Modulation of epithelial sodium channel activity by lipopolysaccharide in alveolar type II cells: involvement of purinergic signaling

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**Pseudomonas aeruginosa** is a gram-negative bacterium that causes chronic infection in cystic fibrosis patients. We reported recently that *P. aeruginosa* modulates epithelial Na⁺ channel (ENaC) expression in experimental chronic pneumonia models. For this reason, we tested whether LPS from *P. aeruginosa* alters ENaC expression and activity in alveolar epithelial cells. We found that LPS induces a ~60% decrease of ENaC apical current without significant changes in intracellular ENaC or surface protein expression. Because a growing body of evidence reports a key role for extracellular nucleotides in regulation of ion channels, we evaluated the possibility that modulation of ENaC activity by LPS involves extracellular ATP signaling. We found that alveolar epithelial cells release ATP upon LPS stimulation and that pretreatment with suramin, a P2Y₂ purinergic receptor antagonist, inhibited the effect of LPS on ENaC. Furthermore, ET-18-OCH₃, a PLC inhibitor, and Go-6976, a PKC inhibitor, were able to partially prevent ENaC inhibition by LPS, suggesting that the actions of LPS on ENaC current were mediated, in part, by the PKC and PLC pathways. Together, these findings demonstrate an important role of extracellular ATP signaling in the response of epithelial cells to LPS.

**protein kinase C; Pseudomonas**

**ACTIVE SODIUM TRANSPORT** across the alveolar epithelium in the lung has been demonstrated to be important for the reabsorption of fetal lung fluid around birth (36) and for the resolution of pulmonary edema (6, 33). This active Na⁺ transport involves the amiloride-sensitive epithelial Na⁺ channel (ENaC), located at the apical surface of alveolar epithelial cells, as well as Na⁺-K⁺-ATPase, located at the basolateral surface (6, 33). Although several types of Na⁺ channels and cotransporters are expressed in alveolar epithelial cells (30), experimental evidence indicates that ENaC plays a prominent role in the lung liquid clearance process. ENaC comprises three homologous subunits (α, β, and γ), of which α-ENaC is essential for channel activity (9, 31). Although ENaC inhibition by amiloride decreases the Na⁺ transport and liquid clearance process across the alveolar epithelium (33), the importance of ENaC was definitively proven in an α-ENaC-knockout mouse model. These mice developed respiratory distress and died within 40 h of birth because of their inability to clear their alveolar space (23). This reveals the crucial role of ENaC in alveolar liquid clearance. Downregulation of ENaC expression and activity is, therefore, associated with susceptibility to lung edema (38, 49).

Inflammatory cytokines and growth factors, such as IL-1β (47), IL-4 (20), interferon-γ (19), and transforming growth factor-β1 (18), present during lung inflammation and linked with acute respiratory distress syndrome, have been found to modulate ENaC expression and activity in lung epithelial cells. We reported recently that TNF decreased ENaC expression and activity in primary cultures of alveolar epithelial cells (13) and that dexamethasone, a synthetic glucocorticoid, inhibited the action of TNF on ENaC (12). Moreover, we discerned changes of ENaC expression in mouse lungs infected with *Pseudomonas aeruginosa* (14). Although *P. aeruginosa* and *P. aeruginosa* flagellin alter Na⁺ absorption in airway epithelial cells (17, 26), the mechanisms involved are not clearly defined. LPS, a glycolipid that constitutes the major portion of the outermost membrane of gram-negative bacteria, is a potent proinflammatory molecule that provokes local acute lung inflammation and, in extreme cases, endotoxic shock. Interaction of the lipid A motif with pattern recognition receptors, such as Toll-like receptors (TLR-4), could result in cellular activation and release of proinflammatory molecules (46). Interestingly, LPS was shown in collecting duct principal cell models to down-regulate α-ENaC transcript and channel activity via the nuclear transcription factor NF-κB and serum- and glucocorticoid-regulated kinase (SGK1) signaling pathways (15).

In light of these findings and the effect of *P. aeruginosa* on ENaC expression in mouse lungs, we studied whether LPS from *P. aeruginosa* could regulate ENaC activity in alveolar type 2 (ATII) cells and tested the signaling pathways that could be associated with such responses. We noted that LPS has an impact on ENaC activity and that this effect is modulated by ATP secretion and activation of the purinergic receptor signaling pathway.

**MATERIALS AND METHODS**

**Materials.** MEM and FBS were purchased from Invitrogen (Burlington, ON, Canada); porcine pancreatic elastase from Worthington Biochemical (Lakewood, NJ); amiloride, trypsin, suramin, pyridoxal phosphate-6-azophenyl-2′,4′-disulfonate (PPADS), Brilliant Blue G (BBG), trinitrophenyl-ATP (TNP-ATP), 2-methylthioadenosine-5′-O-triphosphate (2-MeSATP), ATP, ATP-γ-S, amphotericin B, neomycin, and monoclonal antibody against β-actin (Ac-74) from Sigma (St. Louis, MO); EZ-Link sulfo-NHS-LC-biotin, NeutrAvidin agaorse resin, secondary antibody, and chemiluminescence reagents from Thermo Scientific Pierce Protein Research Products (Rockford, IL); primary antibodies against α-, β-, and γ-ENaC from Affinity Bioreagents (Golden, CO); and ET-18-OCH₃, Go-6976, and wortmannin from Calbiochem (San Diego, CA).
Alveolar epithelial cell isolation and experimental conditions. Alveolar epithelial cells were isolated from male Sprague-Dawley rats, as described previously (12) and according to a procedure approved by our institutional Animal Care Committee. Perfused lungs were digested with elastase, and the cells were purified by a differential adherence technique on bacteriological plastic plates coated with rat immunoglobulin G. The cells were maintained in MEM containing 10% FBS, 0.08 mg/ml gentamicin, 0.2% NaHCO3, 0.01 M HEPES, and 2 mM l-glutamine. They were plated at 1 × 10^6 cells/cm^2 on polycarbonate filters (Corning Costar Transwell, Torrance, CA) and cultured at 37°C with 5% CO2 in a humidified incubator. The medium was supplemented with Septra (3 μg/ml trimethoprim and 17 μg/ml sulfamethoxazole) for the first 3 days. Then the medium was replaced, and the cells were cultured without Septra.

Short-circuit current measurements in an Ussing chamber. Cells grown on 4-cm^2 Costar Transwell filters were cultured for 3 days and treated for 4 h with 15 μg/ml LPS, as described above. For short-circuit current (Isc) measurements, the filters were mounted in an Ussing chamber and bathed on the apical and basolateral sides with warm physiological buffer (140 mM NaCl, 5 mM KCl, 1 mM MgCl2, 5 mM glucose, and 10 mM HEPES, pH 7.4). The transepithelial potential difference was clamped to zero by an external voltage-clamp amplifier (apical and basolateral sides of the monolayer connected via KCl agar-calomel half-cells and Ag-AgCl electrodes). The resulting Isc was recorded by a voltage-clamp amplifier (model VCC MC2, Physiological Instruments, San Diego, CA) linked to a data acquisition and analysis system (4sp PowerLab, ADInstruments, Grand Junction, CO). Resistance was quantified from the current needed to clamp voltage from 0 to 1 mV for 1 s every 10 s. When Isc was stabilized, the cell monolayers were treated with 10 μM amiloride on the apical side for measurement of amiloride-sensitive current in control and LPS-treated cells. Basolateral permeabilization with amphotericin B was performed to evaluate ENaC activity in the presence of a 5.6× apical-basolateral Na+ gradient. Briefly, cells cultured on Costar Transwell filters were mounted in an Ussing chamber and bathed on the apical side with Na+ physiological buffer (141 mM NaCl, 5.4 mM KCl, 0.78 mM NaH2PO4, 1.8 mM CaCl2, 0.8 mM MgCl2, 5 mM glucose, and 15 mM HEPES; pH 7.4, 37°C). On the basolateral side, 116 mM NaCl was replaced by an equivalent amount of N-methyl-d-glucamine chloride in the presence of 7.5 μM amphotericin B. After stabilization of the Isc, amiloride-sensitive current was measured by the addition of 10 μM amiloride. For the measurement of LPS impact on ENaC activation by extracellular proteases, the filters were bathed with physiological buffer, and when the current reached a plateau, amiloride was added to the apical side at 10 μM final concentration. Amiloride on the apical side of the cell was washed away, and when the current reached a new plateau, trypsin was added on the apical side of the cells to reach a concentration of 10 μg/ml. After current stabilization at its highest point, 10 μM amiloride was again added on the apical side.

Protein extraction and immunoblotting. ATII cells cultured on Costar Transwell filters in the presence or absence of 15 μg/ml LPS were washed twice with PBS and incubated for 1 h at 4°C under agitation in lysis buffer (1% Triton X-100, 150 mM NaCl, 5 mM EDTA, and 50 mM Tris, pH 7.5) supplemented with a protease inhibitor cocktail (1 mM PMSF, 10 μg/ml aprotinin, and 500 ng/ml leupeptin). The cells were subsequently scraped with a rubber policeman, collected, and centrifuged at 12,000 g for 5 min. Protein concentration of the supernatant was evaluated by the Bradford method (Bio-Rad Life Science, Mississauga, ON, Canada). After solubilization in sample buffer (50 mM Tris-HCl, 2% SDS, 0.1% bromphenol blue, 10% glycerol, and 125 mM DTT), 50 μg of total proteins were subjected to SDS-PAGE and transferred electrophoretically onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were blocked for 16 h at 4°C with 5% (wt/vol) skim milk in TBS-Tween buffer (500 mM NaCl, 20 mM Tris-HCl, and 0.1% Tween 20, pH 7.4) and then incubated in the same buffer for 2 h at room temperature with a 1:1,000 dilution of α,β,γ-ENaC antibody (Affinity Bioreagents), a 1:1,000 dilution of pan-phosphorylated PKC (Cell Signaling Technology, Beverly, MA), or a 1:1,000 dilution of pan-PK-C antibody (Abcam, Cambridge, MA). The membranes were washed then incubated in TBS-Tween and incubated for 1 h at room temperature with a 1:2,500 dilution of horseradish peroxidase-linked secondary antibody (Cell Signaling Technology). After they were washed in TBS-Tween, the membranes were incubated with Pierce chemiluminescence reagent (Pierce Biotechnology, Rockford, IL) for 5 min before the luminescent signals were recorded with X-Omat BT film (Kodak, Rochester, NY). The X-ray films were then scanned, and band densitometry was quantified by MultiGauge software (FujiFilm, Mississauga, ON, Canada). Western blots were then probed with antibodies extracted from cells purified from different rats.

Cell surface biotinylation. The cell surface pool of ENaC subunits was quantified by surface biotinylation, as described elsewhere (8). Briefly, ATII cells cultured on Costar Transwell filters were washed three times with ice-cold Ca2+- and Mg2+-containing PBS with agitation on ice to remove growth media. Apical membrane proteins were biotinylated in borate buffer (85 mM NaCl, 4 mM KCl, and 15 mM Na2B4O7, pH 9) containing 1.5 mg/ml NHS-ss-biotin (Pierce Biotechnology) for 25 min on ice. Biotinylation was quenched by three washes with Ca2+- and Mg2+-containing PBS + 100 mM glycine. The monolayers were then rinsed three times with Ca2+- and Mg2+-containing PBS and lysed in cell lysis buffer (see above). To harvest biotinylated protein, 500 μg of protein were incubated overnight at 4°C under agitation with NeutrAvidin agarose resin (Pierce Biotechnology). After centrifugation and several washes of the beads with protein lysis buffer, the samples were eluted in 2× sample buffer containing 10% β-mercaptoethanol and incubated for 10 min at 95°C. They were then subjected to SDS-PAGE and transferred to membranes, and the immunoblots were exposed to an enhanced chemiluminescence system (see Protein extraction and immunoblotting). An equal amount (50 μg) of nonbiotinylated cell lysates was analyzed by Western blotting in parallel with the biotinylated fraction to quantify the intracellular α,β,γ-ENaC pool.

ATP assay. The extracellular ATP level following LPS treatment was measured in alveolar epithelial cells grown on 12-mm Costar filters. Since MEM interferes with the luciferase assay, on the day of the experiment the medium was replaced with a physiological buffer (140 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 10 mM glucose, and 10 mM TES, pH 7.4). Since medium change is required, the NaCl was replaced by an equivalent amount of N-methyl-d-glucamine chloride in the presence of 10% β-mercaptoethanol and incubated for 1 h at 37°C. After stabilization of the luciferase activity by extracellular proteases, the filters were bathed with physiological buffer, and when the current reached a plateau, amiloride was added to the apical side at 10 μM final concentration. Amiloride on the apical side of the cell was washed away, and when the current reached a new plateau, trypsin was added on the apical side of the cells to reach a concentration of 10 μg/ml. After current stabilization at its highest point, 10 μM amiloride was again added on the apical side.

Protein extraction and immunoblotting. ATII cells cultured on Costar Transwell filters in the presence or absence of 15 μg/ml LPS were washed twice with PBS and incubated for 1 h at 4°C under agitation in lysis buffer (1% Triton X-100, 150 mM NaCl, 5 mM EDTA, and 50 mM Tris, pH 7.5) supplemented with a protease inhibitor cocktail (1 mM PMSF, 10 μg/ml aprotinin, and 500 ng/ml leupeptin). The cells were subsequently scraped with a rubber policeman, collected, and centrifuged at 12,000 g for 5 min. Protein concentration of the supernatant was evaluated by the Bradford method (Bio-Rad Life Science, Mississauga, ON, Canada). After solubilization in sample buffer (50 mM Tris-HCl, 2% SDS, 0.1% bromphenol blue, 10% glycerol, and 125 mM DTT), 50 μg of total proteins were subjected to SDS-PAGE and transferred electrophoretically onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were blocked for 16 h at 4°C with 5% (wt/vol) skim milk in TBS-Tween buffer (500 mM NaCl, 20 mM Tris-HCl, and 0.1% Tween 20, pH 7.4) and then incubated in the same buffer for 2 h at room temperature with a 1:1,000 dilution of α,β,γ-ENaC antibody (Affinity Bioreagents), a 1:1,000 dilution of pan-phosphorylated PKC (Cell Signaling Technology, Beverly, MA), or a 1:1,000 dilution of pan-PK-C antibody (Abcam, Cambridge, MA). The membranes were then washed in TBS-Tween and incubated for 1 h at room temperature with a 1:2,500 dilution of horseradish peroxidase-linked secondary antibody (Cell Signaling Technology). After they were washed in TBS-Tween, the membranes were incubated with Pierce chemiluminescence reagent (Pierce Biotechnology, Rockford, IL) for 5 min before the luminescent signals were recorded with X-Omat BT film (Kodak, Rochester, NY). The X-ray films were then scanned, and band densitometry was quantified by MultiGauge software (FujiFilm, Mississauga, ON, Canada). Western blots were then probed with antibodies extracted from cells purified from different rats.

Statistical analysis. Values are means ± SE. Comparisons among groups were analyzed by unpaired t-test and one-factor analysis of variance with post hoc comparison (Fisher’s protected least significant difference). The level of significance was defined to be P < 0.05.

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LPS decreases Na\(^+\) transport across rat lung epithelial cell monolayer. Exposure to 15 μg/ml LPS on the apical side of the cell monolayer for 4 h significantly decreased (P < 0.05) total and amiloride-sensitive transepithelial current across rat alveolar epithelial cells (Fig. 1A). Amiloride-sensitive current in LPS-treated cells decreased 50%, from 20.57 ± 1.3 to 9.63 ± 0.81 μA/cm\(^2\), compared with the controls (Fig. 1B). Dose-response curves (not shown) demonstrate that alveolar epithelial cells are very sensitive to LPS, since LPS at 12.5 ng/ml still significantly reduces the amiloride-sensitive current in these cells. To determine whether LPS was affecting ENaC, we evaluated the amount of amiloride-sensitive ENaC current at the apical membrane after amphotericin B permeabilization of the basolateral membrane in the presence of an apical-to-basolateral Na\(^+\) gradient where N-methyl-D-glucosamine chloride replaced 82% of Na\(^+\) in the basolateral solution (Fig. 1C). In these conditions, LPS reduced amiloride-sensitive (ENaC) current to 41% of the controls: I\(_{sc}\) decreased from 25.07 ± 1.65 to 10.34 ± 3.13 μA/cm\(^2\) (Fig. 1D; P < 0.05).

Membrane and intracellular pool of α,β,γ-ENaC protein are not affected by LPS. We investigated whether LPS had an impact on the apical membrane and intracellular pool of α,β,γ-ENaC protein in alveolar epithelial cells treated with 15 μg/ml LPS for 4 h. Immunodetection of ENaC subunits by Western blotting detected one or two broad bands between 70 and 90 kDa (as expected by the manufacturer’s datasheet) in the membrane biotinylated surface fraction and in the intracellular pool (Fig. 2). The presence of a double band in the biotinylated fraction represents the glycosylated form of the subunits, which cannot be observed in the intracellular fraction and is more pronounced for γ-ENaC. Together, we observed no significant modulation of α-, β- or γ-ENaC protein expression in response to LPS compared with the control condition.

Trypsin cannot alleviate transepithelial amiloride-sensitive Na\(^+\) transport in LPS-treated cells. ENaC activity is also modulated by membrane-bound or extracellular proteases. For this reason, we tested the effects of trypsin on transepithelial amiloride-sensitive current in control and LPS-treated cells. Although trypsin elevated amiloride-sensitive current in control and LPS-treated cells (P < 0.05), the current after trypsin treatment was still downregulated in LPS-treated cells compared with controls (P < 0.05; Table 1).

ATP secretion and involvement of purinergic receptors in ENaC modulation by LPS. LPS was reported to promote ATP secretion and ATP-dependent MUC5AC overproduction in nasal epithelial cells (48). To investigate whether purinergic signaling could also be involved in the ENaC/LPS response, we tested the effect of pretreatment with suramin, a broad-spectrum purinergic receptor inhibitor, on the modulation of ENaC current by LPS. As shown in Fig. 3, 15 min of apical

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**RESULTS**

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pretreatment with 500 μM suramin inhibited the action of LPS on transepithelial amiloride-sensitive current, implicating purinergic receptors in the modulation of ENaC by LPS. The ATP level was significantly elevated in the apical medium of LPS-treated cells after 30 and 60 min compared with untreated cells (P < 0.05).

P2Y2 receptors are mainly involved in response to LPS and are implicated in ENaC modulation. The purinergic receptor subtype involved in ENaC modulation by LPS was further characterized with different antagonists. Pretreatment of the cells with 100 μM PPADS, a broad-spectrum P2Y receptor antagonist (P2Y1, P2Y4, P2Y12, and P2Y14), or with 100 μM BBG or TNP-ATP, two P2X receptor antagonists (P2X1, P2X2, P2X3, P2X4, P2X5, and P2X7), could not inhibit the LPS-induced decrease in amiloride-sensitive current (Fig. 4B).

Finally, 4 h of treatment with 100 μM ATPγS, a nonhydrolyzable ATP analog known to specifically activate P2Y2 receptors, reproduced the effects of LPS on apical amiloride-sensitive (ENaC) current (Fig. 4C), whereas 2-MeSATP, a broad-spectrum P2X receptor agonist, had no impact.

Early, but not late, suramin treatment inhibits the influence of LPS on amiloride-sensitive current. ATP is known to exert an acute action on ENaC function after P2Y2 receptor stimulation. Since the ATP release we detected after LPS treatment was somewhat earlier than the decrease in ENaC current we detected at 4 h, we tested how control and LPS-treated alveolar epithelial cells reacted to ATP and followed the time course of the response to suramin. Alveolar epithelial cells were treated on the apical side with 100 μM ATP, and transepithelial amiloride-sensitive current was recorded in the Ussing chamber. As illustrated in Fig. 5, A and B, ATP induced a rapid decrease (~30%) of amiloride-sensitive current in control cells, from 25.6 ± 3.13 to 16.72 ± 1.91 μA/cm² (P < 0.05). In 4-h LPS-treated cells, ATP still induced a reduction of amiloride-sensitive current, from 10.35 ± 2.27 to 4.54 ± 0.4 μA/cm² (Fig. 5, C and D; P < 0.05). Amiloride-sensitive current was, therefore, modulated similarly in control and LPS-treated cells after ATP treatment, indicating that the current reduction after LPS treatment probably involved other mechanisms besides the acute inhibition of amiloride-sensitive current by extracellular ATP. To test this hypothesis, the amiloride-sensitive current was recorded after 4 h of LPS treatment following two alternative pretreatment protocols with suramin. In addition to the pretreatment protocol when suramin is added before LPS treatment, we also tested a late suramin treatment 15 min before Ussing chamber recording to test whether late inhibition of the purinergic receptor could be sufficient to restore the current. As reported in Fig. 6, although early suramin treatment was effective in inhibiting the LPS-induced decrease in current, late treatment was not.

Decrease of ENaC apical current induced by LPS is mediated, in part, by the PLC and PKC pathways. It is known that activation of purinergic receptors can lead to breakdown of PIP2 via PLC and activation of PKC, two pathways known to downregulate ENaC current. For this reason, we investigated whether these pathways were involved in the signaling response linking LPS to the modulation of apical ENaC current. Pretreatment of the cells with 40 μM ET18-OCH3, a PLC inhibitor, had a partial, but significant, suppressive effect on LPS-induced current reduction (Fig. 7A). ATP has been shown to downregulate ENaC by affecting the phosphatidylinositol 4,5-bisphosphate (PIP2) level at the membrane (25). To determine whether the LPS-induced modulation could involve a modulation of PIP2 level, alveolar epithelial cells were treated for 5.5 h with 10 μM wortmannin, which inhibits phosphatidylinositol 4-kinase at this concentration. This treatment is known to decrease the PIP2 level at the membrane. The decrease in ENaC apical current mediated by wortmannin was similar to that mediated by LPS (Fig. 7B). When LPS was added to wortmannin-pretreated cells, LPS was still able to decrease the current by a further 54% compared with wort-
pretreated cells, LPS could not decrease the current further (Fig. 7C).

We then tested how PKC could be involved in the LPS-induced modulation of ENaC current. Immunoblots against PKC showed that LPS activated the PKC pathway in cells, increasing the level of phosphorylated PKC isoforms above untreated control values after 30 or 60 min of treatment (P < 0.05; Fig. 8A). Pretreatment of the cells with Go-6976, a PKC inhibitor, prevented the effect of LPS on amiloride-sensitive transepithelial current (Fig. 8B) and prevented, in part, the impact of LPS on ENaC apical current (Fig. 8C). In ATPγS-treated cells, Go-6976 restored apical ENaC current, demonstrating that PKC is also involved in the downregulation of apical ENaC current by ATP (Fig. 8D).

**DISCUSSION**

In light of previous data from our laboratory (14), we studied the impact of LPS from *P. aeruginosa* on ENaC modulation in alveolar epithelial cells. We found that 4 h of treatment with LPS leads to a decrease in ENaC current that is not related to modulation at the surface or in the intracellular pool of ENaC protein or its capacity to be modulated by extracellular proteases. We further showed that ATP release and activation of suramin-sensitive pathways were involved in this response. Finally, our results pointed out the involvement of the PKC signaling pathway in the mechanisms of ENaC downregulation by LPS.

Our results disclosed that treatment of rat alveolar epithelial cells with LPS from *P. aeruginosa* induced a decline of total and amiloride-sensitive transepithelial current (Fig. 1). Because transepithelial Na⁺ transport is the result of Na⁺ channel activity at the apical membrane, as well as Na⁺-K⁺-ATPase and K⁺ channel activity on the basolateral side, we tested whether ENaC was implicated in the current decrease evoked by LPS. To address this question, the basolateral membrane was permeabilized with amphotericin B in the presence of an apical-to-basolateral Na⁺ gradient, as reported previously (12). These experiments revealed that LPS downregulated amiloride-sensitive ENaC current at the apical membrane by ~60% (Fig. 1). These changes in ENaC current could be secondary to its altered expression and trafficking or modifications in the regulation of channel activity. To study this question, the level of each ENaC subunit, α-, β-, and γ-ENaC proteins, was measured in biotinylated membrane fractions and in the corresponding nonbiotinylated intracellular pool. These immunoblots showed no variation of α-, β-, or γ-ENaC protein level at the membrane surface or in the intracellular compartment, suggesting that LPS does not modulate the amount of ENaC in the cells (Fig. 2). Since it has been shown that extracellular proteases modulate ENaC current in airway epithelial cells (2, 35), we determined whether LPS could influence ENaC activation by extracellular proteases. We found that trypsin treatment increases ENaC current in control and LPS-treated cells. These results are different from those of Planes et al. (40); they reported that trypsin treatment of rat alveolar epithelial cells could not increase ENaC current in the absence of aprotinin. This discrepancy could be explained by differences in the culture conditions between the two groups. Planes et al. treated the cells with dexamethasone, a modulator of ENaC expression in alveolar epithelial cells (11). In the absence of dexametha-

![](https://example.com/image.png)

Fig. 4. LPS-dependent ATP release and effects of different purinergic agonists and antagonists on amiloride-sensitive *Iₑ*. A: extracellular modulation of ATP after LPS treatment (15 μg/ml) assessed by luciferase assay of apical supernatant collected at 15, 30, and 60 min. Modulation of ATP level was calculated as luciferase activity relative to time 0 (T₀) for each independent well. Three wells per animal were studied for each condition and time point. Values are means ± SE (n = 6). *P < 0.05 vs. Ctrl for each time point (by paired t-test). B: amiloride-sensitive *Iₑ* in alveolar epithelial cells pretreated for 15 min with different purinergic antagonists [pyridoxal phosphate-6-azophenyl-2'-4'-disulfonate (PPADS), Brilliant Blue G (BBG), or triiodonucleotide-ATP (TPN-ATP)] at 100 μM before 4 h of LPS treatment. Treated cells were bathed with an Na⁺ gradient buffer in the presence of 7.5 μM amphotericin B for study of apical ENaC current. Values are means ± SE (n = 4). *P < 0.05 (by Fisher’s PLSD). C: impact of 15 min of pretreatment with 100 μM purinergic agonists [ATPγS or 2-methylthioadenosine-5′-O-triphosphate (2-MeSATP)] on ENaC activity shown as amiloride-sensitive *Iₑ*. Values are means ± SE (n = 5). *P < 0.05 (by t-test).
sone, such as the culture condition used in the present study, ENaC activation is probably not maximal, and trypsin treatment allows an increase of ENaC current. Although trypsin increases the level of ENaC current in LPS-treated cells, this current is still lower than the current of trypsin-treated control cells \((P < 0.05)\). It indicates that trypsin cannot compensate for the effect of LPS and that trypsin and LPS probably modulate ENaC current through different pathways (Table 1). Together, these data show that the effects of LPS on ENaC current are not linked to modulation of the channel’s protein level or protease activation but, rather, result from the reduction of ENaC activity at the apical membrane.

Kunzelmann et al. (26) recently demonstrated, in human bronchial epithelial cells, that luminal exposure to *P. aeruginosa* flagellin leads to ENaC inhibition via an ATP-dependent mechanism. For this reason, we investigated the potential role of purinergic signaling in LPS modulation of ENaC current. First, we examined whether suramin, a purinergic receptor antagonist, could influence the LPS effects on ENaC. We found that apical pretreatment with 500 μM suramin abrogated the actions of LPS on transepithelial amiloride-sensitive current (Fig. 3), indicating that it indeed might be mediated by purinergic receptors. Next, we demonstrated that LPS promotes a transient elevation of ATP accumulated in the apical medium of alveolar epithelial cells. The ATP levels in the bulk of the apical medium represent a steady-state between two processes: cellular ATP release and ATP degradation by cell surface ecto-ATPases (39). We reported previously that a significant fraction (40%) of released ATP was hydrolyzed rapidly when the ectoenzymes were not inhibited (50). This rapid degradation may contribute to the moderate (2-fold) ATP elevation detected above basal level following LPS treatment (Fig. 3). Several groups, using cell surface-attached luciferase, showed that ATP concentration is much higher at the cell surface at the sites of release (24, 37). This suggests that ATP concentration at the cell surface could reach levels sufficient to stimulate P2Y receptors after LPS treatment. All these data suggest that, as with flagellin (26), the LPS effect on ENaC current in alveolar epithelial cells involves the purinergic pathway. Interestingly, LPS exposure has been recently reported to modulate surfactant secretion in alveolar epithelial cells (21) and mucus secretion in human nasal epithelial cells (48) via P2Y2 receptors and the purinergic system. Our findings, therefore, reinforce the concept of ATP as an LPS inflammation-related signal that affects ENaC current.

Different activators or antagonists of the purinergic pathway were tested to identify the type of receptor involved in ENaC activation.

![Diagram](https://example.com/diagram.png)

**Fig. 5.** Acute effect of ATP on amiloride-sensitive $I_{sc}$. Alveolar epithelial cells plated for 3 days on Costar Transwell filters were cultured as usual or exposed for 4 h to 15 μg/ml LPS. Filters were mounted in an Ussing chamber, and cells were bathed with physiological buffer. A and C: acute action of 100 μM ATP on amiloride-sensitive current in control and LPS-treated cells, respectively. When the current was stabilized, the first amiloride dose was added on the apical side at 10 μM final concentration for measurement of baseline ENaC activity in control and LPS-treated conditions. After amiloride was washed off the apical side, ATP was added, and the second 10 μM amiloride dose was added. B and D: influence of ATP on ENaC activity, shown as amiloride-sensitive $I_{sc}$, before and after ATP in control and LPS-treated cells, respectively. Values are means ± SE (n = 4). *$P < 0.05$ (by Fisher’s PLSD).

![Diagram](https://example.com/diagram.png)

**Fig. 6.** Sensitivity of LPS to different suramin treatment modes. Transepithelial amiloride-sensitive current was recorded in cells treated with 500 μM suramin 15 min before 4 h of LPS treatment (Sur pre) and in cells treated with suramin after LPS treatment [15 min before Ussing chamber recording (Late Sur)]. Values are means ± SE (n = 4). *$P < 0.05$ vs. basal (by Fisher’s PLSD).
 modulation. Suramin was the only antagonist that could prevent the effects of LPS on ENaC current (Fig. 3B). One explanation for why suramin inhibits the effect of LPS on ENaC current, whereas PPADS, a broad-spectrum P2Y receptor antagonist, could not, is that suramin, in addition to inhibiting P2Y2 receptors, could also inhibit secondary targets involved in the purinergic response. We found, for instance, that apyrase, an ATP scavenger, can decrease ENaC current to a level similar to that found in LPS-treated cells (data not shown). This result suggests that, in addition to ATP, other purinergic metabolites produced secondary to ATP secretion by LPS could modulate ENaC current. Suramin has been reported to be an inhibitor of ecto-ATPase (7). This action, in addition to P2Y2 receptor inhibition, could explain why suramin can inhibit the effects of LPS on ENaC current. Furthermore, to support the hypothesis that P2Y2 receptors are implicated in the ENaC response to LPS, we demonstrated that ATPyS, a specific P2Y2 receptor agonist, mimics LPS effects on ENaC activity (Fig. 4C). This relationship between the P2Y2 receptor pathway and ENaC regulation has been reported recently by Pochynyuk et al. (41) in the rat collecting ducts of P2Y2−/− mice. Since mice lacking the P2Y2 receptor (P2Y2−/−) develop salt-resistant arterial hypertension (45), these authors suggest that the ENaC hyperactivity and hypertension observed in this mouse model are due to a loss of intrinsic ENaC regulation by ATP in an autocrine/paracrine manner.

P2Y receptors are G protein-coupled receptors that activate PLC, an enzyme that hydrolyzes PIP2 to inositol trisphosphate and diacylglycerol, after activation of the receptor (52). The involvement of the P2Y2 receptor pathway in ENaC modulation by LPS was tested by inhibiting PLC activity with ET-18-OCH3 (Fig. 7) or U-73122 (data not shown). Pretreatment with ET-18-OCH3 significantly, but not totally, inhibited the effects of LPS on apical ENaC current. ATP has been shown to downregulate ENaC by affecting the PIP2 level at the membrane (25). For this reason, two strategies were used to evaluate the role of PIP2 in ENaC modulation after LPS treatment. Phosphatidylinositol 4-kinase inhibition with 10 μM wortmannin, a phosphatidylinositol 4-kinase inhibitor at this concentration, was used to inhibit PIP2 synthesis. Although wortmannin decreased apical ENaC current, LPS was still able to decrease the current by a further 54% compared with wortmannin alone (Fig. 7B). This result suggests that the membrane level of PIP2 is not modulated by LPS. To test the hypothesis that LPS could decrease the binding of PIP2 to ENaC, we used neomycin-pretreated cells to segregate PIP2 from ENaC and measured its impact on ENaC current in an Ussing chamber. Neomycin and LPS had the same effect on ENaC apical current. In neomycin-pretreated cells, LPS could not decrease the current further. Together with the results with the PLC inhibitor, these findings suggest that ENaC modulation by LPS is indirectly modulated by PIP2. This modulation probably involves a mechanism different from that associated with the acute modulation of ENaC following ATP and P2Y2 receptor activation.

Fig. 7. Involvement of PLC and phosphatidylinositol 4,5-bisphosphate (PIP2) in modulation of apical ENaC current by LPS. A: ENaC apical Iₐ measured in the Ussing chamber after basolateral membrane permeabilization with amphotericin B in an apical-to-basolateral Na⁺ gradient. Cells were left untreated or treated with LPS for 4 h in the basal condition or after 30 min of pretreatment with 40 μM ET-18-OCH3, a PLC inhibitor. Values are means ± SE (n = 4). *P < 0.05 (by Fisher’s PLSD). B: ENaC apical current after amphotericin B permeabilization as described in A. Cells were treated with 10 μM wortmannin for 5.5 h to inhibit PIP2 formation. For double treatment (Wort + LPS), cells were pretreated for 1.5 h with wortmannin before 4 h of LPS treatment. Wortmannin significantly (P < 0.05) decreased ENaC current. ENaC current was further significantly decreased by wortmannin + LPS compared with LPS alone. Values are means ± SE (n = 3). *P < 0.05 (by Fisher’s PLSD). C: ENaC apical current in cells treated with neomycin (5 mM for 6 h) to segregate phosphatidylinositol 4,5-bisphosphate (PIP2). For double treatment (Neo + LPS), cells were pretreated for 2 h with neomycin before 4 h of LPS treatment. Neomycin and LPS alone or in combination significantly (P < 0.05) decreased ENaC current. There was no further significant decrease of ENaC current in Neo + LPS compared with LPS. Values are means ± SE (n = 4). *P < 0.05 (by Fisher’s PLSD).
Fig. 8. Involvement of PKC in modulation of apical ENaC current by LPS and ATPyS. A: immunodetection of PKC activation in response to LPS. Activation of the PKC signaling pathway was assessed by quantitation of phosphorylated PKC (p-PKC) protein level relative to total PKC (T-PKC) protein level detected by Western blotting. A representative chemiluminescent reaction is illustrated. Values are means ± SE (n ≥ 5), *P < 0.05 (by Fisher’s PLSD). B and C: impact of PKC inhibition on amiloride-sensitive transepithelial current (B) and ENaC apical current (C). Alveolar epithelial cells plated for 3 days on Costar Transwell filters were cultured as usual or treated with 5 μM Go-6976 30 min before addition of 15 μg/ml LPS for 4 h. Filters were mounted in an Ussing chamber. Amiloride-sensitive current and ENaC apical current were recorded as described above. D: effect of PKC inhibition on amiloride-sensitive ENaC apical current in cells treated for 4 h with 100 μM ATPyS, instead of LPS. Values are means ± SE (n = 4), *P < 0.05 (by Fisher’s PLSD).

Since PKC is one of the signal transduction pathways activated by the receptor after PLC, we investigated whether LPS could modulate PKC phosphorylation. Immunoblots with an antibody against all phosphorylated PKC isoforms show that LPS activated the PKC pathway in the cells, increasing the level of phosphorylated PKC isoforms after 30 and 60 min of treatment (Fig. 8). This activation is detected when ATP is increased in the apical medium of LPS-treated cells (Fig. 4A). Pretreatment of the cells with Go-6976, a PKCα and PKCβI inhibitor, suppressed the effect of LPS on amiloride-sensitive transepithelial current (Fig. 8B) and the action of LPS on ENaC apical current (Fig. 8C). Ro-31-8220, a wide-spectrum inhibitor of different PKC isoforms, has the same effect (data not shown). PKCα and PKCβI are activated by diacylglycerol and Ca²⁺, the by-product of PLC activation, suggesting that these isoforms are activated by ATP. The antibody used for detection of PKC phosphorylation was a wide-spectrum antibody. For this reason, we cannot exclude the involvement of other PKC isoforms in the ENaC current modulation. In ATPyS-treated cells (Fig. 8D), Go-6976 almost completely restored ENaC apical current, indicating that the PKC pathway is involved in the delayed ATP modulation of ENaC current by the LPS pathway. Although we cannot exclude that other pathways besides ATP and PKC could also be involved, inhibitors of MAPK extracellular signal-regulated kinases (ERK1/2) and epidermal growth factor receptors, two transduction pathways known to suppress ENaC, failed to slow the downregulation evoked by LPS (data not included). Several studies have shown that PKC can inhibit ENaC function (4, 10). We reported previously that PKC activation by PMA could decrease ENaC activity in alveolar epithelial cells (54). The present data suggest that a PKC signaling pathway is involved in the LPS-induced modulation of ENaC. However, it is not clear how PKC could modulate ENaC, since the channel is not phosphorylated by the kinase (28). In A6 cells, ENaC inhibition by TNF does entail PKC-dependent externalization of phosphatidylinerse (5), and in other cell systems, PKC has been observed to suppress renal outer medulla K⁺ and G protein-gated inwardly rectifying K⁺ channels by reducing the level of PIP₂ in the membrane (55, 56). A similar process could also occur in alveolar epithelial cells upon LPS treatment.

Few reports have documented the effects of LPS on ENaC current. Although culture supernatants of LPS-treated macrophages have been found to downregulate ENaC mRNA expression and activity in fetal distal lung epithelial cells (16), the present work is the first to note a direct action of LPS on ENaC current in alveolar epithelial cells and the first to link it to ATP stimulation. Studies of different epithelia (colon (32), kidney

Fig. 9. Signaling pathways involved in ENaC modulation by LPS. Exposure of alveolar epithelial cells on the apical side to 15 μg/ml LPS leads to ATP secretion and stimulation of purinergic/nucleotide receptors sensitive to suramin. It activates a signaling pathway involving the PLC-PKC cascade. As a result, PKC inhibits ENaC at the cell membrane with a decrease in ENaC current. Impact of LPS on ENaC current can be inhibited with suramin, a P2Y purinergic receptor inhibitor, by ET-18-OCH₃, a PLC inhibitor, or by Go-6976, a PKC inhibitor.
(53), distal lung (44), and nasal airway (43)] have shown that ATP modulates ENaC activity through activation of the purinergic receptor signaling pathway. One of the major differences between the present investigation and the work published previously lies in the putative cellular mechanisms involved. Previous experiments have linked the purinergic modulation of ENaC activity to PLC kinase activation and changes of PIP2 levels at the cell membrane. Since ENaC activity depends on PIP2 binding (25, 27, 42) onto the positively charged region in the NH2 terminus of β- and γ-ENaC (42), the decrease in membrane PIP2 level by PLC activation after purinergic activation results in a reduction of ENaC activity. This acute effect of ATP on ENaC activity has been observed to be inhibited by suramin and PLC blockers (25, 42). The data presented here suggest that another mechanism is involved in the ATP-dependent modulation of ENaC elicited by LPS. First, although the decrease in ENaC activity could be inhibited by PLC blockers, this suppression was also sensitive to PKC. Second, the addition of extracellular ATP at 100 μM further decreased amiloride-sensitive current in control, as well as LPS-treated, cells, indicating that the action of LPS on amiloride-sensitive current implicates a mechanism other than that linking acute ENaC modulation by ATP. Finally, the finding that only suramin pretreatment before LPS stimulation could inhibit the effect of LPS on the current (Fig. 6) suggests that the ENaC current decrease after 4 h of LPS treatment results from a delayed action of ATP on ENaC, but not a direct acute outcome of ATP on the channel, which should have been inhibited by later suramin administration (Fig. 6). This response is also consistent with the time course of ATP release detected after 30 min of stimulation with LPS. Although it is known that P2Y2 receptor stimulation increases the activity of Goαq(11) protein, which activates PKC, this is the first time that the pathway has been shown to be involved in ENaC down-regulation, since purinergic activation in mouse cortical collecting ducts does not lead to ENaC down-regulation via PKC (51). The ATP-dependent downregulation of ENaC current in response to LPS is, therefore, not the result of acute regulation of the channel via PIP2 breakdown but, rather, ATP-dependent activation of the PKC signaling pathway, including other potential pathway(s) yet to be identified.

Although the purpose of this investigation was not to identify the mechanisms potentially playing a role in the effect of LPS on ATP release, it is possibly mediated through specific LPS TLR-4, which have been shown to elicit the secretion of inflammatory mediators by alveolar cells (3). However, intracellular TLR (22), other TLRs, such as TLR-2 (29), or other types of receptors, such as AsialoGM1 (1, 34), could be responsible. The influence of the TLR and ASGM1 systems in the modulation of ENaC by LPS needs further investigation.

In summary, the data presented here reveal that P. aeruginosa LPS affects ENaC activity in ATII cells through the purinergic signaling pathway. Four hours of incubation with LPS induces ATP release, which, in turn, could stimulate P2Y purinergic receptors, which leads to subsequent activation of the PLC-PKC pathway, with decreased ENaC apical activity at the membrane. Figure 9 describes the mechanism proposed to be implicated in ENaC modulation by LPS. This mechanism could be potentially important in modulating ion transport and lung epithelial cell surface liquid regulation (21).

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