Syntaxin 1A Regulates ENaC via Domain-specific Interactions*

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The epithelial sodium channel (ENaC) is a heterotrimeric protein responsible for Na⁺ absorption across the apical membranes of several absorptive epithelia. The rate of Na⁺ absorption is governed in part by regulated membrane trafficking mechanisms that control the apical membrane ENaC density. Previous reports have implicated a role for the t-SNARE protein, syntaxin 1A (S1A), in the regulation of ENaC current (Iₙa). In the present study, we examine the structure-function relationships influencing S1A-ENaC interactions. In vitro pull-down assays demonstrated that S1A directly interacts with the C termini of the α-, β-, and γ-ENaC subunits but not with the N terminus of any ENaC subunit. The H3 domain of S1A is the critical motif mediating S1A-ENaC binding. Functional studies in ENaC expressing Xenopus oocytes revealed that deletion of the H3 domain of co-expressed S1A eliminated its inhibition of Iₙa, and acute substitution of a GST-H3 fusion protein into ENaC expressing oocytes inhibited Iₙa, to the same extent as S1A co-expression. In cell surface ENaC labeling experiments, reductions in plasma membrane ENaC accounted for the H3 domain inhibition of Iₙa. Individually substituting C terminus-truncated subunits for their wild-type counterparts reversed the S1A-induced inhibition of Iₙa, and oocytes expressing ENaC comprised of three C terminus-truncated subunits showed no S1A inhibition of Iₙa. C terminus truncation or disruption of the C terminus β-subunit PY motif increases Iₙa by interfering with ENaC endocytosis. In contrast to subunit truncation, a β-ENaC PY mutation did not relieve S1A inhibition of Iₙa, suggesting that S1A does not perturb Nedd4 interactions that lead to ENaC endocytosis/degradation. This study provides support for the concept that S1A inhibits ENaC-mediated Na⁺ transport by decreasing cell surface channel number via direct protein-protein interactions at the ENaC C termini.

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† The abbreviations used are: ENaC, epithelial Na⁺ channel; S1A, syntaxin 1A; SNARE, soluble N-ethylmaleimide-sensitive factor attachment.
SIA-ENaC Domain Interactions

EXPERIMENTAL PROCEDURES

Materials—mMESSAGE mMACHINE™ T7 or T3 complimentary RNA synthesis kits were purchased from Ambion Inc. (Austin, TX). DNA Mini-Prep kits, MIDI-Prep kits, and nickel-nitritolactric acid beads were obtained from Qiagen. Restriction enzymes, T4 DNA Ligase, T4 polynucleotide kinase, and Plu I polymerase were purchased from New England Biolabs. Glutathione-Sepharose 4B was purchased from Amer sham Biosciences. Complete Protease Inhibitor Mixture tablets were obtained from Roche. All other reagent grade chemicals were obtained from Sigma. Plasmids encoding human ENaC α-, β-, and γ-subunits were kindly provided by Dr. Michael Welsh (University of Iowa). Mouse ENaC constructs were generously provided by the laboratory of Dr. Thomas Kleymen (University of Pittsburgh). Subunit truncations were generated by introducing a stop codon by PCR at the designated amino acid (see below).

Construction of His-tagged ENaC Cytoplasmic Domains—The N and C termini of the human α-, β-, and γ-ENaC subunits were produced in bacteria as His fusion proteins. The appropriate cDNA fragments were synthesized by PCR with a subunit-specific primer pair and the corresponding template, pBS/KS/ENaCs. The sense and antisense primer pairs were as follows: α-ENaC, 5'-CATATGGAAAGGAAC- AAGCTCAG-5'; 5'-GGATCCCTAGAAGGCTTCTGATCAG-3'; β-ENaC, 5'-CATATGTCCTGCAAGAAGTTC and 5'-GGATCCTCAGGGCCCCC-CCAG; β-ENaC, 5'-CATATGGAAAGGAAC-AAGCTCAG-5'; 5'-GGATCCCTAGAAGGCTTCTGATCAG-3'; γ-ENaC, 5'-CATATGGACGCGCGAGAGTAGCTG and 5'-GGATCCCTCAGGGCCCCC-CCAG; γ-ENaC, 5'-CATATGGAAAGGAAC-AAGCTCAG-5'; 5'-GGATCCCTAGAAGGCTTCTGATCAG-3'. PCR amplicons were cloned into the plasmid vector pGEX-6p-1 (Amersham Pharmacia Biotech) and then digested with Sal I and Not I and ligated into the pGEX-6p-1 vector. Other S1A deletion mutants (see below) were constructed in a similar manner. All constructs were verified by microsequence analysis.

Glutathione S-Transferase-S1A Fusion Proteins—Syntaxin 1A fusion proteins containing GST at the N terminus were produced in BL21 competent cells. GST-H3 contains the S1A H3 domain, S1A1–129, GST-H3-TM adds the transmembrane domain, S1A129–2200, and GST-A H3 truncates both the H3 and TM domains from full-length S1A, S1A1–129. These constructs were employed in in vitro pull-down assays with the His6-tagged ENaC C termini (domain deletions are also provided with the results; see Fig. 2A). Conditions for bacterial growth and purification of GST fusion proteins have been described previously (16).

Generation of His6-tagged ENaC C and N Termini—Fresh bacterial BL21 colonies harboring His6-tagged ENaC cDNAs were cultured in 20 ml of LB medium containing ampicillin (50 μg/ml). Overnight cultures were diluted with pre-warmed LB medium at a ratio of 1:10, cultured at 37°C for 1-1.5 h and then induced with 1 mM isopropyl-β-D-thiogalactopyranoside at 37°C with shaking for 3-3.5 h until the A600 was 0.35-0.38. Bacterial pellets were resuspended in sonication buffer (100 mM Tris, 500 mM KCl, 8 mM MgCl2, 1 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture, 1 tablet/50 ml). Cells were lysed by sonication and incubated at 4°C for 30 min. Lysates were clarified by centrifugation at 12000 × g at 4°C and subsequently incubated with pre-equilibrated nickel-nitritolactric acid beads at room temperature for 30 min. Beads were washed at least three times in Buffer A (20 mM HEPES, 200 mM KCl, 2 mM β-mercaptoethanol, 0.5 mM Na3ATP, 10% glycerol, 30 mM imidazole). Fusion proteins were eluted by three washes with 250 mM imidazole in Buffer A. Purified His6-tagged ENaC C-terminal or N-terminal proteins were dialyzed in phosphate-buffered saline for 36 h at 4°C. The purified proteins were verified using Coomassie Blue-stained SDS-PAGE or by Western blotting with monoclonal anti-polylhistidine antibodies (Sigma).

Pull-down Assays—These assays were performed as described previously (16), with modifications. Briefly, 10 μg of GST-S1A fusion protein was immobilized on glutathione-Sepharose 4B and incubated with 10 μg of His6-tagged ENaC proteins in 200 μl of a modified DIGNAM D buffer (20 mM HEPES, 50 mM KCl, 2.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.2% Triton X-100, 20 μM CaCL2, 1 tablet of protease inhibitor mixture/50 ml of buffer, pH 7.0, with KOH) at 4°C with shaking. The next day, samples were pelleted by centrifugation, washed three times with DIGNAM D buffer at 4°C, and resolved using 15% SDS-PAGE. His6-tagged ENaC proteins were detected by use of poly- histidine antibodies (see above). The effect of adding ENaC-S1A interactions was assessed by varying the ion concentrations of the binding buffer; solutions containing 0.1 or 10 μM free Ca2+ were generated by EGTA buffering, as described (17).

Complimentary RNA (cRNA) Transcription and Oocyte Injection—Vectors containing mouse ENaC human, ENaC, and rat syntaxin 1A inserts were linearized, and cRNAs were synthesized in vitro by use of T7 or T3 cRNA synthesis kits. Oocyte isolation and RNA injection were performed as described previously (18). Briefly, 0.5 ng of cRNA of each ENaC subunit was injected into stage V or VI oocytes. For experiments investigating the effect of S1A co-expression on ENaC function, oocytes were co-injected with 5 ng of S1A. Expression proceeded at 18°C for 16-24 h in sodium-free ND96 solution before experiments. GST fusion proteins were injected in a volume of 50 nl (estimated final concentration, 50 ng/μl) into oocytes expressing ENaC, and currents were recorded 1 h after injection. Protein binding studies (above) were performed using cytoplasmic domains derived from human ENaC, whereas the functional studies generally employed mouse ENaC subunit expression. Amino acid identity within the C-terminal cytoplasmic domains of human and mouse ENaC is >70% (this region is important in SIA-ENaC interactions; see below). In addition, functional experiments with co-expressed S1A, performed using C terminus human ENaC truncation, yielded results identical to those performed with mouse ENaC (Fig. 3), confirming that these effects are splice-specific.

Electrophysiology—Two-electrode voltage clamp recordings were performed as described (18) using 3 mM KCl-filled micropipettes connected to a GeneClamp 500 amplifier (Axon Instruments, Foster City, CA). Oocytes were bathed continuously in ND96 solution as follows (in mM): 96 NaCl, 1 KCl, 1.8 CaCl2, 1 MgCl2, and 5 HEPES, pH 7.4. In some experiments, 10 mM ND96, equimolar sodium chloride, and 10 mM NaCl replaced NaCl. After impalement, membrane potentials were allowed to stabilize before voltage clamping at −100 mV; amiloride-sensitive Na+ currents were recorded as the difference in current before and after addition of 10 μM amiloride.

Co-immunoprecipitation—To detect ENaC labeling, the general approach was based on that of Zerangue et al. (19). Briefly, oocytes expressing mouse α-, β-FLAG, and γ-ENaC subunits for 2 days were blocked for 30 min in MBS supplemented with 1 mg/ml of bovine serum albumin (MBS-BSA) and then exposed to MBS-BSA plus 1 μg/ml of a mouse monoclonal anti-FLAG antibody (M2, Sigma) at 4°C for 1 h. β-ENaC contained the
FLAG epitope (DYDKKKD) at the extracellular loop position defined by Firsov et al. (20), which did not alter I\textsubscript{Na}, relative to wt ENaC expression. This was confirmed in parallel current measurements performed prior to antibody labeling. After primary antibody labeling the oocytes were washed six times with MBS-BSA at 4°C and then incubated with MBS-BSA supplemented with 1 mg/ml horseradish peroxidase-conjugated secondary antibody for 1 h at 4°C (peroxidase-conjugated AffiniPure F(ab’\textsubscript{2}) fragment goat anti-mouse IgG; Jackson Immunoresearch Laboratories, West Grove, PA). After 12 additional washes, individual oocytes were placed in 100 μl of SuperSignal ELISA Femto solution (Pierce, Rockford, IL) and incubated at room temperature for 1 min. Chemiluminescence was quantitated in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA) where the signal was integrated over a 60-s interval and is provided in relative light units. These and other results are expressed as mean ± S.E.; statistical differences were assessed by analysis of variance.

RESULTS

S1A Interacts with ENaC Subunit C Termi—Our previous results (14) and those of Saxena et al. (15) demonstrated that S1A co-expression reduced ENaC currents expressed in Xenopus oocytes. A physical interaction of S1A with ENaC subunits was also demonstrated in co-immunoprecipitation assays (14, 15). We reasoned that the ENaC cytoplasmic domains would be the potential binding sites for S1A, a type 2 plasma membrane protein that has no significant structure within the extracellular compartment. To evaluate the cytoplasmic domains of ENaC as potential binding sites for S1A, GST-S1A\textsubscript{4–267} protein was employed in pull-down experiments with His\textsubscript{6}-tagged ENaC C or N termini. As in similar protein interaction studies, a truncated syntaxin 1A (S1A\textsubscript{1–267}) lacking the transmembrane domain was employed in pull-down experiments with His\textsubscript{6}-tagged ENaC C or N termini. As in similar protein interaction studies, a truncated syntaxin 1A (S1A\textsubscript{1–267}) lacking the transmembrane domain was employed for its enhanced solubility and purification properties and because the last 21 amino acids of the S1A C terminus constitute the transmembrane domain, which is not expected to be involved in cytosolic protein-protein interactions. In addition, previous studies (14) have demonstrated that ENaC currents are inhibited to the same extent by co-expression of either full-length S1A or a soluble S1A\textsubscript{1–267}, lacking the transmembrane domain. As shown in the upper panel of Fig. 1, the C termini of ENaC α, β, and γ subunits interacted with GST-S1A. In contrast with these results, no interaction could be detected between S1A and the N terminus of any ENaC subunit (Fig. 1, lower panel). These findings demonstrate that S1A binds to the ENaC subunit cytoplasmic C termini.

S1A Domain of S1A Binds the ENaC C Termini—As the H3 helical region of S1A is an important domain for protein-protein interactions within the SNARE fusion complex (21) and has been shown to interact with other ion channels (22, 23), we determined whether the H3 domain of S1A was involved in interactions with ENaC. Three deletion constructs (Fig. 2A) containing N terminus GST fusions were derived from a full-length S1A clone for comparison with the data from the GST-S1A used in Fig. 1. These constructs were employed in in vitro pull-down assays with the His\textsubscript{6}-tagged ENaC C termini. The results (Fig. 2B) show that GST fusions containing the syntaxin 1A H3 domain (GST-H3 and GST-H3-TM) generally exhibited a stronger interaction with the ENaC subunit C termini than did the same amount of GST-S1A. This is not unexpected, because prior work (24) has demonstrated that regions N-terminal to the H3 domain have an autoinhibitory function and can reduce H3 domain interactions with substrates. Deletion of the H3 domain abolished the interactions of syntaxin with the ENaC C termini (Fig. 2B, lane 5). As a negative control, GST alone had no significant affinity for the ENaC C termini. These results indicate that the H3 domain of S1A is the critical site for ENaC C terminus binding.

H3 Domain Truncations Reduce S1A Inhibition of ENaC Currents—To determine whether the physical interaction between S1A and the ENaC C termini is involved in the inhibition of ENaC current observed with S1A co-expression, we evaluated the effect of S1A on Na\textsuperscript{+} currents expressed from subunits with C terminus truncations. In agreement with previous studies (14, 15), S1A co-expression significantly decreased the amiloride-sensitive Na\textsuperscript{+} current in oocytes expressing wt ENaC (Fig. 3, A–D). This inhibition averaged 75%. Substitution of the wt α-subunit by α-H613X had no significant effect on the magnitude of the amiloride-sensitive I\textsubscript{Na}, in agree-
ment with prior studies of \( \alpha \)-ENaC truncation (12). However, oocytes expressing \( \alpha \)-H9251-H613X ENaC together with S1A had significantly greater currents than those expressing wt ENaC plus S1A. Under these conditions the S1A inhibition was reduced to 38% (Fig. 3A).

Qualitatively similar results were obtained from individual C terminus truncations of the \( \alpha \)- and \( \gamma \)-ENaC subunits. In contrast to the \( \alpha \) truncation, expression of the \( \gamma \)-R583X significantly increased \( I_{Na} \) relative to controls, also consistent with prior findings and the stimulation of \( I_{Na} \) observed with Liddle’s mutations that truncate \( \gamma \)-ENaC (12). Fig. 3B shows that, when co-expressed with \( \beta \)-R583X ENaC, S1A did not significantly inhibit \( I_{Na} \), indicating that S1A inhibition may be reversed by truncation of the \( \beta \)-ENaC C terminus alone. Truncation of the \( \gamma \)-ENaC C terminus resulted also in a gain of function consistent with mutations producing Liddle’s disease. S1A co-expression did not inhibit \( \gamma \)-R583X ENaC \( I_{Na} \) as potently as in wild-type controls (Fig. 3C); the inhibition was reduced to 51% with \( \gamma \)-ENaC truncation. These results suggest that S1A can interact with each ENaC subunit C terminus to produce an inhibition of \( I_{Na} \), because truncation of individual subunit C termini fully or partially reverse the S1A inhibitory effect. The elimination of S1A inhibition by \( \beta \)-ENaC truncation alone suggests that a selective interaction at this site may be sufficient to account for the inhibitory effect of S1A. Finally, we expressed a functional ENaC comprised of three C terminus truncated subunits and determined the influence of S1A co-expression on \( I_{Na} \) (Fig. 3D). S1A inhibition was reversed completely under these conditions, as there was no significant difference between the combined \( \alpha \beta \gamma \) truncated ENaC currents and those with co-expressed S1A. These findings suggest that the S1A inhibition of \( I_{Na} \) involves functional interactions of syntaxin with the C termini of all ENaC subunits, which is consistent with the protein binding data.

An ENaC PY Mutant Is Inhibited by S1A—The PY motif located in the C terminus of the \( \beta \) - and \( \gamma \)-ENaC subunits is involved in important interactions with the ubiquitin ligase Nedd4 (7). To examine whether the PY motif may also influence S1A binding and subsequent ENaC inhibition, we measured \( I_{Na} \) in oocytes co-expressing a \( \beta \)-ENaC PY mutant,
β-Y618A, together with S1A. As described above and shown in Fig. 4, S1A inhibited wt ENaC current, which was reversed upon truncation of the β-ENaC C terminus. Amiloride-sensitive currents were approximately doubled in β-Y618A ENaC-expressing oocytes relative to wt ENaC controls (Fig. 4), as has been demonstrated previously for β- or γ-ENaC PY mutations (25). However, in contrast to the β-ENaC C terminus truncation, co-expression of S1A with β-Y618A ENaC resulted in a 76% inhibition of the I$_{Na}$ (Fig. 4). This value is quantitatively similar to the inhibition obtained for S1A co-expression with wt ENaC (Fig. 3), indicating that the PY motif itself is not involved in the functional interaction with S1A.

The H3 Domain Is Necessary for Inhibition of ENaC Current—The H3 domain of S1A has been shown to regulate other ion channels, and our results demonstrate that it interacts physically with the ENaC C terminus (Fig. 2). To test the hypothesis that the H3 domain interaction with ENaC regulates channel function, we co-expressed a S1A H3 deletion mutant (ΔH3) together with ENaC in oocytes and measured amiloride-sensitive I$_{Na}$. Fig. 5A shows that wt S1A significantly inhibited I$_{Na}$ whereas S1A ΔH3 had no effect. These results indicate that functional inhibition of ENaC requires the H3 domain of S1A. To verify this, we examined the effect of acutely injecting various S1A fusion proteins on I$_{Na}$; current recordings were obtained 1 h after injection. As shown in Fig. 5B, injection of GST alone had no effect on I$_{Na}$. However, injection of GST-H3 significantly attenuated the amiloride-sensitive I$_{Na}$; the inhibition obtained from acute injection of GST-H3 was quantitatively similar to that resulting from co-expression of full-length S1A. This acute effect of the H3 domain suggests that S1A is important in its regulation of ENaC activity.

H3 Reduces Cell Surface ENaC Expression—Previously (14), we found that co-expression of S1A reduced cell surface ENaC localization without changing total protein expression levels. This finding suggests that S1A may interfere with ENaC trafficking to the plasma membrane. In light of the fairly rapid effect of H3 domain injection on I$_{Na}$ shown above, we sought to determine whether this short-term action of GST-H3 also affected the amount of ENaC expressed at the cell surface; alternatively, H3 injection may decrease I$_{Na}$ by influencing channel gating. FLAG-tagged surface ENaC expression was assessed using an enzyme-linked luminescence assay developed previously (19) for cell surface K$^+$ channel expression; the results are summarized in Fig. 6. The cell surface signal from β-FLAG ENaC was about eight times the background level, and the

![Fig. 4. Mutation of the PY motif does not affect S1A inhibition of ENaC. Oocytes were injected with α-, β-, and γ-ENaC subunit cRNAs with or without S1A cRNA. Channels expressed with β C terminus mutants are designated by the single mutated subunit and were expressed with complementary wild-type subunits. Wt and β-R564X ENaC were used as positive and negative controls, respectively. Amiloride-sensitive Na$^+$ currents were measured as described for Fig. 3. Data are expressed as the fraction of amiloride-sensitive current relative to wt ENaC controls (I/I$_{wt}$), n = 13–15; N = 4 (see legend for Fig. 3).](Image)

![Fig. 5. The S1A H3 domain is required for inhibition of ENaC currents. A, oocytes were injected with α-, β-, and γ-ENaC subunit cRNAs with or without full-length S1A or S1A H3 deletion mutant (S1A ΔH3, S1A$_{ΔH3}$) cRNAs. Amiloride-sensitive Na$^+$ currents were measured as described for Fig. 3. Data are expressed as the fraction of amiloride-sensitive current relative to wild-type ENaC controls (I/I$_{wt}$) obtained for each animal, n = 12; N = 4. B, oocytes were injected with α-, β-, and γ-ENaC subunit cRNAs; expression proceeded for 18–36 h. 1 h prior to recording, ENaC-expressing oocytes were injected with GST-H3 or GST-ΔH3 fusion proteins or GST (estimated final concentration, 50 ng/μl; see Fig. 2 for definitions). Amiloride-sensitive Na$^+$ currents were measured as described for Fig. 3. Data are expressed as the fraction of amiloride-sensitive current relative to the wild-type ENaC controls (I/I$_{wt}$), n = 12; N = 4 (see legend for Fig. 3).](Image)
latter was determined using oocytes expressing wt ENaC lacking FLAG epitope. Injection of GST-H3 1 h prior to surface labeling reduced this signal by 70% (corrected for background), whereas prior injection of S1A fusion protein lacking the H3 domain had no effect. This reduction in surface labeling correlates with the 75% reduction in \( I_{Na} \) observed in similar H3 injection experiments (Fig. 5B) or when S1A was co-expressed with ENaC (Fig. 3). The data suggest that, even within this time frame, the primary action of the H3 domain is on expression of ENaC at the cell surface.

**The ENaC-S1A Interaction Is Salt-sensitive**—Increased cellular Na\(^+\) concentration elicits a decrease in ENaC-mediated apical membrane conductance in epithelial cells. This cellular protective mechanism balances apical Na\(^+\) entry with basolateral Na\(^+\) extrusion (1). To determine whether the S1A-ENaC interaction may be involved in feedback regulation of Na\(^+\) entry, we determined the effect of ambient Na\(^+\) and K\(^+\) concentrations in the binding buffer on the physical interaction between syntaxin and ENaC determined in *vitro*. As shown in Fig. 7, the binding of the \( \beta \)-ENaC C terminus to GST-S1A decreased with increasing Na\(^+\) concentration in the binding buffer, such that 100 mM Na\(^+\) virtually abolished this interaction. There was also a marked reduction in binding at 50 mM Na\(^+\). Increasing buffer K\(^+\) concentration also reduced the interaction of S1A with the \( \beta \)-ENaC C terminus, but elevated K\(^+\) was less disruptive than Na\(^+\) (Fig. 7, lower panel). Similar results were obtained for the interaction of S1A with the \( \alpha \)- and \( \gamma \)-ENaC C termini (data not shown). These data suggest thationic forces are involved in the ENaC C terminus interaction with S1A and that binding is particularly sensitive to ambient Na\(^+\).

**Ca\(^{2+}\) Sensitivity of S1A-ENaC Interactions**—Apical Na\(^+\) conductance is also inhibited when cellular free Ca\(^{2+}\) concentration rises (1). We evaluated the Ca\(^{2+}\) and ATP dependence of the ENaC-S1A interactions using *in vitro* pull-down assays performed in the presence of 0.1 or 10 \( \mu \)M free Ca\(^{2+}\) (EGTA-buffered). We also tested the effect of ATP on the S1A-ENaC interaction *in vitro*. The results in Fig. 8 show that 10 \( \mu \)M Ca\(^{2+}\) abolished the S1A-ENaC interaction. The presence of 2.5 mM ATP in the low Ca\(^{2+}\) binding buffer did not influence the interaction of ENaC with S1A.
The H3 domain of syntaxin was critical for this functional effect. Its deletion restored INa to values not significantly different from expression of wild-type ENaC alone, and acute injection of a GST-H3 fusion protein, but not a S1A GST-ΔH3 fusion protein, inhibited ENaC currents and cell surface ENaC to the same degree as S1A co-expression. Previous studies (14) showed that co-expression of a soluble S1A lacking the transmembrane domain was as inhibitory for ENaC currents as the full-length protein, in agreement with the inhibitory effect of the soluble H3 domain shown here. Increasing the Na⁺ or K⁺ concentrations of the binding buffer reduced ENaC-S1A binding, suggesting that electrostatic forces are involved in these interactions. Although the ENaC C terminus was the structural target for the inhibitory effect of S1A, a Liddle’s disease-related mutation in the C terminus PY motif did not obviate the S1A inhibition of ENaC current. This result, together with previous findings (14), has implications for the mechanism of S1A inhibition that will be discussed below.

The rate of Na⁺ entry across the apical membranes of absorptive epithelial cells is determined by the number and open probability of apical ENaC channels. Control over apical ENaC density is a key component in the regulatory actions of vasoconstriction that will be discussed below.

Previous studies of the functional effect of co-expressed syntaxin 1A on ENaC currents in Xenopus oocytes demonstrated an inhibition of amiloride-sensitive Na⁺ entry that could not be ascribed to a nonspecific effect on ENaC protein expression (14, 15). The study of Qi et al. (14), attributed the decrease in ENaC current to a reduction in the number of cell surface channels, detected by antibody labeling of non-permeabilized oocytes expressing extracellular FLAG-tagged ENaC subunits. Nevertheless, in a conceptually similar study Saxena et al. (15) found that co-expression of S1A increased cell surface ENaC. This result would require a proportionately greater decrease in channel open probability to override the apparent increase in channel number. However, the surface labeling conditions employed in these experiments are questionable. After fixation of the oocytes in 3% formaldehyde, they were labeled sequentially with primary and secondary antibodies, each for 1 h at 37 °C. Given these conditions, the blotchy ENaC staining detected at low magnification may reflect access of the antibody to intracellular epitope. In the experiments of Qi et al. (14), antibody labeling was performed without fixation or permeabilization at 4 °C, and the resulting ENaC staining pattern was uniform. In the present study, we used luminometry to detect ENaC at the cell surface. The S1A H3 domain suppressed ENaC INa by 75% approximately 1 h after its injection (Fig. 5), and this relatively rapid effect could be attributed to a quantitatively similar reduction in ENaC expression at the cell surface (Fig. 6).

Previous studies (7, 13) have presented evidence for a relatively rapid turnover of channel protein at the plasma membrane, and they have identified the mechanisms responsible for endocytic retrieval of ENaC. Studies in Xenopus oocytes examining the decay of INa following inhibition of ENaC traffic to the plasma membrane estimate the half-life of cell surface ENaC to be about 1 h. Accordingly, the 70% reduction in surface ENaC plasma membrane estimate the half-life of cell surface ENaC to be about 1 h. Accordingly, the 70% reduction in surface ENaC insertion, to about 1 h after its injection (Fig. 5), and this relatively rapid effect could be attributed to a quantitatively similar reduction in ENaC expression at the cell surface (Fig. 6).

Inferences regarding the mechanism whereby S1A would reduce ENaC surface expression can be made also from the ENaC mutation analysis. Studies of amiloride-sensitive, genetic hypertension (reviewed in Ref. 6) have implicated structures in the C termini of ENaC subunits in the control of plasma membrane channel number, perhaps by two mechanisms. First, the PPPXXXL motif within the subunit C termini may serve as an internalization or endocytic motif (13). Second, this motif participates in a physical interaction with the ubiquitin ligase, Nedd4–2, which binds to PY motifs in the ENaC C termini and decreases ENaC current by reducing cell surface channel number (7, 8). Nedd4-mediated ubiquitination of the ENaC N termini promotes channel internalization by endocytosis and its degradation in lysosomes (38).

Syntaxin 1A binds to the ENaC C termini, and its inhibition of ENaC current was eliminated by C terminus truncations.
These findings could be consistent with the concept that SIA promotes ENaC retrieval by a mechanism that depends on Nedd4-mediated ENaC endocytosis/degradation. Similar to C terminus truncations, mutants in the PY motif augment ENaC currents (see Figs. 3 and 4) by increasing cell surface ENaC; Nedd4 binding to ENaC is disrupted by C terminus truncation or by mutation of the PY motif (7, 25). However, a mutant that disrupts the PY motif in β-ENaC, Y618A, was strongly inhibited by SIA co-expression. The persistence of SIA inhibition in the PY mutant therefore suggests that the action of SIA is not related to Nedd4 binding or to the role of the PY motif in ENaC endocytosis, although we cannot formally rule out stimulation of an endocytic process by SIA that does not involve PY. Nevertheless, it seems unlikely that the influence of SIA on cell surface ENaC is because of stimulation of ENaC removal from the cell surface, because the effects of truncation and PY mutation would be expected to affect SIA inhibition similarly. It is more likely that the reduction in cell surface channel number detected here, and in the studies of Qi et al. (14), arises from inhibition of the insertion of ENaC channels into the plasma membrane.

Recent findings have implicated Nedd4 in the inhibition of Na\(^+\) entry that is associated with increased intracellular Na\(^+\) concentration (39). In addition, truncation of the ENaC C termini attenuates this feedback effect of intracellular Na\(^+\) (40). These findings led us to evaluate the influence of increased salt concentrations and Ca\(^{2+}\) on the physical interaction between S1A and the ENaC C termini. Increasing Na\(^+\) or K\(^+\) concentration of the binding buffer decreased this interaction, and Na\(^+\) was a more potent disruptor of SIA binding than K\(^+\), as would be expected from a Na\(^+\)-selective feedback event. However, inasmuch as the interaction of ENaC with expressed SIA is itself inhibitory, it seems difficult to infer a role for Na\(^+\)-mediated disruption of this interaction in the process of feedback inhibition. Disrupting an inhibitory interaction should increase Na\(^+\) transport. However, this reasoning assumes that the physiological action of endogenous SIA is inhibitory. Rather, if endogenous syntaxin 1A normally mediates apical ENaC insertion, or if it positively regulates trafficking reactions that deposit more ENaC in the plasma membrane, then a Na\(^+\)-induced disruption of the S1A-ENaC interaction could decrease cell surface ENaC and reduce Na\(^+\) currents. Similar conclusions would apply to the inhibition of S1A-ENaC binding observed at physiologically high Ca\(^{2+}\) concentrations (10\(^{-5}\) M), because increased intracellular Ca\(^{2+}\) is also inhibitory to ENaC currents (1). Our findings concerning the cation dependence of S1A interactions indicate that electrostatic forces are involved in their physical association. However, elucidating a potential role for SIA in ENaC regulatory effects that involve changes in cellular composition will require a better understanding of the physiological role of endogenous syntaxin 1A in regulating apical ENaC density.

How does the effect of overexpressed SIA relate to its physiological action on sodium transport? On one hand, the inhibition of ENaC current associated with SIA overexpression may result from disruption of normal SNARE-mediated ENaC trafficking mechanisms that are responsible for channel insertion into the plasma membrane. The influence of overexpressed syntaxins on specific trafficking pathways is commonly used to infer a physiological role for a specific syntaxin isoform in a specific trafficking pathway. For example, the selective inhibition of endoplasmic reticulum to Golgi traffic observed with exogenous syntaxin 5 expression reflects its physiological role as the t-SNARE in this step of protein secretory pathway traffic (36). Syntaxin 5 overexpression is sometimes used as an experimental means of interfering with vesicle-mediated protein transport between endoplasmic reticulum and Golgi (37). Other examples of selective syntaxin isoform inhibition are provided in Qi et al. (14). However, according to this view, the effect of exogenously expressed syntaxin would arise from disruption of the stoichiometric protein interactions necessary for membrane fusion, a general mechanism that should lack specificity for vesicle cargo, in this case, ENaC. Conversely, the protein binding studies and the elimination of SIA inhibition by ENaC submit truncation argue for a more selective phenomenon, related to the physical presence of ENaC in the trafficking vesicles. The present findings suggest that domain-specific interactions between the ENaC C-terminal cytoplasmic tails and S1A are involved in regulating plasma membrane channel number. It remains to be determined whether apical SIA is the principal t-SNARE that determines the insertion of ENaC containing vesicles into the epithelial cell apical membrane or whether it may compete with another syntaxin isoform that mediates the insertion of ENaC channels.

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