Interaction of Syntaxins with the Amiloride-sensitive Epithelial Sodium Channel

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Amiloride-sensitive sodium channels mediate sodium entry across the apical membrane of epithelial cells in variety of tissues. The rate of Na\(^+\) entry is controlled by the regulation of the epithelial sodium channel (ENaC) complex. Insertion/retention of the ENaC complex into the apical membrane as well as direct kinetic effects at the single channel level are recognized mechanisms of regulation. Recent data suggest that the syntaxin family of targeting proteins interact with and functionality regulate a number of ion channels and pumps. To evaluate the role of these proteins in regulating ENaC activity, we co-expressed rat ENaC cRNA (\(\alpha, \beta, \gamma\) subunits) with syntaxin 1A or 3 cRNAs in Xenopus oocytes. Basal ENaC currents were inhibited by syntaxin 1A and stimulated by syntaxin 3. Both syntaxin 1A and syntaxin 3 could be co-immunoprecipitated with ENaC subunit proteins, suggesting physical interaction. Interestingly, immunofluorescence data suggest that with either syntaxin isoform the ENaC-associated epitfluorescence on the oocyte surface is enhanced. These data indicate that (i) both syntaxin isoforms increase the net externalization of the ENaC channel complex, (ii) that the functional regulation is isofrom specific, and (iii) suggest that ENaC may be regulated through mechanisms involving protein-protein interactions.

The epithelial sodium channel (ENaC)\(^1\) provides the rate-limiting step in the absorption of sodium ions in the distal nephron and functions to maintain Na\(^+\) ion homeostasis (1, 2). Such channels have been characterized in amphibian skin and in mammalian tissues, including urinary bladder, renal collecting duct, distal colon, sweat and salivary glands, lung, and taste buds. They mediate the first step of active Na\(^+\) reabsorption and play a major role in the maintenance of electrolyte and water homeostasis. The ENaC complex is specifically blocked by the diuretic drug amiloride and its analogues (3), is composed of three homologous \(\alpha, \beta, \gamma\) subunits (4, 5), and is involved in the pathogenesis of human low renin, salt-sensitive hypertension. For example, Liddle syndrome is an autosomal dominant form of human hypertension in which the cytosolic region of either the \(\beta\) or \(\gamma\) subunit is truncated or altered by missense mutations in a critical PPPnY domain in their cytosolic carboxyl termini (6, 7).

\(\text{Na}^+\) entry through the apical membrane of epithelial cells is tightly regulated by several known mechanisms (8). These include changes in protein expression, the relative distribution of protein between intracellular pools and the apical membrane, and changes in unitary conductance properties of the channel. Mutations associated with Liddle syndrome lead to both an increase in channel density at the cell surface and an increase in open probability (9, 10). Channel density at the cell surface could be modulated by retrieval of the channel complex from the plasma membrane and subsequent degradation. The \(\alpha\) and \(\gamma\) subunits of ENaC are ubiquitinylated and hence targets for degradation through the lysosomal pathway (11). Clathrin-mediated endocytosis has also been implicated in the control of ENaC half-life and density of cell surface expression (12).

Another mechanism for ENaC regulation is through protein-protein interactions, although few data exist to date on this possibility. There is a direct interaction between PPPnY domains in the carboxyl termini of the ENaC subunits and the WW domain of the Nedd-4 protein (13, 14). Another family of proteins that could mediate such functional interactions is the syntaxins, plasma membrane localized t-SNARES that are hypothesized to mediate vesicle trafficking (15, 16). Several lines of evidence raise the possibility of an interaction between syntaxin and ENaC: (i) each member of the syntaxin family contains several domains predicted to form \(\alpha\)-helical coiled-coiled structures (16), regions likely to be involved in protein-protein interactions; (ii) several syntaxin isoforms (1A, 3, and 4) have been localized to the apical membrane in cultured epithelial cells (17) and principal cells of the rat cortical collecting tubule (18), and thus could be co-localized with ENaC; and (iii) syntaxin 1A has been shown to interact with and functionally regulate a number of ion channels including Cu\(^2+\) channels (19, 20), CFTR Cl\(^-\) channels (19–22), and GABA transporters (23). Syntaxins 3 and 4 have a high degree of homology with syntaxin 1A. The role of these synatxins in plasma membrane fusion reactions and regulation of ion channel activity has not yet been determined.

In the present report, we show that syntaxins 1A and 3 interact with and functionally regulate rat ENaC complexes when they are co-expressed in Xenopus oocytes. We found that these syntaxin isoforms enhance the surface expression of ENaC complexes but had opposite effects on ENaC function. Similar functional results were observed when syntaxin 1A was co-expressed with an ENaC complex containing a COOH-terminal truncated \(\beta\) subunit (as occurs in Liddle’s syndrome (7)). Co-immunoprecipitation studies demonstrated that syn-
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Effect of Syntaxins 1A and 3 on Amiloride-sensitive Currents—To test the hypothesis that syntaxins functionally regulate ENaC, amiloride-sensitive currents were assayed in *Xenopus* oocytes expressing ENaC with or without co-expression of syntaxin 1A and 3 (Fig. 1). At −100 mV, oocytes expressing blocked with 5% non-fat dry milk and probed with M2 monoclonal antibody. The blots were visualized using the enhanced chemiluminescence technique.

**Materials**—Rat ENaC α, β, and γ subunits with or without FLAG™ inserts were kind gifts from Dr. Bernard Rossier (University of Lausanne, Switzerland). The kit for cRNA preparation (mMessage mMachine™) was from Ambion (Austin, TX). Enhanced chemiluminescence kits for developing the Western blots was obtained from Amer sham Pharmacia Biotech. Goat anti-mouse IgG-alexa 488 was purchased from Molecular Probes (Eugene, OR). The M2 monoclonal anti-FLAG antibody was obtained from Eastman Kodak Co. Anti-syntaxin 3 antibody (24) was kindly provided by Dr. M. A. Knepper (National Institutes of Health). The anti-syntaxin 1A monoclonal antibody (HPC-1) and other antibodies and reagents were obtained from Sigma. ND96 buffer was used for oocyte incubation and contained in mM NaCl, 96; KCl, 2; CaCl2, 1.8; MgCl2, 2; HEPEs, 5; pH 7.4. RIPA buffer contained 20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5% deoxycholate, 1% Nonidet P-40, 5 mM EDTA, pH 8.0, and 0.5% Triton X-100. DIGNAM D buffer (20 mM HEPEs, pH 7.8, 100 mM KCl, 0.2 mM EDTA, and 0.5 mM sodium dodecyl sulfate) was used for digestion with the restriction enzymes. RIPA and DIGNAM D buffers also contain 1 mg/ml phenylmethylsulfonyl fluoride and 2 μg each of pepstatin, leupeptin, apro tinin, and soybean trypsin inhibitor with 0.2% Triton X-100. For statistical analyses, two-sample comparisons were performed using the Student’s t test.

**K100 Chimera**—The COOH terminus truncated chimeric β subunit (rat plus human) was constructed by replacing the COOH-terminal region of the full-length rat β subunit with human COOH-terminal sequences. Human β subunit COOH-terminal sequences were obtained by PCR amplification using cDNAs prepared from peripheral blood lymphocytes from an individual in the original Liddle’s kindred (6). PCR primers were designed so that the PCR products contained artificially introduced Xhol or AccI sites at the ends. The forward primer corresponded to nucleotides 1609–1631 (numbered according to GenBank™ data base, X77932) with the XhoI restriction site, and the reverse primer corresponded to nucleotides 1790–1814 with two nucleotide substitutions (GG to CA at 1807–1808) to create an AccI restriction site. The amplified PCR products were digested with XhoI and AccI and ligated into the full-length rat β subunit cDNA (pβENaC) in pSPORT. The Xhol site in pβENaC was created at the corresponding site of the human β subunit by PCR-directed mutagenesis, and an extra AccI site at nucleotide position 2 (numbered according to GenBank™, X77932) in pβENaC was removed by a restriction with SalI, followed by blunt-end ligation. The sequence of the K100 chimera was confirmed by restriction map analysis as well as DNA sequencing. Nucleotide sequences were determined using an ABI 373 automated DNA sequencer at the University of Alabama for Cited Research Core.

**RNA Transcription and Oocyte Injection**—Vectors containing ENaC and syntaxin inserts were linearized, and complimentary RNA templates (cRNA) were synthesized in vitro using the mMessage mMachine™ kit. For most experiments, 5 ng each of the rat ENaC subunit cRNAs (α, β, γ subunits) with either full-length syntaxin constructs (Syn3 or Syn1A) or syntaxin constructs lacking the transmembrane domain (Syn3Ac or SynIAC) in a 1:3 ratio. Amiloride-sensitive currents were determined as the difference in currents before and after the addition of 10 μM amiloride to oocytes at a holding potential of −100 mV. The data represent the mean of eight experiments carried out with different sets of cRNA preparations at different times under comparable conditions. Experimental conditions that resulted in a significant change (p < 0.05) from the relevant control values are denoted by an asterisk.

**Immunoprecipitation**—Oocytes were injected with ENaC cRNAs with or without syntaxin 1A or 3. For control assays, some oocytes were injected with wild-type rat α, β, γ cRNAs without inserted FLAG epitopes. Oocytes were homogenized in DIGNAM D buffer with a glass homogenizer on ice. Cell debris was removed by centrifugation, and the supernatant fraction was obtained. The lysates were incubated with anti-syntaxin antibody for 4–6 h. 50 μl of a 50% slurry of protein A-Sepharose beads was added to the sample, and the preparation was gently shaken for 2–4 h. The beads were then washed three times with DIGNAM D. The proteins were then eluted in Laemmli sample buffer and electrophoresed on 10% SDS-polyacrylamide gels and transferred to PVDF membrane in Towbin transfer buffer for 2 h. The PVDF membrane was then blocked with 5% non-fat dry milk and probed with M2 monoclonal antibody. The blots were visualized using the enhanced chemiluminescence technique.

**Immunofluorescence**—Oocytes were fixed in 3% formaldehyde in PBS, pH 7.4, for 30 min. Non-specific sites were blocked with 1% bovine serum albumin in PBS. The oocytes were then incubated with M2 monoclonal antibody directed against the FLAG epitope. The blots were visualized using the enhanced chemiluminescence technique.

**Digital Confocal Epifluorescence Microscopy**—Oocytes were optically sectioned on an Olympus IX70 inverted epifluorescence microscope equipped with step motor filter wheel assembly (Ludl Electronics Products Ltd., Hawthorne, NY) and filter set 83000 (Omega Optical, Brattleboro, VT). Images were captured with a SenSys cooled CCD, high resolution, monochromatic, digital camera (Photometrics, Tucson, AZ). Deconvolution of optical sections and merging of resultant sections were done with a PowerMac 9500/132 computer supplied with IP Lab Spectrum software (Scanalytics, Fairfax, VA) and Power Microtome software (VayTek, Inc., Fairfield, IA).

**RESULTS**

**Effect of Syntaxins 1A and 3 on Amiloride-sensitive Currents**—To test the hypothesis that syntaxins functionally regulate ENaC, amiloride-sensitive currents were assayed in *Xenopus* oocytes expressing ENaC with or without co-expression of syntaxin 1A and 3 (Fig. 1). At −100 mV, oocytes expressing
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that resulted in a significant change (p < 0.05) from the relevant control values are denoted by an asterisk.

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**FIG. 4.** Dose-dependent modulation of ENaC currents by syntaxins 3 or 1A. The regulation of ENaC activity by both syntaxins 3 and 1A is dose-dependent. 5 ng of ENaC (1.66 ng each of α, β, and γ subunit cRNA) was co-injected in Xenopus oocytes with increasing concentration of syntaxin cRNA (0, 5, 10, 15, 20, and 25 ng). A, the dose-dependent activation of ENaC currents with increasing amounts of injected syntaxin 3 cRNA. B, similar experiments were also conducted with syntaxin 1A. A decline in amiloride-sensitive currents was observed with increasing amount of syntaxin 1A full-length cRNA. The data are from three separate experiments. The numbers in the parentheses indicate the number of oocytes used. Experimental conditions that resulted in a significant change (p < 0.05) from the relevant control values are denoted by an asterisk.

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**FIG. 5.** Syntaxins co-immunoprecipitate with ENaC. Oocytes were injected with ENaC (E) cRNAs (rat α, β^{LAM}, γ^{LAM}), with or without syntaxin cRNA (syntaxin 3, S3, syntaxin 1A, S1A). For control assays, oocytes were injected with rat α, β, γ cRNAs without FLAG constructs. Oocytes were homogenized in DIGNAM D buffer, cell debris was removed, and the supernatant fraction was precipitated with antisyntaxin isoform-specific antibodies. The proteins were eluted in Laemmli sample buffer. A, β^{LAM} and γ^{LAM} ENaC subunits were visualized by Western blot using M2 monoclonal antibody, and the blots were developed with enhanced chemiluminescence. B, in another set of experiments, the proteins were first immunoprecipitated with the M2 monoclonal antibody, and the blots were developed with isoform-specific anti-syntaxin antibodies. Both proteins, syntaxin 3 (A) and 1A (B) could be detected in increased amounts by increasing the amount of their injected cRNA.

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**FIG. 6.** Syntaxins interact physically with ENaC. Oocytes were injected with ENaC cRNAs (5 ng comprising rat α, β^{LAM}, γ^{LAM}) with increasing amounts (5, 10, 15, 20, 25 ng) of syntaxin cRNA (syntaxin 3 or syntaxin 1A). Proteins were first immunoprecipitated with the M2 monoclonal anti-FLAG antibody, and the blots were developed with isoform-specific anti-syntaxin antibodies. Both proteins, syntaxin 3 (A) and 1A (B) could be detected in increased amounts by increasing the amount of their injected cRNA.

The principal findings of the studies include: (i) ENaC can be

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**FIG. 7.** Interaction of Syntaxins with the Amiloride-sensitive ENaC. The regulation of ENaC activity by both syntaxins 3 and 1A is dose-dependent. 5 ng of ENaC (1.66 ng each of α, β, and γ subunit cRNA) was co-injected in Xenopus oocytes with increasing concentration of syntaxin cRNA (0, 5, 10, 15, 20, and 25 ng). A, the dose-dependent activation of ENaC currents with increasing amounts of injected syntaxin 3 cRNA. B, similar experiments were also conducted with syntaxin 1A. A decline in amiloride-sensitive currents was observed with increasing amount of syntaxin 1A full-length cRNA. The data are from three separate experiments. The numbers in the parentheses indicate the number of oocytes used. Experimental conditions that resulted in a significant change (p < 0.05) from the relevant control values are denoted by an asterisk.

more bound syntaxin 1A or syntaxin 3 with ENaC (Fig. 6, A and B). These data suggest that ENaC and syntaxins physically associate in the oocyte expression system.

**Immunofluorescence**—Since the syntaxin family of membrane proteins has been implicated in membrane trafficking events, we next tested the hypothesis that the increase in amiloride-sensitive ENaC activity in the presence of syntaxin 3, and the decrease in amiloride-sensitive currents in the presence of syntaxin 1A was due to correlated changes in surface ENaC expression. We used FLAG epitope constructs (inserted into the extracellular loop of β and γ subunits) of ENaC, which we could label and detect on the oocyte surface by epifluorescence. As expected, we observed an increase in epifluorescence on the oocyte membrane surface of oocytes co-injected with syntaxin 3 (Fig. 7). Surprisingly, syntaxin 1A co-expression also caused an increase in surface epifluorescence, even though the functional activity of ENaC was reduced. The carboxy-terminal deletion constructs of syntaxins 1A and 3 did not affect surface labeling of ENaC complexes, confirming the importance of the carboxy-terminal membrane-spanning region of syntaxins for interactions with membrane transport proteins. These data demonstrate that both syntaxin isoforms increase the net outward trafficking of ENaC proteins. The fact that syntaxin 1A increased surface ENaC epifluorescence but decreased ENaC currents suggests that factors other than surface expression, per se, can regulate ENaC function.

**Total Cell ENaC Determination**—Since we observed increased ENaC surface labeling in oocytes co-injected with syntaxins, it was of interest to see if the syntaxins altered the steady-state level of total cell ENaC protein. To answer this question, we extracted total ENaC proteins from oocytes and performed Western blot analysis (Fig. 8). Our data indicate that the protein levels were unchanged in the oocytes preparations expressing ENaC with or without syntaxins 1A or 3. These results suggest that the syntaxins did not affect the ENaC half-life, or synthesis rate, and that the increased surface expression of ENaC observed with co-injection of syntaxins 1A or 3 was due to a redistribution of internal stores of the ENaC complex to the surface membrane.

**DISCUSSION**

We used the Xenopus oocyte expression system to explore the role of syntaxins in the expression of amiloride-sensitive ENaC. The principal findings of the studies include: (i) ENaC can be
Ion channel proteins are targeted to plasma membranes by the translocation of transport vesicles (25). Subsequent vesicular fusion increases channel density at the surface. Important questions remain about the mechanisms by which transport vesicles are targeted to a specific membrane domain, and docking and fusion are regulated. The SNARE hypothesis (26, 27), based on the regulated exocytosis of synaptic vesicles, has expanded this concept. Vesicles may be targeted by membrane-associated proteins that behave as vesicle-targeting receptors or SNAREs. These proteins are associated with vesicular membranes (v-SNAREs) or with the target membrane (t-SNAREs). Syntaxins are a group of t-SNAREs, which participate in intracellular trafficking pathways (26, 27).

By using the oocyte expression system, we have demonstrated that syntaxins 1A and 3 can modulate amiloride-sensitive currents associated with ENaC. Syntaxin 3 is a positive functional regulator, whereas syntaxin 1A acts as a negative regulator of ENaC activity. Both syntaxins physically interact with ENaC as demonstrated by the immunoprecipitation experiments. We were able to reciprocally co-immunoprecipitate ENaC proteins with syntaxin 1A and syntaxin 3 and also demonstrate that the functional interaction is critically dependent upon the carboxyl-terminal membrane-spanning regions of the syntaxins. Truncation of these regions abrogated the ability of syntaxins 1A and 3 to affect either the surface expression or the functional activity of the ENaC complex (Figs. 1 and 7), even though the carboxyl-terminal truncated constructs still co-immunoprecipitated with ENaC (Fig. 5B). This finding is consistent with previous studies, which demonstrated that the carboxyl-terminal membrane-spanning regions of the syntaxins are critical for their localization in the plasma membrane and for their targeting (tSNARE) function (19–23, 28). In addition, the co-immunoprecipitation with the ΔC syntaxin constructs demonstrates that the α-helical regions interact with the ENaC proteins, rather than the carboxyl-terminal domains.

The confocal microscopy data (Fig. 7) clearly demonstrate that ENaC density is increased at the oocyte surface when the ENaC subunits are co-injected with syntaxin 1A or syntaxin 3, thereby implying a role for these proteins in ENaC trafficking. The ΔC syntaxin constructs did not affect the ENaC surface expression. The opposite effects of syntaxins 3 and 1A on ENaC functional activity indicate a complex interaction between the ENaC complex and these syntaxins. We presume that additional components, either of the ENaC complex or other regulator factors, may play a crucial role in regulating ENaC functional activity. It appears likely that the pool of ENaC channels expressed at the membrane surface was labeled in our studies, since the confocal localization used a minor modification of the surface-labeling method described previously (9). Some of the labeled ENaC could be present in subapical vesicles that were unfused with the plasma membrane. Since docking and fusion are distinct functions, the syntaxin–ENaC protein interaction may trigger interactions with other proteins consistent with the large array of proteins associated with syntaxins in SNARE complexes. Interaction between syntaxins and ENaC channel proteins may also result in conformational changes as documented for SNAP-SNARE complex (29) or could affect the stability of interacting components in the ENaC complex once they are assembled and expressed in the surface membrane.

Syntaxin 3 has been cloned and described in various cell systems (16) and enhances CFTR activity when co-injected in the oocyte system (21). Since syntaxin 3 is expressed in principal cells of the collecting tubule (18, 30), it could play a role in the trafficking of ENaC and water channels in these cells. A6 cells (an amphibian renal cell line), which functionally express ENaC activity and are used to study its regulation (31), can also be labeled with syntaxin 3 antibody in our laboratory (data not shown). Syntaxin 3 has approximately 65% homology with...
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Syntxin 1A and also has α-helical domains. Recently, the H3 domain of syntxin 1A has been shown to interact with CFTR (22). A similar homologous H3 domain is present in syntxin 3. Our data demonstrate the physical and functional association of syntxin 3 with ENaC in the oocyte expression system. The presence of this syntxin isoform in native kidney (16, 18) supports the hypothesis that syntxin 3 could participate in the regulation of ENaC expression. In contrast to cultured epithelial cells where syntxin 3 is apically expressed (17, 32), syntxin 3 appears to be localized at the basolateral membrane of principal cells (18), while ENaC is primarily expressed in the apical membrane. Of note, syntxin 4 is clearly expressed at the apical membrane of principal cells of the collecting tubule (33). The potential interactions of syntxin 4 with ENaC have not yet been defined.

ENaC regulates Na+ reabsorption (1) and maintains electrolyte balance. Several mutations in the ENaC subunits cause Liddle’s syndrome, with abnormal regulation of blood pressure and electrolyte balance (1, 6, 7). These mutations increase ENaC functional activity and surface expression (9, 24). Therefore, identification of the mechanisms involved in the regulation of ENaC activity is critical for our understanding of the pathogenesis of human salt-sensitive hypertension. Truncation of the carboxyl terminus of the ENaC β subunit were the originally described mutations in Liddle’s syndrome (6). When such a construct (K100) is co-expressed with wild-type α and γ ENaC subunits, and syntxins 1A, a similar inhibitory effect was observed as with the wild-type ENaC constructs, demonstrating that the functional interaction between syntxin 1A and ENaC does not involve the carboxyl terminus of the β subunit. Since syntxin 1A can still down-regulate the enhanced ENaC activity associated with the K100 construct, it is possible that some sort of protein–protein interaction may provide a therapeutic approach to regulating ENaC activity in human hypertension.

In conclusion, ENaC physically and functionally interacts with at least two syntxins, each of which differentially affect ENaC function. These interactions suggest a role for syntxins in the trafficking and functional regulation of ENaC complexes and may also directly affect channel function as has been described for N-type calcium channel (20) and CFTR (20–22). In view of the variety of syntxin-associated proteins that have been described, it will be important to determine the specific nature of the syntxin interactions with the ENaC complex.

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