to form a negatively charged surface. However, whether the phospholipid asymmetry affects the function of membrane proteins remains largely unknown. Previous studies have shown that one of the anionic phospholipids, phosphatidylinositol 4,5-bisphosphate (PIP2), regulates Na+-Ca2+ exchangers and ATP-sensitive potassium (KATP) channels (1, 2). Convincing evidence suggests that PIP2 directly interacts with the proximal COOH-terminus of inward-rectifier K+ channels (3). Not only PIP2, but also other negatively charged phospholipids such as phosphatidylinositol 4-phosphate (PI-4-P) and phosphatidylinositol 3,4,5-trisphosphate (PIP3) regulate KATP channels (4, 5). A model for the regulation of KATP channels by anionic phospholipids has been proposed, which argues that the negatively charged head group of PIP2, PI-4-P, or PIP3 locks the positively charged carboxyl terminus of KATP channels at a certain position, resulting in the failure of ATP binding to the terminus (6). This model raises an interesting question: can anionic phospholipids interact with the positively charged cytoplasmic termini of other ion channels?

The epithelial sodium channel (ENaC) plays a very important role in regulating total body Na+ homeostasis. Recent studies suggest that PIP2 stimulates ENaC in A6 cells (7) and that a decrease in PIP2 concentration may account for the inhibition of ENaC by luminal purinergic P2Y receptors (8). It is known that ENaC consists of three subunits designated α, β, and γ (9). By examining the first 50 amino acids of the NH2-terminal tails of α-, β-, and γ-ENaC, we found that the NH2-terminal tails of β- and γ-ENaC, but not of α-ENaC, contain significant numbers of positive charges. In fact, the P3geKiKaKiKKnL15 sequence in the γ subunit NH2 terminus is very similar to the pleckstrin homology domain in PLC-δ (10). We hypothesize that these positive charges might interact with anionic phospholipids of the inner leaflet of the plasma mem-}

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1 The abbreviations used are: PIP2, phosphatidylinositol 4,5-bisphosphate; PI-4-P, phosphatidylinositol 4-phosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; ENaC, epithelial sodium channel; PLC, phospholipase C; PS, phosphatidylserine; PC, phosphatidylcholine; PI 3-kinase, phosphoinositide 3-kinase.
line (PC), and poly-L-lysine were obtained from Sigma. PIP2, PIP3, and phosphatase inhibitor mixture were purchased from Calbiochem. Monoclonal anti-PIP2 antibody was from Assay Designs. NaCl bath solution contained (in mM): 100 NaCl, 3.4 KCl, 1 CaCl2, 1 MgCl2, and 10 HEPES, at a pH of 7.4. KCl bath solution contained (in mM): 100 KCl, 5 NaCl, 1 MgCl2, 10 HEPES, and 50 mM Ca2+ (after titration with 2 mM EGTA), at a pH of 7.4. All the concentrations throughout this article are shown as the final concentration.

**Patch Clamp Inside-Out Recordings**—Immediately before use, a Snapwell insert was thoroughly washed with NaCl bath solution (see “Chemicals and Solutions”) and transferred into the patch chamber mounted in the stage of a Leitz inverted microscope. Using patch clamp techniques, inside-out recordings were established on the apical membrane of A6 cells with polished micropipettes with tip resistance of 2.5–5 megohms. Under the above culture conditions, a patch seal (seal resistance > 20 gigohms) was usually formed after releasing positive pressure in the patch pipette or after applying a slightly negative pressure. After establishing the cell-attached mode, only patches containing channel activity without base-line drift were used for experiments. Before forming inside-out patches, NaCl bath solution in the patch chamber was replaced with KCl bath solution. Single-channel currents were obtained with +40-mV applied pipette potential (i.e., Vm = −40 mV), filtered at 1 kHz, and recorded on video tapes with a modified Sony PCM video converter (Vetter Instruments). Before digitization with pClamp 8 software (Axon Instruments), single-channel records were low-pass filtered at 100 Hz. The total numbers of functional channels (N) in the patch were estimated by observing the number of peaks detected on the current amplitude histograms. As a measure of channel activity, NP, (number of channels × the open probability, P, was calculated by using at least 2 min of a single-channel record as we described previously (13). Experiments were conducted at 22–23 °C.

**Two-electrode Voltage Clamp Recordings**—Oocytes were excised from adult female Xenopus frogs and treated with collagenase. Stage V–VI oocytes were injected with cRNAs for wild-type Na+/K+ pump α-subunit and then were incubated at 18 °C in modified Leibovitz medium. Electrophysiological recordings were performed 24–48 h after the injections using two microelectrodes filled with 3 mM KCl and inserted into the oocyte, as described previously (14). A voltage step protocol from −120 to +40 mV in increments of 20 mV was used.

Between voltage steps the membrane was voltage-clamped at a holding potential of −40 mV. The macroscopic ENaC currents were verified by application of 10 μM amiloride to the bath. The net amiloride-sensitive currents were used to represent ENaC activity. After recording control ENaC currents, the oocytes were taken out of the chamber and injected with 3 mM H2O, PIP2 (30 μM), or PIP3 (30 μM), respectively. Phosphatase inhibitor mixture (2 μM) was included in each injection. 30 min after these injections, amiloride-sensitive currents were re-measured in these oocytes and compared with the currents before these injections.

**Evaluation of ENaC Surface Expression by Confocal Microscopy**—Using confocal microscopy, the surface expression of ENaC after each experimental manipulation was evaluated; rat β and γ-ENaC subunits were tagged in the extracellular loops with the FLAG epitope (DYKDDDK), which can be recognized by M2 monoclonal antibody, as described previously (15). The FLAG-tagged β-, γ-, and α-ENaC cRNAs were injected into Xenopus oocytes. Fluorescent imaging analysis of the expression level by confocal microscopy was carried out, as we described previously (16). The oocytes were then secondary injected with H2O, PIP2 or PIP3, as described above. The effects of PIP2 and PIP3 on ENaC surface expression were evaluated using the confocal fluorescent imaging methods.

**Statistical Analysis**—A paired t test or analysis of variance for multiple comparisons was used for statistical analysis, as we described previously (13). A p value less than 0.05 was considered significant.

### RESULTS AND DISCUSSION

**A Decrease in PIP2 Concentration Appears to Mediate Inhibition of ENaC by the P2Y2 Receptor**—The G protein-coupled P2Y2 receptor is expressed in renal epithelial cells (17). Therefore, we hypothetize that a decrease in PIP2 concentration might mediate the P2Y2 receptor-induced inhibition of ENaC. To test this hypothetesis, inside-out patch experiments were performed as shown in Fig. 1. We found that ENaC activity in inside-out patches was stable for the initial 4 min. However, a sudden rundown occurred during the period from 4 to 5 min. Interestingly, the channel rundown was clearly prevented when the cytoplasmic bath contained 5 μM PIP2 (second trace), 100 nM anti-PIP2 antibody (third trace), or 0.5 unit/ml phosphatidylinositol-specific PLC (fourth trace) or when the patch pipette contained 100 μM ATP (fifth trace), respectively. Downward events show channel openings. “C” shows the baseline when channels are closed. B, summary plots of NPo under above conditions, showing that ENaC has a sudden rundown during the period from 4 to 5 min under control conditions (open circles), which was prevented by PIP2 (open squares) and accelerated by anti-PIP2 antibody (solid triangles), PLC (open triangles), or ATP (solid circles).

**An Increase in PIP3 Concentration May Mediate Stimulation of ENaC by Corticosteroid Receptors and Insulin**—It is known that KATP channels are not only regulated by PIP2, but also by PIP3 (4). However, the role of PIP3 has been neglected, because the plasma membrane does not contain PIP3 under normal conditions. Nevertheless, PIP3 can be generated by activation of phosphoinositide 3-kinase (PI 3-kinase). Interestingly, recent studies have shown that both aldosterone and insulin enhance Na+ transport by activating PI 3-kinase in A6 cells and that inhibition of PI 3-kinase will block their stimulatory effect on ENaC activity (19–21). To test whether PIP3 could affect ENaC activity, the inside-out patch configuration was used.
B 

A representative single-channel recordings of ENaC under control conditions (first trace) or when the cytoplasmic bath contained 5 μM PIP2 (second trace), 20 μM PS (third trace), 10 μg/ml poly-L-lysine (fourth trace), or 20 μM PC (fifth trace). Downward events show channel openings. “C.” shows the base line when channels are closed. B, summary plots of ENaC activity under conditions showing that ENaC rundown (solid circles) was prevented by either PIP2 (open triangles) or PS (solid squares) and accelerated by poly-L-lysine (solid triangles), but not affected by PC (open circles).

FIG. 2. Effects of PIP2, PS, poly-L-lysine, and PC on ENaC activity in inside-out patches. A, representative single-channel recordings of ENaC under control conditions (first trace) or when the cytoplasmic bath contained 5 μM PIP2 (second trace), 20 μM PS (third trace), 10 μg/ml poly-L-lysine (fourth trace), or 20 μM PC (fifth trace). Downward events show channel openings. “C.” shows the base line when channels are closed. B, summary plots of ENaC activity under conditions showing that ENaC rundown (solid circles) was prevented by either PIP2 (open triangles) or PS (solid squares) and accelerated by poly-L-lysine (solid triangles), but not affected by PC (open circles).

FIG. 3. Stimulation of amiloride-sensitive ENaC current by PIP2 and PIP3. A, summary plots of amiloride-sensitive currents before (blank bars) and after injections with H2O, PIP2, or PIP3 (hatched bars). Oocytes used in this set of experiments were injected with cRNAs encoding rat α-, β-, and γ-ENaC subunits. Two-electrode voltage clamp recordings were carried out during the period of 24–48 h after the injection. Amiloride-sensitive currents were the currents at a potential of −100 mV under control conditions subtracted by the currents after addition of 10 μM amiloride. After initial recordings of amiloride-sensitive currents as a control, the oocytes were then injected with H2O, PIP2, or PIP3. Fluorescent labeling was performed 30 min after these injections. B, no obvious change in ENaC surface expression after injection of PIP2 or PIP3. Oocytes used in this set of experiments were injected with cRNAs encoding FLAG-tagged rat α-, β-, and γ-ENaC subunits. After ENaC was significantly expressed (24–48 h after injection with cRNAs of ENaC), the oocytes were then injected with H2O, PIP2, or PIP3. Fluorescent labeling was performed 30 min after the secondary injections.

ponent with the results as shown in Fig. 1, ENaC activity in inside-out patches was steady during the initial 4–5 min before a sudden rundown occurred. In contrast, the channel activity was maintained without rundown when the cytoplasmic bath contained 5 μM PIP2 (Fig. 2). Because the concentration of PIP3 is elevated in response to aldosterone (19), the effect of PIP2 on ENaC activity may account in part for the regulation of ENaC by aldosterone. To test whether other anionic phospholipids can also regulate ENaC activity, inside-out patches were examined when negatively charged PS (20 μM) was applied to the cytoplasmic bath. Similar to the effect of PIP2 and PIP3, anionic PS also prevented ENaC rundown, suggesting that the effect of phospholipids on ENaC activity may be related to their anionic composition. To test whether negative charges are important for the effect of PIP2, PIP3, and PS on ENaC activity, the effect of the positively charged molecule, poly-L-lysine, on ENaC activity was examined. It appears that addition of poly-L-lysine (10 μg/ml) accelerated ENaC rundown. However, neutral PC had no effect on ENaC activity (Fig. 2). These data suggest that an increase in PIP3 concentration may mediate stimulation of ENaC by corticoid receptors and insulin at the level of interaction of the ENaC complex with the inner leaflet of the plasma membrane.

PIP2 and PIP3 Enhance ENaC Current in Xenopus Oocytes—In addition to A6 cells that natively express ENaC when conditioned by aldosterone, the Xenopus oocyte system was also used to test the role of PIP2 and PIP3 in regulating ENaC activity. Using two-electrode voltage clamp techniques exogenously expressed ENaC activity was evaluated with amiloride-sensitive currents following injection of rat α-, β-, and γ-ENaC cRNAs. Amiloride-sensitive currents were compared in the same oocyte before and 30 min after injection of equal volume of H2O (as a control), PIP2 (30 μM), or PIP3 (30 μM), respectively. Amiloride-sensitive currents were not changed in the oocytes injected with H2O (−728 ± 84 nA versus −750 ± 72 nA; n = 7). In contrast, amiloride-sensitive currents were increased, from −797 ± 40 nA to −1091 ± 69 nA (p < 0.001; n = 14) after injection with PIP2 and from −735 ± 31 nA to −1077 ± 73 nA (p < 0.001; n = 10) after injection with PIP3. To further determine whether the increase in amiloride-sensitive currents were related to ENaC trafficking, the density of ENaC on the surface of the plasma membrane was evaluated with confocal surface labeling techniques. The oocytes that expressed FLAGTM-tagged ENaC were injected with equal volume of H2O (as a control), PIP2 (30 μM), or PIP3 (30 μM), respectively. Fluorescent labeling was carried out 30 min after these injections. The data demonstrated that there was no difference in ENaC surface density between each group of oocytes injected with H2O, PIP2, or PIP3 (Fig. 3), indicating that PIP2 and PIP3 up-regulate ENaC through a mechanism that appears to be independent of ENaC trafficking as it affects the density of surface expression. Although PIP2, PIP3, and PS failed to enhance ENaC activity in A6 cells, but only maintain the channel activity, it is likely that the stimulatory effect of anionic phospholipids on ENaC activity may be already saturated in A6 cells, which are continuously cultured in the presence of aldosterone. Further experiments will address this hypothesis by using ENaC-expressing renal epithelial cells cultured either in the absence or in the presence of aldosterone. Presumably, without prestimulation by aldosterone, anionic phospholipids will increase the low basal level of ENaC activity in such cells.
Conclusion and Potential Significance—We (8) and others (18) have recently demonstrated that stimulation of the P2X family, probably the P2Y2 receptor, inhibits ENaC activity in A6 distal nephron cells and amiloride-sensitive I_{Na} in mouse cortical collecting duct principal cells via a pathway that appears to occur independently of an increase in [Ca^{2+}]. The present study demonstrates that anionic phospholipids activate endogenously expressed ENaC in A6 cells and exogenously expressed ENaC in Xenopus oocytes. Since both chelation of endogenous PIP_2 with anti-PIP_2 antibody and hydrolysis of endogenous PIP_2 with exogenous PLC or extracellular ATP that presumably activates endogenous PLC could reduce ENaC activity (Fig. 1), a decrease in PIP_2 concentration in the inner leaflet of the plasma membrane may explain inhibition of ENaC by the P2Y2 Receptor. In addition, we have found that PIP_3 also regulates ENaC activity (Fig. 2). With the recognition of the role of PIP_3 and PI 3-kinase in the responses to aldosterone and insulin on ENaC activity in A6 cells (19–21), and the recent recognition of other phosphatidylinositol kinases (22), the role of anionic phospholipids in the tonic regulation of ENaC activity at the level of the plasma membrane may well be of general importance. The response to aldosterone is pleotropic and involves sgk kinase as well as changes in PI 3-kinase (23–26). It appears that aldosterone-mediated activation of sgk kinase rapidly stimulates translocation of ENaC to the apical membrane (27), while the experiments described in the legend to Fig. 3, using anionic phospholipids as the putative downstream effectors of the aldosterone response, demonstrate activation of ENaC in situ rather than recruitment or translocation of ENaC complexes to the plasma membrane in the oocyte system. Therefore, an increase in PIP_3 concentration in the inner plasma membrane may account in part for the stimulatory effects of aldosterone and insulin on ENaC activity at the level of the inner leaflet of the plasma membrane.
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