Arachidonic Acid Regulates Surface Expression of Epithelial Sodium Channels*

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Epithelial Na⁺ channels (ENaCs) are regulated by the phospholipase A₂ (PLA₂) product arachidonic acid. Pharmacological inhibition of PLA₂, with arachidonic acid, induced a significant increase in amiloride-sensitive currents in Xenopus oocytes expressing ENaC. Arachidonic acid or 5,8,11,14-eicosatetraynoic acid (ETYA), a non-metabolized analog of arachidonic acid, induced a time-dependent inhibition of Na⁺ transport. These effects were also observed by co-expression of a calcium-independent or a calcium-dependent PLA₂- Channels with a truncated α, β, or γ C terminus were not inhibited by arachidonic acid or ETYA. Furthermore, mutation of Tyr⁴¹⁸ in the PY motif of the β subunit abrogated the inhibitory effect of ETYA, suggesting that intact PY motifs participate in arachidonic acid-mediated ENaC inhibition. Analyses of channels expressing a series of β subunit C-terminal truncations revealed a second region N-terminal to the PY motif (spanning residues βVal⁵⁸⁰–βGly⁵⁹⁹) that allowed for ETYA-mediated ENaC inhibition. Analyses of both ENaC surface expression and ENaC trafficking with mutants that either gate channels open or closed in response to [(2-trimethylammonium) ethyl] methanethiosulfonate bromide, or with brefeldin A, suggest that ETYA reduces channel surface expression by inhibiting ENaC exocytosis and increasing ENaC endocytosis.

Epithelial Na⁺ channels (ENaCs) are expressed in apical plasma membranes of principal cells in the distal nephron, airway, and alveolar epithelia in the lung, surface cells in the distal colon, urinary bladder epithelia, and other tissues including ducts of salivary and sweat glands. ENaC is formed by three subunits, termed αβγ, arranged with a subunit stoichiometry of 2α:1β:1γ (1, 2), although an alternative 3α:3β:3γ stoichiometry has been proposed (3). The three subunits are similar in overall structure, with intracellular N and C termini, two transmembrane spanning domains, and a large extracellular domain. Several hormones, such as aldosterone, arginine vasopressin, insulin, as well as selected kinases, modulate ENaC activity. ENaC activity can be regulated by two distinct mechanisms: (i) changes in single channel gating properties (i.e. open probability) or (ii) changing the number of Na⁺ channels in the apical membrane. The number of Na⁺ channels expressed at the cell surface reflects a balance of delivery of channels to the plasma membrane and internalization of channels from the plasma membrane.

Arachidonic acid is found in the sn-2 position of membrane phospholipids, where it can potentially be liberated by the deacylating action of different lipases. Arachidonic acid and its metabolites have been implicated in the regulation of a number of important physiologic processes in the kidney, including water and Na⁺ reabsorption and K⁺ secretion (4). Phospholipase A₂ (PLA₂) is the principal enzyme responsible for arachidonic acid production in most mammalian cells (5). PLA₂ can be classified in reference to its intracellular (calcium-independent (iPLA₂) or calcium-dependent (cPLA₂)) or extracellular (secretory (sPLA₂)) localization. iPLA₂ is a membrane-associated enzyme that has been implicated in phospholipid remodeling and signal transduction (6). Overexpression of iPLA₂ increases the spontaneous release of fatty acids including arachidonic acid (7). The 85-kDa ePLA₂ is a ubiquitously expressed enzyme and is the only PLA₂ that preferentially hydrolyzes the sn-2 position of phospholipids to produce arachidonic acid. The liberation of arachidonic acid occurs by activation of cPLA₂ in response to several agonists and cell-specific intracellular signals that involve G-proteins, increases in cytosolic Ca²⁺, and activation of kinases such as mitogen-activated protein kinases and protein kinase C (8, 9). Recent findings suggest the participation of PLA₂ in the control of trafficking and surface expression of integral membrane proteins including the α and β subunits of Na⁺, K⁺-ATPase, and aquaporin-2 (10, 11).

Early work in toad urine bladder suggested a regulatory effect of PLA₂ and prostaglandin synthetase on transepithelial Na⁺ transport under both control and aldosterone-stimulated conditions (12). In addition, the epithelial cell line A6 derived from Xenopus laevis kidney, grown on non-permeable supports, expressed apical Na⁺ channels that were regulated by the activities of phospholipase and lipoxygenase enzymes (13). A recent study reported that inhibition of PLA₂ by the addition of arachidonic acid to the apical bath increased amiloride-sensitive transepithelial Na⁺ transport in A6 cells and was associated with a reduction in the production of arachidonic acid. 5,8,11,14-Eicosatetraynoic acid (ETYA), a non-metabolized analog of arachidonic acid, antagonized this effect through a reduction in ENaC open probability (14). In contrast, the addi-
tion of blockers of PLA₂ or cyclooxygenase to the basolateral bath inhibited transpithelial Na⁺ transport. This decrease in Na⁺ transport was reversed by prostaglandin E₂ (14, 15).

The oocyte expression system has been extensively used to study ENaC regulation and to identify regions within ENaC involved in gating and trafficking. We used this system to explore mechanisms by which arachidonic acid regulates ENaC. We demonstrated that both arachidonic acid and ETYA induced a time-dependent reduction in the functional expression of ENaC by reducing surface expression of channels in association with altering the rates of delivery and internalization of functional channels. Furthermore, we have identified two distinct C-terminal domains that were required for ETYA-mediated inhibition of ENaC surface expression.

**MATERIALS AND METHODS**

**DNA Constructs**—Point mutations or truncations of mENaC subunits were generated by site-directed mutagenesis with the sequential PCR method using Pfu DNA polymerase (Stratagene, La Jolla, CA) (16). PCR-amplified fragments containing the desired mutations were ligated into wild type mENaC cDNA. C-terminal truncations were generated by the introduction of stop codons at the desired positions. Mutations that were generated included ΔG542/H9262, Δβ580X, β660X, β8620X, β8161A, and β8588X.βmENaC was tagged with the FLAG reporter octapeptide in the extracellular loop between Thr137 and Arg138 as described by Firsov et al. (17). PCR-amplified fragments were sequenced by automated DNA sequencing at the University of Pittsburgh Sequencing Facility to confirm the desired mutation or insertion. Mouse iPLA₂ (GenBank™ accession number BC003847) and mouse cPLA₂ (GenBank™ accession number BC003816) were obtained from Openbiosystems (Livermore, CA).

**Oocyte Expression**—cRNAs for α, β, and γ mENaC subunits, iPLA₂, and cPLA₂ were synthesized with T3, T7, or SP6 mMessage mMachine® (Ambion, Austin, TX). Stage V–VI X. laevis oocytes were pretreated with 1.5 mg/ml type IV collagenase and injected with 0.5–2 ng/subunit of mENaC cRNAs. Some oocytes were co-injected with mENaC cRNAs for ENaC subunits, iPLA₂, and cPLA₂. Oocytes were transferred to fresh wells and incubated at room temperature with either Me₂SO control (1:1000 dilution) or freshly made drugs (final concentration 50 μM). ENaC-mediated Na⁺ currents were subsequently determined by perfusion with TEV solution containing benzamid (100 μM) and with or without ETYA (50 μM) in the bath solution. Rates of ENaC Internalization—To examine ENaC removal from plasma membranes, oocytes were injected with αSS800Cβγ. Covalent modification of this mutant by MTSET resulted in a channel open probability that approached 1 (20), and subsequent decreases in whole cell Na⁺ currents should reflect channel internalization. Oocytes expressing αSS800Cβγ were perfused for 4 min in TEV solution, and whole cell currents were monitored for 4 min with a TEV solution containing 1 μM MTSET to modify accessible cysteine residues. Oocytes were then perfused with a TEV solution with or without ETYA (50 μM). ENaC-mediated Na⁺ currents were subsequently determined by perfusion with TEV solution containing benzamid (100 μM) and with or without ETYA (50 μM). ENaC-mediated Na⁺ currents were then blocked by perfusion with TEV solution containing benzamid (100 μM). A second assay used to examine whether ETYA enhanced rates of channel internalization was based on the interruption of delivery of newly synthesized channels to the plasma membrane by brefeldin A (BFA) (21). Thirty h following co-injection of ENaC cRNAs, amiloride-sensitive currents were measured under bath solution containing 10 mM HEPES, pH 7.4 supplemented with 10 μM sodium penicillin, 10 μM streptomycin sulfate, and 100 μg/ml gentamicin sulfate.

**Two-electrode Voltage Clamp**—Two-electrode voltage clamp (TEV) was performed using a Geneclamp 500B amplifier (Axon Instruments, Union City, CA). Data were acquired through Clampfit 7.0 using a DigiData 1200 interface at 1 kHz and stored on a hard disk of a 233-MHz Pentium II PC. Pipettes had resistances of 0.5–5 megohms when filled with 3M KCl. Oocytes were maintained in a recording chamber with 1 ml of bath solution and continuously perfused with TEV solution containing (in mM) 110 NaCl, 2 KCl, 1.54 CaCl₂, 10 Hepes, pH 7.4. Oocytes currents were allowed to stabilize over 10 min before reagent addition. Only oocytes with stable currents during the stabilization period were used in TEV experiments. After this period, a series of voltage steps (300 ms) from −140 to +60 mV in 20-mV increments were performed every 2 min. Mean currents between 100 and 500 ms were calculated at a clamp potential of −100 mV. ENaC-mediated Na⁺ current amplitudes were defined as the current difference in the absence and presence of amiloride (100 μM) or benzamid (100 μM) in the bath solution.

**Arachidonic Acid Release**—Oocytes were co-injected with ENaC (2 ng/subunit) with our without iPLA₂ (5 ng). Oocytes expressing ENaC currents were sorted 14–16 h after injection by TEV and incubated for 20 h in 6-well plates (31 oocyte/well) in MBS supplemented with 1 μCi/ml [²H]arachidonic acid (ICN Biomedicals, Irvine, CA) in a total volume of 3 ml/well, to allow [²H]arachidonic acid to be incorporated into the cellular lipid pool. Oocytes were then transferred to fresh wells and were washed four times with 6 ml of MBS supplement with 0.5% (w/v) bovine albumin (fatty acid-free, Sigma). Three additional washes were performed over 15 min. The release of [²H]arachidonic acid from oocytes was measured by placing groups of 10 oocytes in 3 ml of MBS supplement with 0.5% of bovine albumin (fatty acid-free). After 30 min, the incubation media were removed, and fresh media were added. This was repeated at 60 and 120 min. Oocytes were then homogenized in 3 ml of fresh media. Radioactivity in the incubation media and in the cell lysate was measured by scintillation counting. The release of [²H] was expressed as the total radiolabel of the total oocytes.
RESULTS

Arachidonic Acid Regulates ENaC Expressed in Xenopus Oocytes—TEV was performed in Xenopus oocytes expressing αβγ mENaC. A, representative recordings in an oocyte expressing αβγ ENaC under control conditions, 10 min following addition of aristolochic acid (100 μM), and after addition of amiloride (100 μM). Whole cell currents were obtained by clamping the oocyte in 20-mV steps from −140 to 60 mV. B, relative changes in amiloride-sensitive currents as a function of time are illustrated. Oocytes were continuously perfused with the TEV bath solution with (circle, n = 9) or without (triangle, n = 11) the addition of aristolochic acid (100 μM) at time 0 min. Relative amiloride-sensitive currents at a holding potential of −100 mV were plotted as a function of time. Initial whole cell amiloride-sensitive currents (time 0 min) were −4.48 ± 0.98 μA (aristolochic acid) and −8.18 ± 1.19 μA (control). The effect of aristolochic acid on ENaC was not dependent on ENaC expression levels. Statistically significant differences when controls were compared with aristolochic acid-treated oocytes are indicated as *, p < 0.02 and **, p < 0.001 (Mann-Whitney test). C, relative changes in amiloride-sensitive currents as a function of holding potential (V) in oocytes treated with aristolochic acid (100 μM) (n = 9) are shown.

Fig. 1. Aristolochic acid, a PLA₂ inhibitor, increases amiloride-sensitive whole cell currents in Xenopus oocytes expressing αβγ mENaC. TEV was performed 18–48 h after injection of αβγ mENaC cRNAs. A, representative recordings in an oocyte expressing αβγ ENaC under control conditions, 10 min following addition of aristolochic acid (100 μM), and after addition of amiloride (100 μM). Whole cell currents were obtained by clamping the oocyte in 20-mV steps from −140 to 60 mV. B, relative changes in amiloride-sensitive currents as a time function are illustrated. Oocytes were continuously perfused with the TEV bath solution with (circle, n = 9) or without (triangle, n = 11) the addition of aristolochic acid (100 μM) at time 0 min. Relative amiloride-sensitive currents at a holding potential of −100 mV were plotted as a function of time. Initial whole cell amiloride-sensitive currents (time 0 min) were −4.48 ± 0.98 μA (aristolochic acid) and −8.18 ± 1.19 μA (control). The effect of aristolochic acid on ENaC was not dependent on ENaC expression levels. Statistically significant differences when controls were compared with aristolochic acid-treated oocytes are indicated as *, p < 0.02 and **, p < 0.001 (Mann-Whitney test). C, relative changes in amiloride-sensitive currents as a function of holding potential (V) in oocytes treated with aristolochic acid (100 μM) (n = 9) are shown.

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ENaC Regulation by Arachidonic Acid

effects of arachidonic acid and ETYA on ENaC activity, oocytes were co-injected with \( \alpha \beta \gamma \text{ENaC} \) cRNAs and increasing amounts of iPLA\(_2\) cRNA. After 18 h amiloride-sensitive Na\(^+\) currents were determined, and currents were normalized to control oocytes expressing \( \alpha \beta \gamma \text{ENaC} \) alone. A dose-dependent reduction in amiloride-sensitive Na\(^+\) currents was observed in response to increasing amounts of iPLA\(_2\) cRNA injected. Relative amiloride-sensitive whole cell currents measured in oocytes co-injected with \( \alpha \beta \gamma \text{ENaC} \) and 5 ng of iPLA\(_2\) cRNAs were 68.0 ± 6.9% of the current measured in oocytes expressing \( \alpha \beta \gamma \text{ENaC} \) alone (\( p < 0.05; n = 50–68 \); see Fig. 4B). Co-injection of oocytes with \( \alpha \beta \gamma \text{ENaC} \) cRNAs and 5 ng of cPLA\(_2\) cRNA also led to a significant reduction in expression of amiloride-sensitive whole cell current, compared with oocytes expressing \( \alpha \beta \gamma \text{ENaC} \) alone (relative amiloride-sensitive whole cell currents: 0.72 ± 0.07 (ENaC + cPLA\(_2\)) versus 1.00 ± 0.07 (ENaC); \( p < 0.01, n = 54 \)).

ENaC C-terminal Domains Are Required for Arachidonic Acid-mediated Inhibition of Na\(^+\) Currents—To identify domains in ENaC that are required for arachidonic acid-mediated channel down-regulation, deletions of the C-terminal intracellular regions of each subunit (\( \alpha \text{H613X}, \beta \text{R564X}, \) or \( \gamma \text{R583X} \)) were generated. Expression of mENaCs with C-terminal deletions of the \( \alpha, \beta, \) or \( \gamma \) subunit prevented the arachidonic acid or ETYA-mediated inhibition of ENaC currents (Fig. 5), suggesting that regions present in the C terminus of each subunit are involved in arachidonic acid-mediated channel down-regulation. One possible candidate is the PY motif, PPPXXYYXX, a conserved tract present in the three ENaC subunits that interacts with WW domains within the ubiquitin protein ligase Nedd4 (26) and facilitates channel ubiquitination and endocytosis (27). This tract also has a hydrophobic internalization motif, YXXL, as defined previously (28). To examine further the role of the PY motif in arachidonic acid-mediated ENaC down-regulation, \( \alpha \beta \gamma \text{Y618A} \) was expressed in oocytes. Previous studies have shown that this mutation abrogates Nedd4 binding to ENaC and reduces rates of ENaC internalization from the plasma membrane (29, 30). ETYA did not significantly inhibit amiloride-sensitive Na\(^+\) currents in oocytes expressing \( \alpha \beta \gamma \text{Y618A} \) (\( n = 11 \), \( p = 0.01 \), Fig. 6). A series of C-terminal truncations of the \( \beta \) subunit (\( \beta \text{V580X}, \beta \text{E600X}, \) and \( \beta \text{S620X} \)) were generated and co-expressed with wild type \( \alpha \) and \( \gamma \) mENaCs to define further the regions involved in arachidonic acid-mediated regulation of ENaC (Fig. 6). No significant inhibitory effect of ETYA on amiloride-sensitive Na\(^+\) currents was observed in oocytes expressing a C-terminal deletion of the terminal 19 amino acids of the \( \beta \) subunit (\( \beta \text{S620X} \)) that truncates the \( \beta \) subunit just beyond Tyr\(^{618}\) and within the YXXL motif (\( n = 8, p = 0.001 \), \( \alpha \beta \gamma \text{S620X} \gamma \) versus \( \alpha \beta \gamma \text{Y618A} \)). Surprisingly, deletion of 20 additional residues (\( \beta \text{E600X} \)) of the \( \beta \) subunit restored ETYA-mediated inhibition of ENaC (\( n = 15, p < 0.01 \)) whereas the inhibitory effect of ETYA was not observed with deletion of the C-terminal 59 amino acids (\( \beta \text{V580X}, n = 6, p = 0.01 \)).

ETYA Reduces ENaC Cell Surface Expression—Our results demonstrate that arachidonic acid is directly involved in the control of ENaC functional expression in oocytes. Mutations or deletions of the PY motif prevented ETYA-mediated inhibition of ENaC, suggesting that this regulation involves ENaC trafficking. To corroborate that the inhibition of functional ENaC expression by arachidonic acid and related analogs reflects a reduction in the number of channels at the cell surface, we examined surface expression of \( \alpha \beta \gamma \) and \( \alpha \beta \gamma \text{Y618A} \) mENaCs in oocytes using a FLAG epitope-tagged \( \beta \) subunit and a chemiluminescence-based assay originally described by Zerangue et al. (18). The assay background measured in non-epitope-tagged

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**Fig. 2. Time-dependent inhibition of amiloride-sensitive whole cell Na\(^+\) currents by arachidonic acid and ETYA.** Whole cell currents were recorded in oocytes expressing \( \alpha \beta \gamma \text{ENaC} \) and perfused with the TEV bath solution alone (circle, \( n = 11 \)) or with the TEV bath solution supplemented with arachidonic acid (50 \( \mu \)M, square, \( n = 8 \)) or ETYA (50 \( \mu \)M, triangle, \( n = 16 \)) at time = 0 min. Amiloride (100 \( \mu \)M) was added to the bath at time = 20 min, and relative amiloride-sensitive currents (at -100 mV) were plotted as a function of time. Initial amiloride-sensitive whole cell currents (time = 0 min) were -8.18 ± 1.19 \( \mu \)A (control), -8.04 ± 1.71 \( \mu \)A (arachidonic acid), and -6.44 ± 0.80 \( \mu \)A (ETYA). Statistically significant differences are indicated as **, \( p < 0.05 \) (control versus arachidonic acid) and +, \( p < 0.01 \) (control versus ETYA; **, \( p < 0.01 \) (control versus ETYA and control versus arachidonic acid)). Statistical comparisons were performed by Kruskal Wallis test (nonparametric ANOVA) followed by Dunn's multiple comparisons post test.

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Inhibits both the cyclooxygenase and lipoxygenase pathways of arachidonic acid metabolism (23–25), these data suggest that arachidonic acid per se induced a time-dependent inhibition of ENaC. Relative amiloride-sensitive currents measured in \( \alpha \beta \gamma \text{ENaC} \)-expressing oocytes after 20 min in TEV solution alone (0.89 ± 0.03, \( n = 11 \)) were not significantly different from relative amiloride-sensitive currents measured in ENaC-expressing oocytes after 20 min in TEV solution plus vehicle (\( \text{Me}_{2}\text{SO} \) 1:1000 dilution, 0.84 ± 0.04, \( n = 8, \) \( p = 0.01 \). Changes in amiloride-sensitive currents in response to ETYA or arachidonic acid were independent of the clamp voltage. Fig. 3B shows amiloride-sensitive I/V curves obtained before and after treatment of oocytes with ETYA. No changes in reversal potential or induction of rectification of amiloride-sensitive sodium currents were observed following treatment of ENaC-expressing oocytes with ETYA or arachidonic acid (data not shown).

**Co-expression of iPLA\(_2\) or cPLA\(_2\) Inhibits ENaC-mediated Na\(^+\) Currents—**iPLA\(_2\) and cPLA\(_2\) are implicated in the liberation of arachidonic acid from the sn-2 position of membrane phospholipids under physiological conditions (5, 6, 9). We performed an \( ^{3} \text{H} \)arachidonic acid-release assay to determine whether iPLA\(_2\) is functional when co-expressed with ENaC. Oocytes co-injected with ENaC and iPLA\(_2\) showed a 23.6 ± 3.9% increase (at 120 min) in the release of \( ^{3} \text{H} \)arachidonic acid, when compared with oocytes injected with ENaC alone (\( p < 0.001, n = 5–6 \), Fig. 4A). These data suggest that iPLA\(_2\) is functional when expressed in oocytes. To determine whether co-expression of iPLA\(_2\) and ENaC reproduces the inhibitory

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aβγ-injected oocytes was 0.37 ± 0.06 RLU/min. The surface expression of aβ-FLAG-γ ENaC was significant reduced in oocytes treated with ETYA (3.63 ± 0.45 RLU/min, n = 58, p < 0.05) when compared with vehicle controls (5.76 ± 0.77 RLU/min, n = 71, see Fig. 7). Oocytes expressing FLAG-tagged aβY618Aγ mENaC showed a significant increase in surface expression (13.13 ± 1.76 RLU/min, n = 59, p < 0.05) compared with FLAG-tagged aβγ mENaC. However, a significant difference in surface expression was not observed in oocytes expressing aβY618Aγ in response to ETYA (11.88 ± 1.48 RLU/min, n = 48, p = 0.59) when compared with vehicle control (see Fig. 7). These results confirm previous observations that the βY618A mutation results in a large increase in surface expression of Na⁺ channels (31) and suggest that ETYA-mediated inhibition of ENaC is associated with a reduction in surface expression of channels.

Rate of ENaC Exocytosis Is Altered by ETYA—We investigated the delivery and internalization of functional channels in order to delineate mechanisms by which arachidonic acid induced a reduction in ENaC surface expression. Previous stud-
ies (19) have demonstrated that MTSET treatment of oocytes expressing αβγG542C blocks resident channels at the plasma membrane, and the time-dependent recovery of benzamil-sensitive whole cell currents reflects delivery of unmodified channels to the cell surface (32). As the γG542C mutation has a marked increase in the $K_i$ for amiloride to ENaC (1, 33), 100 μM benzamil was used to inhibit Na⁺ currents mediated by this mutant.

**Fig. 4.** Co-expression of calcium-independent PLA₂ (iPLA₂) and ENaC in oocytes inhibits ENaC-mediated Na⁺ currents. A, functional expression of iPLA₂ was assayed by the release of [³H]arachidonic acid (AA). Oocytes were co-injected with ENaC (2 ng/subunit) with (triangle) or without (circle) iPLA₂ (5 ng). Arachidonic acid release was determined as described under "Materials and Methods." Statistical differences in [³H]arachidonic acid release in oocytes co-injected with αβγ ENaC and iPLA₂ (n = 6) compared with oocytes injected with αβγ ENaC alone (n = 5) are indicted as *, $p < 0.01$ and **, $p < 0.001$ (unpaired t test). B, oocytes were co-injected with 2 ng/subunit of αβγ ENaC cRNAs and either various concentrations of iPLA₂ cRNA or vehicle (H₂O). TEV was performed 18 h after injection, and amiloride-sensitive currents were normalized to the mean of the group expressing αβγ ENaC alone. Relative amiloride-sensitive currents at a holding potential of $-100$ mV are shown. Asterisk indicates statistical difference in relative amiloride-sensitive currents compared with oocytes injected with αβγ ENaC alone, $p < 0.05$ (ANOVA followed by Tukey-Kramer multiple comparisons test). Data were obtained from five different batches of oocytes (n = 50–68).
Oocytes expressing αβγG542C were perfused with TEV solution containing MTSET (1 mM) to covalently modify cysteine residues and subsequently perfused with TEV solution in the presence or absence of 50 μM ETYA (see Fig. 8). Benzamil-sensitive whole cell currents fell from 6.50 ± 0.97 pA (before MTSET, n = 13) to −0.32 ± 0.04 μA (after MTSET), indicating that 94.6 ± 0.5% of the benzamil-sensitive currents were inhibited by MTSET. Furthermore, the minimal change in whole cell current observed during a 2-min washout of MTSET indicated that inhibition of αβγG542C by MTSET was not reversible (see Fig. 8). The subsequent increase in whole cell Na⁺ currents as MTSET was washed out of the TEV chamber was likely due to delivery of unmodified channels to the cell surface. The initial rates of increase of whole cell Na⁺ currents, representing delivery of unmodified channels to the cell surface, were significantly reduced in oocytes treated with ETYA (5.4 ± 0.7 × 10⁻³ min⁻¹, n = 6, R = 0.79) when compared with control oocytes (9.1 ± 0.6 × 10⁻³ min⁻¹, n = 7, r = 0.92, p < 0.0002, control versus ETYA). These results suggest that ETYA reduces the delivery of unmodified channels to the cell surface. Amiloride-sensitive Na⁺ currents reached a plateau after ~10 min of TEV perfusion that likely reflects a balance between the delivery to the surface and removal from the surface of unmodified channels. In contrast, oocytes treated

**Fig. 5.** Deletions in intracellular C-terminal domains of α, β, or γ mENaC prevent the down-regulation of amiloride-sensitive Na⁺ transport by arachidonic acid or ETYA. TEV was performed in oocytes expressing either wild type channels or ENaCs with a single subunit lacking its C-terminal domain (αH613Xβγ, αβR564Xγ, or αβR583X). Oocytes were perfused with the TEV bath solution alone (closed bars) or with the TEV bath solution supplemented with arachidonic acid (light gray bars, 50 μM) or ETYA (dark gray bars, 50 μM) at time = 0 min. Amiloride (100 μM) was added to the bath at time = 20 min. Amiloride-sensitive currents at time = 20 min were normalized to amiloride-sensitive currents determined at time = 0. Initial whole cell amiloride-sensitive currents (time = 0 before addition of reagents) were −7.38 ± 0.65 μA (αβγ (wild type)), −6.73 ± 0.99 μA (αH613Xβγ), −14.61 ± 2.48 μA (αβR564Xγ), and −13.93 ± 2.03 μA (αβR583X). Asterisk indicates statistically significant differences in the relative amiloride-sensitive current p < 0.001, wild type control versus ETYA-treated and arachidonic acid-treated (Kruskal Wallis test (nonparametric ANOVA), followed by Dunn’s multiple comparisons post test). The number of experiments was between 5 and 16 per group.
Fig. 6. Identification of domains within the intracellular C terminus of βmENaC that are involved in ETYA-mediated channel inhibition. TEV was performed in oocytes expressing αβγ, αβY618Aγ, or a series of C-terminal truncations, including αβRS64XY, αβV580XY, αβE600XY, and αβS620XY. At time = 0 oocytes were perfused with the TEV bath solution alone or supplemented with ETYA (50 μM). Amiloride-sensitive currents were determined 20 min following addition of treatment and were normalized to currents determined at time = 0. Initial whole cell amiloride-sensitive currents (time = 0 before addition of reagents) were −7.01 ± 0.76 μA (αβγ (wild type)), −9.83 ± 1.68 μA (αβY618Aγ), −10.43 ± 2.25 μA (αβRS64XYγ), −9.90 ± 1.82 μA (αβV580XYγ), and −16.39 ± 1.84 μA (αβE600XYγ). Asterisk indicates statistically significant differences in the relative amiloride-sensitive current, p < 0.001, wild type control versus ETYA-treated wild type; **, p < 0.01, wild type control versus ETYA-treated αβE600XYγ (Kruskal Wallis test (nonparametric ANOVA) followed by Dunn’s multiple comparisons post test).

Fig. 7. Reduction in ENaC surface expression after ETYA treatment in αβ-FLAG-γ but not in αβ-FLAG-Y618Aγ expressing oocytes. Oocytes co-expressing wild type α and β subunits with FLAG-tagged γ subunits with FLAG-tagged β or βY618A were incubated with ETYA (50 μM) or with vehicle alone (Me2SO (1:1000 dilution)) for 20 min at room temperature. Oocytes expressing non-epitope-tagged wild type (αβγ) subunits were used for negative controls. Surface expression of FLAG-tagged channels was determined as described under “Materials and Methods” and was expressed as RLU per min per oocyte. Asterisk indicates statistically significant differences in the surface expression of αβ-FLAG-γ (RLU/min/oocyte), p < 0.05, vehicle control versus ETYA (Mann-Whitney test).

with ETYA showed a subsequent decrease in benzamil-sensitive Na⁺ currents 12 min following MTSET washout, suggesting that rates of channel endocytosis were also increased by ETYA (Fig. 8).

Rate of ENaC Internalization Is Increased by ETYA—A recent study from our group (20) demonstrated that MTSET treatment of oocytes expressing αS580Cβγ led to a change from a low open probability state to a channel open probability approaching 1.0 in conjunction with a reduction in single channel conductance. The time-dependent loss of benzamil-sensitive whole cell Na⁺ currents in oocytes expressing αS580Cβγ and treated with MTSET reflects both the internalization of channels with a high open probability (lower conductance), as well as delivery of unmodified (low open probability, higher conductance) channels to the cell surface, and was used to examine ENaC endocytosis. Oocytes expressing αS580Cβγ were perfused with TEV solution containing MTSET (1 mM) to covalently modify cysteine residues and subsequently perfused with TEV solution in the presence or absence of 50 μM ETYA (see Fig. 9). Immediately following the washout of MTSET, whole cell currents increased in both control and in ETYA-treated oocytes, reaching a plateau after 4 min. These data suggested that MTSET produced a non-covalent inhibition of αS580Cβγ currents that was reversed after washout, in agreement with previous observations (19), in addition to activation of channels by covalent modification (20). The subsequent rate of decrease of benzamil-sensitive whole cell Na⁺ current, representing internalization of channels, was significantly greater in oocytes treated with ETYA (−2.3 × 10⁻² ± 0.2 × 10⁻² min⁻¹, R = 0.87, n = 6) when compared with controls (−0.3 ×
FIG. 8. ENaC trafficking to the plasma membrane is modified by ETYA. Oocytes expressing αβγ-G542C were treated with MTSET to covalently inhibit whole cell Na⁺ currents and subsequently perfused with TEV bath solution with (triangle) or without (circle) ETYA (50 μM). The recovery of benzamil (100 μM)-sensitive currents were plotted as a function of time and were normalized to the benzamil-sensitive whole cell current determined prior to MTSET treatment (−5.15 ± 0.94 μA (control) and −7.67 ± 1.55 μA (ETYA)). Whole cell benzamil-sensitive currents at the completion of MTSET perfusion were −0.37 ± 0.05 μA (control) and −0.26 ± 0.05 μA (ETYA). Initial rates of increase over the linear range were replotted (inset) and fit by linear regression. Slopes were significantly different (p < 0.0002) between oocytes perfused with (5.4 × 10⁻³ ± 0.7 × 10⁻³ min⁻¹, R = 0.79, n = 6) or without (9.1 × 10⁻³ ± 0.6 × 10⁻³ min⁻¹, R = 0.92, n = 7) ETYA. Statistical differences in relative benzamil-sensitive currents between oocytes expressing αβγ-G542C control and ETYA treated are indicated as *, p < 0.05 and **, p < 0.01 (unpaired t test).

FIG. 9. ENaC internalization is enhanced by ETYA. Oocytes expressing αS580Cβγ were treated with MTSET to activate channels at the plasma membrane. Whole cell benzamil-sensitive currents were −1.35 ± 0.52 μA (control) and −2.44 ± 0.86 μA (ETYA (50 μM)) prior to MTSET perfusion and were −2.11 ± 0.58 μA (control) and −4.05 ± 1.16 μA (ETYA) at the completion of MTSET perfusion. Oocytes were subsequently perfused with TEV bath solution (without MTSET) with (triangle) or without (circle) 50 μM ETYA. Relative benzamil-sensitive currents were plotted as a function of time following addition of ETYA (or control). Currents were normalized to currents at either t = 0 or t = 8 min (inset) after addition of ETYA (or control). Rates of decrease over the linear range were replotted (inset) and fit by linear regression. Slopes were significantly different (p < 0.0001) between control oocytes (−0.3 × 10⁻² ± 0.4 × 10⁻² min⁻¹, R = 0.11, n = 8) and oocytes perfused with ETYA (−2.3 × 10⁻² ± 0.2 × 10⁻² min⁻¹, R = 0.87, n = 6). Asterisk indicates statistically significant differences in the relative benzamil-sensitive current between αS580Cβγ control and ETYA-treated oocytes, p < 0.05 (unpaired t test).
Incubated in presence of BFA (5 mM), amiloride-sensitive currents were recorded and plotted as a function of time following reagent addition at time = 0. Asterisk indicates that the relative reductions in amiloride-sensitive currents measured in oocytes incubated in presence of BFA (5 μM) and ETYA (10 μM) were significantly greater than currents in oocytes treated with BFA alone, p < 0.01. Statistical comparisons were performed by Kruskal-Wallis test (nonparametric ANOVA) followed by Dunn’s multiple comparisons post test.

To confirm that ETYA increased rates of channel endocytosis, an alternative approach was used to examine channel endocytosis based on the inhibition of delivery of newly synthesized channels to the plasma membrane by BFA. BFA is a fungal toxin that inhibits the secretion of proteins by disassembly and redistribution of the Golgi complex into the endoplasmic reticulum and inhibits the delivery of newly synthesized proteins to the plasma membrane.

Oocytes were treated with BFA (5 μM) to inhibit selectively the delivery of channels to the plasma membrane. Under these conditions, the reduction in amiloride-sensitive currents reflects retrieval of channels from the plasma membrane. Amiloride-sensitive currents in oocytes treated with BFA and ETYA were significantly lower than in oocytes treated with BFA alone (p < 0.01, see Fig. 10). These data support our conclusion that ETYA reduces ENaC surface expression through an increase in the rate of channel retrieval from the plasma membrane.

As compounds such as arachidonic acid and ETYA may have membrane-perturbing effects, we examined whether arachidonic acid (50 μM) or ETYA (50 μM) altered membrane fluidity by monitoring changes in DPH anisotropy in plasma membranes isolated from Xenopus oocytes. Although changes in anisotropy were observed with 50 μM arachidonic acid over 20 min, no changes in anisotropy were observed with 50 μM ETYA (see Fig. 11). As ETYA and arachidonic acid exerted similar effects on ENaC expression in oocytes, it is unlikely that ETYA or arachidonic acid-mediated inhibition of ENaC represents a nonspecific effect attributable to changes in membrane fluidity.

Due to its intrinsic fluorescence, we were unable to monitor anisotropy in the presence of 100 μM arachidonic acid.

**DISCUSSION**

Previous studies have shown that amiloride-sensitive Na⁺ transport in A6 cells and native tissues is controlled by products of the enzyme PLA₂ (12–15). Our results in Xenopus oocytes expressing αβγ mENaC confirm and extend these observations. Pharmacological inhibition of arachidonic acid production by arachidonic acid led to an increase in ENaC-mediated Na⁺ transport, in agreement with previous observations (14). Arachidonic acid or ETYA (a non-metabolized analog of arachidonic acid) induced a time-dependent decrease in amiloride-sensitive Na⁺ transport. Similar effects were observed by co-expression of ENaC and iPLA₂ (or ENaC and cPLA₂) in oocytes.

Channels with a truncated α, β, or γ C terminus were not inhibited by arachidonic acid or ETYA. These results suggest that domains within the C terminus of each subunit are required for the down-regulation of ENaC by arachidonic acid or ETYA. A mutation in the β subunit PY motif (βY618A) also prevented the down-regulation of ENaC by ETYA, indicating that intact PY motifs have a role in arachidonic acid-mediated ENaC inhibition. Analyses of channels expressing a series of β subunit C-terminal truncations revealed a second region N-terminal to the PY motif (spanning residues βVal⁵⁸⁰–βGly⁵⁹⁹) that allowed for arachidonic acid-mediated ENaC inhibition in the absence of the β subunit PY motif.

A previous study examining the effects of serial deletions of the C terminus of rat ENaC reported that currents measured in oocytes containing a βC595X mutant were modestly lower than currents measured in oocytes containing a βQ589X mutant. Results of alanine scanning mutagenesis within this region in the presence of an intact β subunit PY motif suggested that this region did not participate in the control of amiloride-sensitive currents in channels with otherwise intact C termini (35). However, it is possible that this region is involved in channel regulation under specific conditions, such as in the presence of arachidonic acid.
Changes in surface expression of FLAG-tagged ENaC in oocytes treated with or without ETYA (see Fig. 7) indicated that ETYA-mediated inhibition of ENaC was associated with a reduction in surface expression of Na\(^{+}\) channels. The reduction in surface expression of FLAG-tagged ENaC in oocytes after 20 min of perfusion with ETYA (37%, see Fig. 7) was similar in magnitude to the observed decrease in whole cell Na\(^{+}\) currents in response to ETYA (28%, see Fig. 3), suggesting that a significant component of the inhibition of ENaC currents by ETYA was a consequence of a reduction in channel surface expression. Experiments using ENaC mutants that gate open or closed in response to MTSET indicated that the reduction in surface expression of ENaC in response to ETYA occurred in association with a reduction in the rate of channel exocytosis and an increase in the rate of channel endocytosis. The enhanced rate of decrease of amiloride-sensitive currents in oocytes treated with BFA and ETYA, compared with oocytes treated with BFA alone, provided additional evidence that ETYA reduced functional expression of ENaC by increasing rates of channel endocytosis. Furthermore, a PY motif mutation (\(\beta Y618A\)) blocked ETYA-mediated inhibition of ENaC expression and is consistent with previous studies that reported that channels with mutations in the PY motif exhibited increased cell surface expression in association with a reduction in the rate of channel endocytosis (21, 28, 31). Although our results suggest that arachidonic acid inhibits ENaC by reducing surface expression of channels, we cannot discard the possibility that arachidonic acid also reduced channel \(P_{\text{o}}\). A recent report (14) indicated that arachidonic acid reduced ENaC open probability.

In addition to their classic roles in the regulation of second messengers, lipids have been implicated in the regulation of membrane trafficking, vesicular fusion, protein targeting, and synaptic vesicle formation (36, 37). The role of PLA\(_2\) in regulating endocytosis is supported by previous observations (38) indicating that vesicle fusion along the endocytic pathway requires PLA\(_2\) activity. This effect is mediated, in part, by arachidonic acid. Studies (39) of synaptic vesicle fusion with presynaptic membranes in vitro indicated that PLA\(_2\) activity is necessary for fusion, and arachidonic acid potentiated the fusogenic activity of the membrane. Examples of participation of PLA\(_2\) in the trafficking of membrane proteins include studies in proximal tubules, where dopamine increases PLA\(_2\) activity and induces a decrease in Na\(^{+}\),K\(^{+}\)-ATPase activity through internalization of its \(\alpha\) and \(\beta\) subunits into endosomes via a clathrin-dependent pathway (10). In LLC-PK\(_1\) renal epithelial cells, cPLA\(_2\) is involved in the selective and specific control of trafficking of constitutive membrane proteins. Although the trafficking of a Cl\(^{-}/\)HCO\(_3\)\(^{-}\) exchanger to the plasma membrane was not altered in cells overexpressing cPLA\(_2\), significant changes in the trafficking and surface expression of aquaporin-2 and Na\(^{+}\),K\(^{+}\)-ATPase were observed (11). In addition, inhibition of PLA\(_2\) by membrane-permeant antagonists inhibited brefeldin A-stimulated Golgi and trans-Golgi network membrane tubulation, as well as resultant retrograde transport of resident Golgi enzymes to the endoplasmic reticulum (40). It was recently suggested that PLA\(_2\) activity was necessary for transferrin recycling and endosome membrane tubule formation (41).

Exogenous addition of arachidonic acid has marked effects on membrane bilayer structure and organization (42). In addition, during the process of phospholipid hydrolysis by PLA\(_2\), significant changes in the organization of the lipid bilayer may occur (43). These changes (including membrane curvature and fluidity) can modify the fusogenic properties of the membrane and the ability to form vesicles. For example, in the process of synaptic vesicle formation the enzyme endophilin I mediates the transfer of arachidonate to lysophosphatidic acid (44). This process has been proposed to induce a membrane curvature change by converting an inverted cone-shaped lipid to a cone-shaped lipid in the cytoplasmic leaf of the bilayer, and to facilitate synaptic vesicle invagination (44). In our system, a similar mechanism involving modifications of membrane structure mediated by increases in levels of arachidonic acid might work in an analogous manner, facilitating ENaC internalization. Alternatively, arachidonate may activate the endocytic machinery via interactions with specific proteins, including ENaC.

Anisotropy was measured in oocytes membranes to examine whether arachidonic acid or ETYA altered membrane fluidity. While arachidonic acid induced a time-dependent change in anisotropy, no effect was observed with ETYA suggesting that these lipids exert differential effects on membrane fluidity. As ETYA and arachidonic acid exert similar effects on ENaC expression in oocytes, it is unlikely that ETYA- or arachidonic acid-mediated inhibition of ENaC represents a nonspecific effect of membrane fluidity. Our observation that co-expression of ENaC with either iPLA\(_2\) or cPLA\(_2\) led to a reduction in functional ENaC expression is also consistent with the notion that the effects of 50 \(\mu\)M arachidonic acid or ETYA on ENaC expression are not simply related to nonspecific lipid effects on membrane fluidity.

Although our results indicated that arachidonic acid-mediated changes of ENaC functional expression occurred, in part, through changes in ENaC surface expression, it is difficult to determine whether changes in rates of endocytosis and exocytosis of ENaC are independent processes or if changes in one of these processes modifies the other. Stimulation of ENaC endocytosis by arachidonic acid or ETYA appears to be dependent on previously characterized internalization motifs within the C termini of ENaC subunits (21), as substitution of wild type ENaC with the PY mutant \(\beta Y618A\) blocks ETYA effects.

In summary, our data suggest that arachidonic acid has an important role in the control of ENaC surface expression in Xenopus oocytes and that arachidonic acid modulates ENaC trafficking. The control of the number of channels at the membrane mediated by changes in the activity of PLA\(_2\) is a potential regulatory mechanism that is susceptible to modulation in response to several agonists and cell-specific intracellular signals. Channels with truncated \(\alpha\), \(\beta\), or \(\gamma\) C termini or with a mutation in the PY motif (\(\beta Y618A\)) were not inhibited by arachidonic acid (or ETYA). It is unclear whether direct interactions between these lipids and ENaC are necessary to enhance rates of ENaC internalization and reduce rates of ENaC exocytosis, or whether additional proteins have important roles in these processes. We identified a second region N-terminal to the PY motif (spanning residues \(\beta Y618\)–\(\beta G658\)) that allows for arachidonic acid-mediated ENaC inhibition, suggesting that multiple domains within ENaC may participate in arachidonic acid-mediated channel inhibition and that regions, distinct from the PY motif, may regulate ENaC trafficking.

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Arachidonic Acid Regulates Surface Expression of Epithelial Sodium Channels
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