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Src Family Kinases Mediate Epithelial Na^+ Channel Inhibition by Endothelin*

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Elaine S. Gilmore‡, M. Jackson Stutts, and Sharon L. Milgram‡¶

From the Cystic Fibrosis/Pulmonary Research and Treatment Center and ‡Department of Cell and Molecular Physiology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

The epithelial Na^+ channel (ENaC) is implicated in the pathogenesis of salt-sensitive hypertension. Recent evidence from animal models suggests that the vasoactive peptide, endothelin (ET-1), may be an important negative regulator of ENaC *in vivo*. We investigated the signaling pathway involved in endothelin-mediated ENaC inhibition. Experiments were performed in NIH 3T3 cells stably expressing genes for the three (α, β, and γ) ENaC subunits. In whole cell patch clamp experiments, we found that ET-1 treatment induced a dose-dependent decrease in amiloride-sensitive currents. Using receptor-specific antagonists, we determined that the effects of ET-1 were attributed to activation of the ET_B receptor. Moreover, the inhibitory effect of ET-1 on ENaC could be completely blocked when cells were pretreated with the selective Src family kinase inhibitor, PP2. Further studies revealed that basal Src family kinase activity strongly regulates ENaC whole cell currents and single channel gating. These results suggest that Src family kinases lie in a signaling pathway activated by ET-1 and are components of a novel negative regulatory cascade resulting in ENaC inhibition.

Endothelin (ET)[1] 1, a potent vasoactive peptide originally described as an endothelial cell-derived factor, is the founding member of a family of related 21-amino acid peptides (1, 2). Each endothelin (ET-1, ET-2, and ET-3) is encoded by a distinct gene and is processed from an inactive precursor via two proteolytic cleavages to generate a biologically active peptide (3, 4). Endothelins are synthesized in many cell types, including endothelial, epithelial, fibroblast, and cardiac muscle cells, and function as autocrine or paracrine factors to regulate different cellular processes (1, 5–7). The two endothelin receptors, ET_A and ET_B, are broadly expressed with overlapping, but distinct, distributions (8–11). ET_A and ET_B are heterotrimeric G protein-coupled receptors (GPCRs) that can couple with multiple Gα subunits, depending on cell type (12–14). In addition, ET receptors stimulate the activity of nonreceptor tyrosine kinases in some cells (5, 15, 16). Although this activation is generally thought to mediate the mitogenic effects of endothelins, acute activation of NHE3 via ET-1 is blocked ~50% by tyrosine kinase inhibitors (17, 18). The ET-mediated activation of NHE3 occurs specifically via ET_B receptors (19).

Recent evidence indicates that renal ET_B receptors may be important for sodium handling. The function of ET_B has been examined in rats with naturally occurring mutation of the ET_B gene. These studies indicate that ET_B plays an essential role in development of enteric neurons because absence of functional ET_B receptors causes perinatal lethality resulting from megacolon (20, 21). Gariepy *et al.* (22) rescued this phenotype by specifically expressing ET_B only in adrenergic neurons using the dopamine β-hydroxylase promoter (22). On high salt diets, these rats developed salt-sensitive hypertension, which was restored to normal when animals were treated with amiloride, a potent inhibitor of the epithelial Na^+ channel (ENaC) (22, 23). These data indicate that endothelin, acting on ET_B receptors, mediates tonic inhibition of ENaC, and that this inhibition may be required for the maintenance of blood pressure.

ENaC, the product of three distinct genes (α, β, and γ), is a low conductance Na^+-selective channel that provides the rate-limiting step in electrogenic Na^+ transport across the apical membrane of many epithelial tissues including the distal nephron (24–26). Analysis of two human disorders, Liddle syndrome and pseudohypoaldosteronism type 1, indicates that ENaC in renal epithelia plays a critical role in salt homeostasis and the control of blood pressure (27–29). Although all pathways that regulate apical membrane Na^+ transport via ENaC are not known, several hormones stimulate ENaC activity or expression (30, 31). For example, aldosterone acts on cytosolic mineralocorticoid receptors to potently stimulate the absorption of Na^+ across cells of the distal nephron and colon (32). Although this stimulation is accomplished in part via increased transcription of ENaC subunits, aldosterone also induces the transcription of the serum and glucocorticoid-regulated kinase (SGK), which activates ENaC (33, 34). In addition, vasopressin and insulin are known to stimulate the activity or expression of ENaC in some model systems (35–37). In contrast, few hormonal pathways that potently inhibit ENaC activity have been described.

Evidence that rats and mice lacking functional ET_B receptors develop salt-sensitive hypertension supports a role for ET-1 inhibition of ENaC activity. In A6 cells, a *Xenopus* model of the distal nephron, ET-1 dramatically down-regulates ENaC activity, although the pathway linking ET_B receptors to ENaC was not identified (38). Therefore, we sought to identify the signaling pathway linking activation of ET receptors to inhibition of ENaC in mammalian cells. We found that ET_B receptors

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¶ To whom correspondence should be addressed: Dept. of Cell and Molecular Physiology, University of North Carolina, CB 7545, Chapel Hill, NC 27599. Tel.: 919-966-9792; Fax: 919-966-6927; E-mail: milg@med.unc.edu.

[1] The abbreviations used are: ET, endothelin; ENaC, epithelial Na^+ channel; ET_A, endothelin-type A receptor; ET_B, endothelin-type B receptor; GPCR, G-protein-coupled receptor; I, current; I_{Amil}, amiloride current; IV, current-voltage; MCT, mean closed time; MOT, mean open time; NHE3, Na^+/H^+ exchanger isoform 3; SGK, serum and glucocorticoid-regulated kinase; P_o, open probability; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; PP3, 4-amino-7-phenylpyrazol[3,4-*d*]pyrimidine; GST, glutathione *S*-transferase; PKA, cAMP-dependent protein kinase; PAGE, polyacrylamide gel electrophoresis; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid.

act via Src family kinases to potently inhibit ENaC open probability. This provides a novel mechanism of ENaC regulation and explains the inhibitory influence of ET_B on ENaC activity. The identification of signaling proteins that potently inhibit Na^+ absorption in the distal nephron could potentially provide additional therapeutic targets for the treatment of human hypertension.

EXPERIMENTAL PROCEDURES

Cell Culture—Experiments were performed using NIH 3T3 cells infected with retrovirus encoding cDNAs for rat $\alpha\beta\gamma$ ENaC subunits (39). Clones stably expressing ENaC subunits were maintained in a humidified incubator with 5% CO_2, in Dulbecco's modified Eagle's medium (DMEM) with 10% bovine calf serum, 10 μM amiloride, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 300 μg/ml G418, and 1 μg/ml puromycin. Cells for patch clamp experiments were grown on 35-mm culture dishes in standard media and were treated with 2 mM sodium butyrate and 1 μM dexamethasone 24 h prior to recording to induce ENaC expression (39). Cells used for biochemical experiments were maintained at subconfluent states and were moved into serum-free media at least 24 h prior to study.

Electrophysiology—NIH 3T3 cells expressing $\alpha\beta\gamma$ rat ENaC subunits were used in patch clamp analyses. Patch pipettes were pulled from glass capillary tubes using a microelectrode puller and fire-polished to achieve electrode resistances of 5–8 megohms. Pipette (intracellular) solutions for whole cell patch clamp experiments contained, in mM: 120 Tris aspartate, 3 Mg-ATP, 0.3 ADP, 0.1 $CaCl_2$, 7 $MgCl_2$, and 1 EGTA. Additionally, phosphorylated (activating) or nonphosphorylated peptides (control; Calbiochem) modeled on the amino acid sequence of the Src kinase carboxyl terminus (amino acid residues 521–533) were used where indicated, to modulate the activity of endogenous Src family kinases (40). The peptides were dissolved in the whole cell pipette solution at a final concentration of 10 μM. The standard bath solution for whole cell experiments was composed of, in mM: 150 lithium aspartate, 5 TES, 2 $MgCl_2$, and 1 $CaCl_2$. As indicated, amiloride (final concentration of 10 μM) was added to the bath solution during whole cell recordings.

Whole cell and single channel currents were recorded with an Axopatch 1C amplifier (Axon Instruments). Data was acquired at 2 kHz and filtered at 200 Hz during whole cell recordings (50 Hz for single channel recordings) using a low pass four-pole Bessel filter. Data acquisition and subsequent analyses for both whole cell and single channel experiments were performed using the pClamp 8.0 software package (Axon Instruments).

For whole cell patch clamp experiments, cells were voltage-clamped at 0 mV and pulsed to −40 mV every 100 ms for 400-ms intervals. This sequence was repeated for 180 s. Next, cells were voltage clamped at 0 mV and pulsed for 600 ms from −160 mV to +20 mV, in 20-mV increments. Current-voltage (IV) curves were generated from average current measurements starting 200 ms after each voltage pulse. Whole cell amiloride-sensitive currents (I_{amil}) were calculated from the difference in current in the presence and absence of 10 μM amiloride.

Single channel traces were recorded in the cell-attached conformation. The pipette solution for single channel studies contained, in mM: 280 lithium aspartate, 2 $MgCl_2$, 5 TES, and 0.1 $CaCl_2$. The bath solution contained, in mM: 150 Tris gluconate, 1 $CaCl_2$, 2 $MgSO_4$, and 5 TES with CsOH. Cells were preincubated with the Src kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2; 1 μM) or with an inactive control, 4-amino-7-phenylpyrazol[3,4-*d*]pyrimidine (PP3; 1 μM) for several minutes prior to study (41). Channel open probability (P_o), mean open time (MOT), and mean closed time (MCT) were calculated from patches containing only single channel events. Single channel analysis was performed using the pClamp 8.0 software package. To calculate the single channel MOT, the following equation was used.

$$MOT = (T \cdot P_o)/(n/2) \quad \text{(Eq. 1)}$$

T is recording time, P_o is the open probability, and n is the number of channel transitions during the recording time (38, 42). The single channel MCT was calculated with the following equation, where the variables are defined as for Equation 1.

$$MCT = [(T)/(n/2)] - MOT \quad \text{(Eq. 2)}$$

Western Blots—NIH 3T3 cells were maintained in media without serum for 24 h prior to study. Cells were treated with 10 nM ET-1 or vehicle control for 10 min, washed twice in ice-cold phosphate-buffered saline with phosphatase and protease inhibitors, and lysed in cold lysis buffer (50 mM Tris, 150 mM NaCl, 0.5% deoxycholate, pH 8.0) with serine/threonine and tyrosine phosphatase inhibitors (Sigma). Lysates were passed several times through a 27.5-gauge needle to shear chromosomal DNA. Homogenates were spun at 313,000 × *g*, and the soluble fraction was used for Western blotting and immunoprecipitations. For phosphotyrosine Western blots, lysates were separated by SDS-PAGE, transferred to Immobilon-P, and blotted with monoclonal anti-phosphotyrosine antibody (clone 4G10, Upstate Biotechnology).

In Vitro Kinase Assays—Kinases were immunoprecipitated from NIH 3T3 cell lysates using an antibody directed against the conserved COOH terminus of the Src family kinase sequence that recognizes all Src kinase family members (rabbit polyclonal antibody; Santa Cruz), or using antibodies to specific Src family kinases (α-Src kinase, Oncogene; α-Yes kinase, Wako). Lysates were incubated with antibody at 4 °C for 3–4 h before a 1:1 mixture of Protein A/G-agarose was added. After 1 h of incubation with Protein A/G-agarose at 4 °C, immunoprecipitates were washed in binding buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% Triton X-100, pH 7.4), and resuspended in a modified pipette solution/kinase assay buffer (150 mM Tris aspartate, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM orthovanadate, 4 μCi of [γ-^{32}P]ATP) with an exogenous Src family kinase substrate, rabbit muscle enolase (10 μg, Sigma). Additionally, we tested whether ENaC cytosolic domains were substrates for Src family kinases. GST-ENaC fusion proteins were generated by subcloning the cDNAs encoding the NH_2- and COOH-terminal subunits of human α, β, and γ ENaC into the pGEX-2TK (Amersham Pharmacia Biotech) expression vector, which contains an engineered PKA phosphorylation site. Purified fusion proteins were immobilized on glutathione-Sepharose in the standard kinase assay buffer described above and incubated with purified PKA catalytic subunit (Promega) or purified Src kinase (Upstate Biotechnology). Samples were incubated at 30 °C for 45 min, and reactions were stopped by the addition of Laemmli sample buffer. Samples were subjected to SDS-PAGE and visualized by phosphorimage analysis.

RESULTS

ET-1 Inhibition of ENaC—We used a previously described NIH 3T3 fibroblast cell model system that stably expresses $\alpha\beta\gamma$ ENaC subunits (39). Similar to those reports, we observed ENaC current density that ranged from 10 to 30 pA/pF (at −160 mV) under whole cell patch clamp conditions. Specific ENaC currents were detected as the difference current (I_{amil}) in the presence and absence of 10 μM amiloride, a selective ENaC channel blocker (Fig. 1*A*). Generally, treatment with amiloride decreased whole cell currents by 50–70%, reflecting the expression level and activity of ENaC in these cells. ET-1 treatment markedly inhibited amiloride-sensitive current. Full inhibition of I_{amil} was observed in cells treated with 10 nM ET-1 (Fig. 1*B*). Concentrations of ET-1 in the range of 1 pM to 10 nM produced a dose-dependent inhibition of I_{amil} (Fig. 1*C*).

ET-1 Activation of ET_B Inhibits ENaC—The ET-1 concentration that resulted in 50% inhibition of I_{amil} was ~50 pM, in a range consistent with ET-1 activation of ET_B receptors. To test this possibility, cells were pretreated with 1 μM BQ-788, a selective ET_B receptor antagonist. Under these conditions, BQ-788 prevented the inhibition of ENaC currents by 100 pM ET-1 (Fig. 2*A*). In contrast, 100 pM ET-1 was fully inhibitory in the presence of 1 μM BQ-123, a selective ET_A receptor antagonist (Fig. 2*B*). These results demonstrate that, in NIH 3T3 cells, ET_B receptor signaling is responsible for ET-1 inhibition of ENaC.

Src Inhibitor Prevents ET-1 Inhibition of ENaC—We have demonstrated previously that Src family kinases potently inhibit ENaC when coexpressed in *Xenopus* oocytes.[2] Therefore, we investigated whether Src family kinases were involved in mediating the effects of ET-1 on ENaC. We used a cell-permeable specific Src family kinase inhibitor, PP2, in whole cell patch clamp experiments. PP2 competes with ATP for binding

[2] Donaldson, S. H., Boucher, R. C., and Stutts, M. J., unpublished observations.

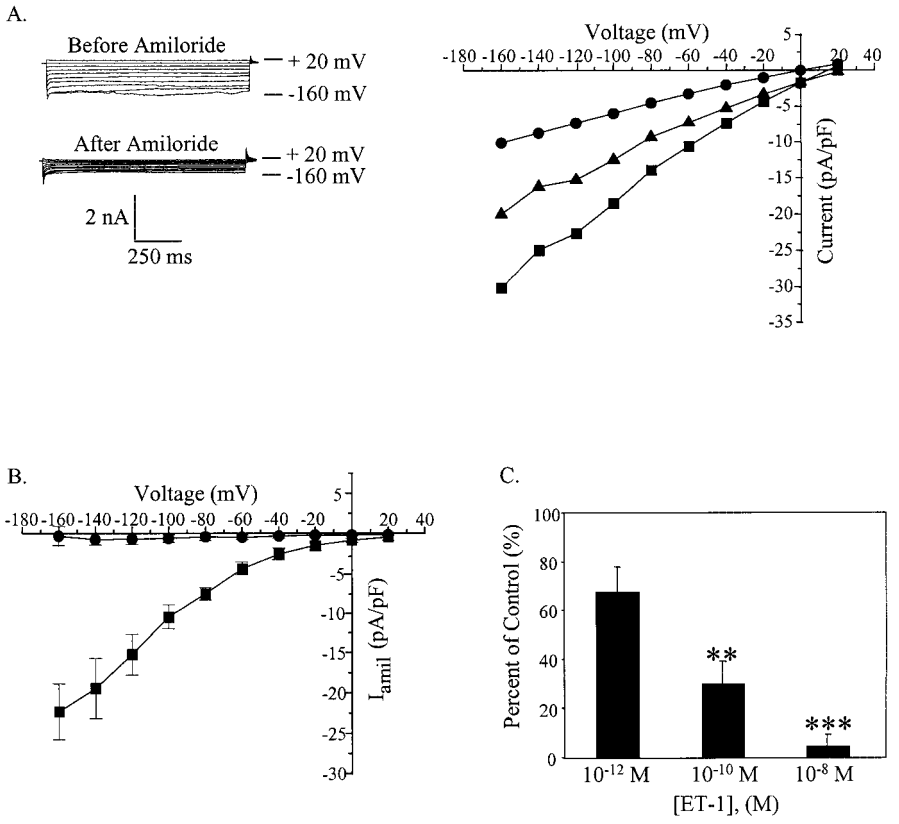

FIG. 1. **Endothelin-1 potently inhibits its amiloride-sensitive currents.** *A*, raw traces from IV curves generated from whole cell recordings in the presence and absence of 10 μM amiloride confirm the expression and activity of ENaC in NIH 3T3 cells infected with $\alpha\beta\gamma$ ENaC genes. The amiloride-sensitive component (▲ = I_{amil}) of the whole cell currents is calculated from the difference in current before (■) and after (●) treatment with amiloride. *B*, treatment of NIH 3T3 cells with 10 nM ET-1 (●) reduces I_{amil} significantly below control (■). Cells were studied in the presence (■) or absence (●) of 10 nM ET-1 for 3–8 min. Results are average currents ± S.E. for six experiments under each condition. *C*, ET-1 inhibition of ENaC is dose-dependent. Cells were treated with 1 pM, 100 pM, or 10 nM ET-1 as in *B*, and full IV curves were generated. Shown is I_{amil} for ET-1-treated cells expressed as a percentage of the control (no treatment) amiloride-sensitive current at −120 mV. Results represent the average currents ± S.E. for four to six experiments. Statistics were performed using Student's *t* test (**, $p < 0.005$; ***, $p < 0.0005$).

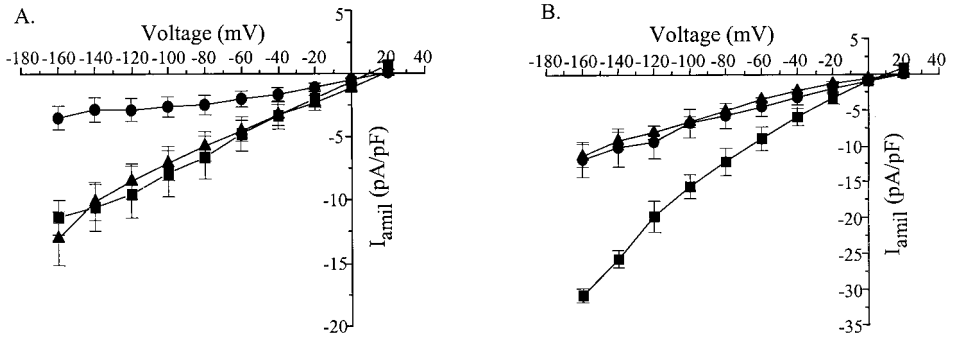

FIG. 2. **ET_B receptors mediate ET-1 inhibition of ENaC.** *A*, whole cell currents were recorded from cells treated with 100 pM ET-1 in the presence (▲) or absence (●) of 1 μM BQ-788, a selective ET_B receptor antagonist. Control cells (■) were treated with vehicle alone. In the presence of BQ-788, ET-1 did not significantly alter I_{amil}. Results are the average ± S.E. of six experiments. *B*, whole cell currents were recorded from cells treated with 100 pM ET-1 in the presence (▲) or absence (●) of 1 μM BQ-123, a selective ET_A antagonist. Control cells (■) were treated with vehicle alone. In the presence of BQ-123, ET-1 fully inhibited I_{amil}. Results are the average ± S.E. of four experiments.

to Src family kinases (41). In *in vitro* kinase assays, purified Src kinase did not phosphorylate a known substrate, enolase, in the presence of 1 μM PP2 (Fig. 3*A*, *lane 2*). The negative control compound PP3 shares a similar structure but does not affect Src kinase activity and did not inhibit the phosphorylation of enolase (Fig. 3*A*, *lane 3*). Cells were incubated in bath solutions containing 1 μM either PP2 or PP3 and subsequently treated with 100 pM ET-1. In cells incubated in PP3, ET-1 inhibited I_{amil} as observed previously (Fig. 3*B*). In contrast, in cells pretreated with PP2, I_{amil} was not inhibited by 100 pM ET-1. This observation implicates Src family kinase activity in the inhibition of ENaC by ET-1.

To demonstrate biochemically that Src family kinases are involved in ET-1 signaling, we examined changes in phosphotyrosine levels in response to ET-1 stimulation. Cells were treated with 10 nM ET-1 for 10 min, lysed, subjected to SDS-PAGE, and transferred to membranes. Total cell lysates were blotted with anti-phosphotyrosine antibody (monoclonal antibody clone 4G10; Fig. 3*C*). We observed a dramatic increase in tyrosine phosphorylation in response to ET-1. In particular, tyrosine phosphorylation of ~85-, 65-, 48-, and 43-kDa proteins were enhanced by ET-1 treatment. Additionally, *in vitro* kinase assays measuring the activity of Src family kinases show a significant increase in kinase activity in cells treated with 10 nM ET-1 (72 ± 20% increase over control; $p < 0.05$; Fig. 3*D*). These results indicate that ET-1 signaling activates tyrosine kinase pathways that include Src family kinases.

Activation of Endogenous Src Family Kinases Inhibit ENaC—To further show that activation of Src family kinases mediates inhibition of ENaC, we used peptides modeled on the conserved Src family kinase COOH terminus to activate the endogenous kinases. c-Src is inactivated by phosphorylation of tyrosine 527, by COOH-terminal Src kinase (CSK; Ref. 43). Intramolecular interactions between the Src homology 2 domain and this phosphotyrosine residue (Tyr^{527}) maintain c-Src kinase in an inactive state. Introduction of phosphorylated peptides containing the sequence of the Src carboxyl terminus disrupts the intramolecular interactions that hold the kinase in

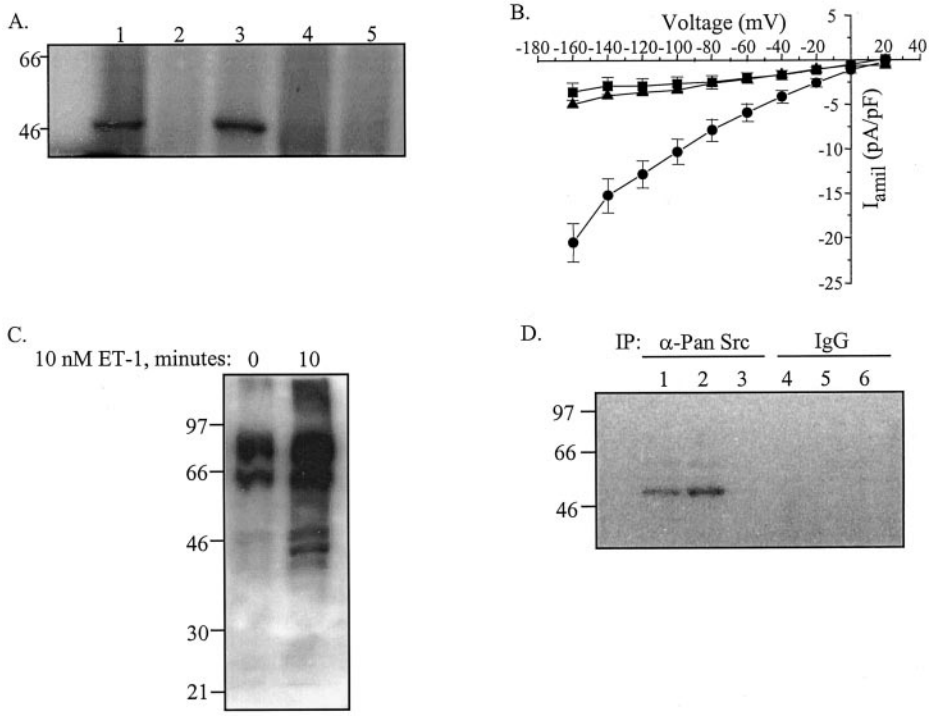

FIG. 3. **Src family kinase inhibitors prevent ET-1 inhibition of ENaC.** *A*, *in vitro* kinase assays demonstrate the efficacy of PP2, a specific Src family kinase inhibitor. Purified, active Src kinase was incubated with a Src substrate, enolase, and [γ-^{32}P]ATP in a modified pipette solution buffer. Under control conditions, Src kinase phosphorylated enolase (*lane 1*). In the presence of 1 μM PP2, Src phosphorylation of enolase was inhibited (*lane 2*). Incubation with 1 μM PP3, an inactive analogue of PP2, did not interfere with Src phosphorylation of enolase (*lane 3*). *Lanes 4* and *5* are reactions performed without enolase or Src kinase, respectively. Results are representative of three separate experiments. *B*, whole cell currents were recorded from cells treated with 100 pM ET-1 alone (■) or following pretreatment with 1 μM PP2 (●) or PP3 (▲). Src family kinase inhibition by PP2 completely prevented ET-1 inhibition of I_{amil}, whereas ET-1 was fully inhibitory in the presence of PP3. Results are the average ± S.E. of five experiments. *C*, NIH 3T3 cells were treated with 10 nM ET-1 for 10 min and then lysed. Proteins were separated by SDS-PAGE and transferred to Immobilon-P. Total cellular tyrosine phosphorylation was assessed by Western blot using anti-phosphotyrosine antiserum (monoclonal antibody clone 4G10). An increase in total cellular tyrosine phosphorylation was associated with ET-1 treatment. Results are representative of three separate experiments. *D*, NIH 3T3 cells were treated with 10 nM ET-1 for 10 min, then lysed and immunoprecipitated with 2 μg of α-Src family kinase antisera or rabbit IgG. Immunoprecipitates were subjected to *in vitro* kinase assays, and Src family kinase activity was assessed by phosphorylation of enolase. *Lane 1* represents basal Src family kinase activity (no ET-1 treatment), which is enhanced upon stimulation with 10 nM ET-1 (*lane 2*). Pretreatment with PP2 blocked Src phosphorylation of enolase (*lane 3*). *Lanes 4–6* are corresponding IgG controls. Results are representative of three separate experiments.

the folded, inactive state. Addition of activating phosphorylated peptide to c-Src and c-Yes kinase-specific immunoprecipitates significantly increased phosphorylation of enolase in *in vitro* kinase assays (Fig. 4*A*). As a control, immunoprecipitates incubated with the nonphosphorylated peptide, which does not compete for Src homology 2 binding, show a low level of phosphorylation. These assays demonstrate that c-Src and c-Yes kinase can be activated by the introduction of phosphorylated carboxyl-terminal tail peptides. In whole cell patch clamp experiments, activation of endogenous Src family kinases resulted in a significant decrease in amiloride-sensitive currents (Fig. 4*B*). Dialysis of cells with the nonphosphorylated, control peptide did not alter ENaC activity. These experiments reveal that the cell's endogenous Src family kinases are poised to potently inhibit ENaC when activated by appropriate signals.

Src Family Kinase Inhibitor, PP2, Increases Whole Cell ENaC Currents and Alters Single Channel Gating—We have shown that activation of Src family kinases can dramatically reduce I_{amil} (Fig. 4*B*). However, it is evident that Src family kinase activity exists at a basal level in unstimulated cells (Fig. 3*D*). To examine whether this basal level of kinase activity influences ENaC currents, we measured I_{amil} in the presence of PP2 and PP3. We found that treatment of cells with 1 μM PP2 significantly increased I_{amil} over control (PP3) (Fig. 5). These results suggest that basal Src family kinase activity mediates tonic inhibition of ENaC in this model system.

Previous work in A6 cell lines describes the inhibition of ENaC by ET-1 (38). In that study, ET-1 inhibited ENaC P_o and enhanced mean closed time. Therefore, to test whether Src family kinases could mediate similar effects on ENaC in mammalian cells, we studied ENaC in cell-attached single channel patches in the presence of PP2 or PP3. A representative experiment is shown in Fig. 6*A*. Channel openings, represented by upward deflections in the trace, reveal classical ENaC characteristics, *e.g.* low amplitude and slow gating channel transitions (44). Patches from PP2-treated cells contained ENaC that were open for extended periods (up to tens of seconds), compared with patches from PP3-treated cells. The all-points histograms in Fig. 6*A* are examples of analyses generated from continuous traces from cells treated with PP2 or PP3 and represent the distribution of time that single channel patches resided in the closed or open conformation. Integration of the all-points histograms from the entire data set (1001 s for PP2, 484 s for PP3) shows that cells incubated in PP3 display a low ENaC open probability (P_o = 0.0475 ± 0.0249) as compared with cells treated with PP2 (P_o = 0.531 ± 0.150; $p < 0.02$; Fig. 6*B*). A careful analysis of the channel gating kinetics reveals that incubation with PP2 causes shorter mean closed times (3.42 ± 1.52 s for PP2; 13.7 ± 3.02 s for PP3, $p < 0.02$) between channel events. Additionally, we observed an increase in mean open time under PP2 conditions, but this increase did not reach statistical significance (3.69 ± 1.83 s for PP2; 0.531 ± 0.257 s for PP3, $p = 0.125$). These data suggest that Src family kinase inhibition enhances ENaC P_o by decreasing the closed time

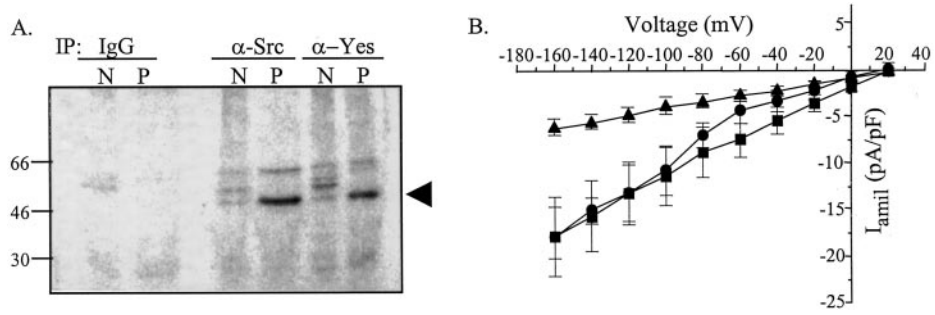

FIG. 4. **Activation of endogenous Src family kinases decreases ENaC currents.** *A*, endogenous Src or Yes kinase was specifically immunoprecipitated from NIH 3T3 cell lysates, and their activities were assessed by *in vitro* phosphorylation of enolase. Immunoprecipitates incubated in the presence of phosphorylated, activating Src tail peptides (*P*) show increased phosphorylation of enolase, consistent with stimulation of Src and Yes kinase activity. Introduction of nonphosphorylated, control peptides (*N*) did not affect Src or Yes kinase activity. *B*, whole cell amiloride-sensitive currents were recorded from cells dialyzed with phosphorylated (▲) or nonphosphorylated (●) Src tail peptides. In the presence of activating, phosphorylated peptides, I_{amil} was significantly decreased as compared with control (■ = no peptide). Results are average ± S.E. of four experiments.

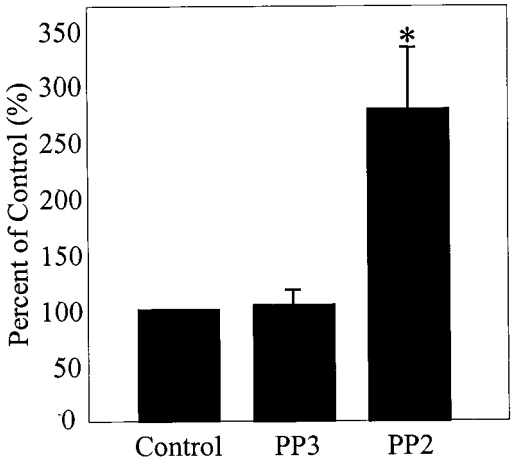

FIG. 5. **Inhibition of basal Src family kinase activity increases whole cell ENaC currents.** Whole cell amiloride-sensitive currents were recorded from cells treated with a standard bath solution or one containing 1 μM PP2 or PP3. Results are plotted as a percentage of control (no treatment) amiloride-sensitive current at −120 mV. Inhibition of basal Src family kinase activity with PP2 significantly increased I_{amil} over control (* $p < 0.05$). Results are the average ± S.E. of four experiments.

between channel opening events.

Src Family Kinases Do Not Regulate ENaC by Direct Phosphorylation of the Channel Subunits—In several instances, Src family kinases regulate ion transport by direct phosphorylation of the ion channel or transporter (53, 54). To investigate this possibility as a mechanism for the regulation of ENaC by Src family kinases, we incubated purified GST fusion proteins encoding the ENaC subunit cytosolic domains with purified Src kinase (Fig. 7). The fusion proteins were generated in-frame with GST from the pGEX-2TK expression vector, which contains an engineered PKA phosphorylation site. As expected, each of the immobilized NH_2- and COOH-terminal ENaC fusion proteins were phosphorylated by exogenous PKA. However, purified, active Src kinase failed to phosphorylate any of the ENaC cytosolic domain fusion proteins. These results demonstrate that ENaC subunit cytosolic domains do not serve as Src family kinase substrates *in vitro*.

DISCUSSION

We have identified Src family kinases as intermediate signaling proteins in a negative regulatory path for ENaC. Previous reports linked ET-1 signaling to inhibition of ENaC, but no information existed concerning the signaling pathway involved (22, 38). Our studies confirmed previous findings that Src family kinases are activated by ET-1 signaling (5, 16). We report the novel finding that Src family kinases potently inhibit ENaC and that this action mediates the inhibitory effect of ET_B on ENaC function. ET-1 is a well known regulator of systemic blood pressure; as such, its action on vascular targets has been intensely studied. Additional physiological roles of this hormone are suggested by ET_A and ET_B receptor localization in nonvascular tissues, such as the nephron (11). Analysis of the ET receptor distribution in the nephron shows that ET_B is expressed at 10-fold higher levels than ET_A, suggesting a dominant role for ET_B in renal tubule epithelial cells (45, 46). Importantly, adult rats lacking functional ET_B receptor activity display enhanced Na^+ reabsorption in the distal nephron, implicating ET_B in regulation of tubular function (22). The elevated blood pressure in these animals was reversed to normal when treated with amiloride, a potent inhibitor of ENaC. Our finding that Src family kinases robustly down-regulate ENaC activity may be critical to understanding how ET_B receptors regulate blood pressure.

Although the phenotype of the ET_B receptor-deficient rat is consistent with an inhibitory effect of ET_B on ENaC activity, the *in vivo* model does not exclude the role of indirect effects from ET_B in the vasculature, the possibility that other hormones may be involved, or that the effects were mediated by changes in ENaC gene expression. Studies in cell culture models provide systems to examine ET-1 regulation of ENaC in isolation, separate from the influences of circulating hormones and complex multisystem regulatory feedback mechanisms. In A6 cells, a *Xenopus* model of the distal nephron, ENaC single channel activity was strongly inhibited by ET-1 (38). This effect was attributed to the activation of ET_B and resulted in decreases in ENaC P_o and increases in channel mean closed time. Our studies in NIH 3T3 cells both confirmed and extended the previously published results. We found that ET-1 potently inhibited ENaC in mammalian cells and that this effect was totally dependent on the activation of Src family kinases (Figs. 1 and 3). Examination of ENaC at the single channel level showed that Src family kinase activity decreases ENaC P_o and mean open time, whereas increasing the channel mean closed time (Fig. 6). Because the regulation of ENaC by ET-1 is robust in cell culture and heterologous expression systems, it is unlikely that the regulation of ENaC by ET-1 observed *in vivo* is a secondary consequence of the actions of other hormones or signals. Instead, the effects of ET-1 on ENaC regulation are most likely caused by direct activation of intracellular signal transduction pathways involving Src kinases that link the receptor to ENaC.

ET_A and ET_B are GPCRs, but several published reports show

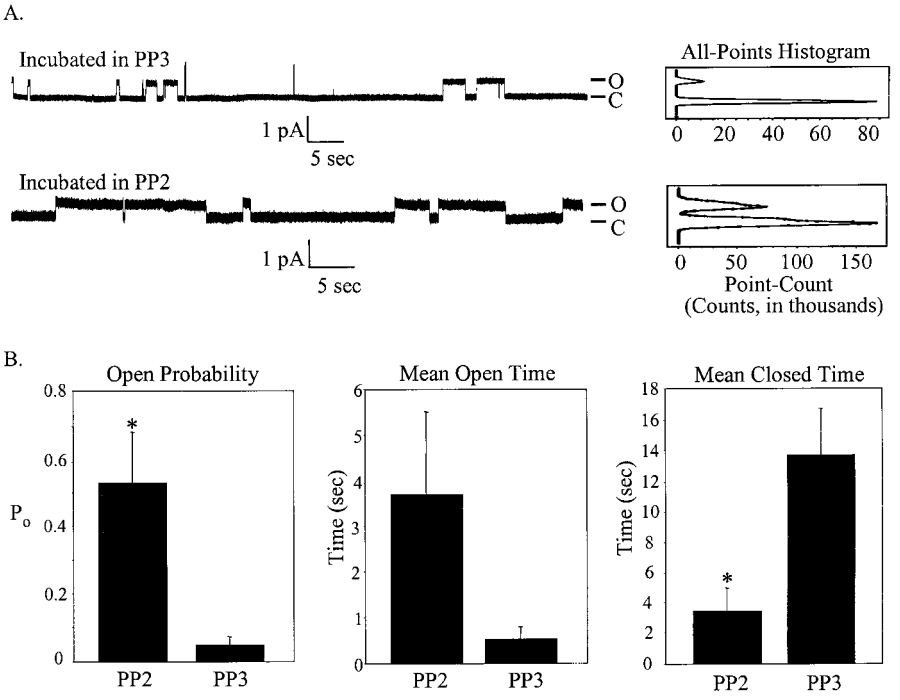

FIG. 6. **Inhibition of Src family kinase activity alters single channel gating in cell-attached patches.** *A*, single channel recordings were obtained from cell-attached patches on cells treated with 1 μM PP2 or PP3. In the presence of the Src family kinase inhibitor, ENaC displayed a higher open probability (P_o) and altered gating kinetics. All-points histograms were generated from continuous traces recorded under conditions of PP2 or PP3 treatment. Integration of the curves shows that Src family kinase inhibition is associated with increased residency time in the open state. *B*, open probability (P_o) and channel mean open and closed times were calculated from single channel recordings of cells incubated in 1 μM PP2 or PP3 as indicated under "Experimental Procedures." Inhibition of Src family kinases is associated with increases in P_o (*, $p < 0.02$) and mean open time (not statistically significant) and decreases in average closed times for ENaC (*, $p < 0.02$).

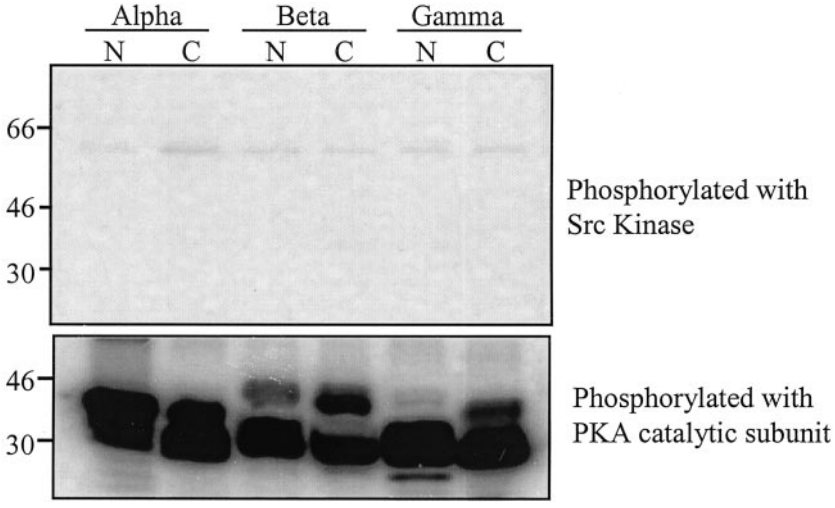

FIG. 7. **Src family kinases do not regulate ENaC by direct phosphorylation of the channel subunits.** Immobilized GST fusion proteins encoding the NH_2- (*N*) and COOH-terminal (*C*) α, β, and γ ENaC subunits were incubated with purified catalytic subunit of PKA or purified Src kinase. Although each of the GST fusion proteins was phosphorylated by PKA (*lower panel*), active Src kinase did not phosphorylate any of the ENaC subunit cytosolic domains (*upper panel*). The faint band observed at 60 kDa in the *upper panel* is Src kinase autophosphorylation.

that ET-1 treatment causes a significant increase in cellular tyrosine phosphorylation and Src kinase activity (5, 47). There are many examples of cross-talk between GPCRs and tyrosine kinases, for instance Src and the calcium-sensitive tyrosine kinase, Pyk2, couple lysophosphatidic acid ($G\alpha_i$-coupled) and bradykinin ($G\alpha_q$-coupled) receptors with the mitogen-activated protein kinase pathway (48). Additionally, inhibitors of tyrosine kinases (herbimycin A) and Src kinases (PP1), block activation of mitogen-activated protein kinase cascades by thrombin receptors ($G\alpha_q$-coupled) (49, 50). The mechanism responsible for linking these signaling cascades is not fully understood, but could involve the activation of Ca^{2+}-sensitive kinases or direct actions of G-protein subunits on tyrosine kinase effectors (48, 51). Here, we showed that treatment of NIH 3T3 cells with 10 nM ET-1 markedly increased total cellular tyrosine phosphorylation and Src family kinase activity, in particular (Fig. 3, *C* and *D*). Further, the inhibitory effects of ET-1 on ENaC activity could be completely blocked if cells were pretreated with Src family kinase inhibitors (Fig. 3*B*). Together, these data strongly argue that Src family kinases contribute to the ET-1 signaling cascade. Src family kinases are potent mitogenic agents, and ET-1-mediated gene expression of atrial natriuretic peptide in primary cardiomyocytes is Src kinase-dependent (5). However, the time frame of our observations eliminates the possibility that changes in expression of ENaC regulatory proteins accounts for the acute effects of Src family kinases on ENaC. Here, we describe a paradigm in which ET-1 rapidly alters ion transport via a transcription-independent mechanism. This effect is similar to previous reports of ET-1 activation of the Na^+/H^+ exchanger 3 (NHE3), in which ET-1 effects (via ET_B) were blocked ~50% by tyrosine kinase inhibitors (17). Additionally, regulation of Na-HCO_3 co-transport by carbachol through activation of muscarinic receptors was completely inhibited by the Src family kinase inhibitor, PP1 (52). These examples provide important evidence that cross-talk between GPCRs and tyrosine kinases can have immediate effects on ion transport, in addition to long term mitogenic effects on gene transcription. Moreover, our *in vitro* studies suggest that the phenotype of the ET_B-deficient rat and mouse may not be explained solely by changes in the expression of ENaC subunit genes or by the involvement of other hormonal systems.

The mechanism by which Src family kinases inhibit ENaC activity remains to be determined. Our single channel data clearly show changes in channel open probability consistent with inhibition of whole cell currents by Src family kinases (Fig. 4). We found that inhibition of Src family kinase activity caused ENaC channels to display short average closed times (Fig. 6). This suggests that active Src family kinases tonically inhibit ENaC by altering the channel gating. The low ENaC P_o associated with Src activation is consistent with the strong inhibition of whole cell currents. Our experiments do not exclude the possibility that Src kinase activity also regulates the number of ENaC channels on the cell surface. However, our single channel data showed a strong inhibition of channel open probability that is sufficient to alone account for the inhibition of whole cell currents by Src family kinases.

We find that Src family kinase activity is required for the inhibition of ENaC by ET-1. Therefore, it will be crucial to identify the relevant proteins that are downstream of Src family kinases to further define the molecular mechanism of this inhibition. Previous reports describing ion transport regulation by Src family kinases include direct phosphorylation of channels or transporters (53, 54). This seems unlikely in the case of ENaC. The *in vitro* phosphorylation of ENaC subunits has been investigated; however, only phosphoserine/threonine, but not phosphotyrosine residues were observed (55, 56). Moreover, the ENaC subunit cytosolic domains do not contain consensus sites for Src kinase phosphorylation. Our data (Fig. 7) support these findings and demonstrate that ENaC cytosolic domains are not substrates for Src kinase *in vitro*. Therefore, it is likely that Src family kinases inhibit ENaC by an indirect mechanism. These findings are similar to the regulation of ENaC by the serum-glucocorticoid-regulated kinase (SGK), where SGK activation increases ENaC activity by enhancing cell-surface expression (34). When coexpressed in *Xenopus laevis* oocytes, SGK increases ENaC activity by stimulating the translocation of ENaC channels to the cell surface in a kinase-dependent manner (34). The mechanism that accounts for enhanced cell-surface expression is unknown, but similar to Src, SGK does not directly phosphorylate ENaC and the target of its kinase activity is unidentified (56). Our results, taken together with these findings on SGK regulation of ENaC, make it clear that unidentified molecular mechanisms exist for control of Na^+ conductance. These mechanisms may vary importantly with cell type and with the upstream hormonal signal involved. Clearly there are intermediary proteins that are required for the regulation of ENaC by SGK and Src family kinases, identification of these proteins will be the subject of future studies.

Several general mechanisms can be envisioned by which Src family kinases may act to indirectly inhibit ENaC activity. First, Src family kinase activation may modulate the activity of a serine/threonine kinase. Insulin stimulates receptor tyrosine kinase activity, increases serine/threonine phosphorylation of β and γ ENaC, and increases ENaC activity (55). Therefore, it is possible that Src kinases may enhance or suppress the activity of a serine/threonine kinase, which in turn, modulates ENaC activity. Second, Src family kinase activation may alter the activity of a phosphatase that subsequently dephosphorylates the channel or an associated regulator. Third, Src family kinases may induce cytoskeletal rearrangements that affect the association of stimulatory or inhibitory proteins with ENaC. Finally, the target of Src family kinase activity could be an ENaC-associated cytosolic protein, whose phosphorylation state influences its association with the channel. Binding or release from the channel may potentially alter channel gating. Given the complexity of ENaC regulation, any of these models could account for the effects of Src family kinases.

The involvement of ET-1 and ET_B in the genesis of salt-sensitive hypertension may provide new insights into the molecular pathophysiology of renal disease. Our novel finding that Src family kinases are required for endothelin-mediated inhibition of ENaC provides further understanding of Na^+ transport regulation, with potential implications for elucidating causes of human hypertension. It will be intriguing to examine the hypertensive population for variations in ET-1, ET_B, and Src family kinase activities in the nephron. Such alterations may help to understand the mechanisms of salt-sensitive hypertension and/or essential hypertension. In additional, identifying the Src family kinase members (Src, Yes, Fyn, etc.) that mediate these effects *in vivo* could provide insights into understanding the functional specificity of Src family kinase signaling. Our identification of Src family kinases as intermediaries of ET-1 modulation of ENaC provides another potential therapeutic target for the treatment of human hypertension.

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