The Activity of the Epithelial Sodium Channel Is Regulated by Clathrin-mediated Endocytosis

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Activity of the epithelial sodium channel (ENaC) is a key determinant of sodium homeostasis and blood pressure. Liddle's syndrome, an inherited form of hypertension, is caused by mutations that delete or alter PY domains in the carboxyl termini of β or γ ENaC subunits, leading to increased channel activity. In this study we investigated the mechanism of this effect by analysis of wild-type and mutant ENaC activity in Xenopus oocytes. By inhibiting insertion of new channels into the plasma membrane with brefeldin A, we demonstrate that the half-life of the activity of channels containing Liddle's mutations is markedly prolonged compared with wild-type channels (t½ of 30 h in mutant versus 3.6 h in wild-type, p < 0.001). We investigated the involvement of clathrin-coated pit-mediated endocytosis by co-expressing a dominant-negative dynamin mutant with wild-type ENaC in oocytes. Expression of this specific inhibitor of endocytosis leads to a large increase in the activity of wild-type channels, demonstrating that normal turnover of this channel is through the clathrin-coated pit pathway. In contrast, co-expression of Liddle's mutations and dynamin mutants leads to no further increase in channel activity, consistent with one of the effects of Liddle's mutations being the loss of endocytosis of these channels. These findings demonstrate the normal mechanism of turnover of ENaC from the cell surface and demonstrate a mechanism that can account for the increased number of channels in the plasma membrane seen in Liddle's syndrome.

Expression of the amiloride-sensitive epithelial sodium channel (ENaC) in the plasma membrane determines the sodium permeability of many epithelia involved in sodium absorption. The channel is regulated by hormones such as aldosterone, insulin, and antidiuretic hormone that increase its activity by diverse mechanisms including increase in channel synthesis, incorporation of channels from an intracellular pool to the plasma membrane, and induction of post-translational modifications of the channel proteins (1). There are fewer known mechanisms that decrease sodium permeability in cells expressing ENaC. Recently, there have been several studies that implicate the carboxyl terminus of the channel subunits in processes that normally down-regulate its activity. Mutations in the human genes of the β and γ subunits cause a form of salt-sensitive hypertension known as Liddle's syndrome (2, 3). These mutations introduce frameshifts or premature stop codons that delete the intracellular carboxyl-terminal domains of the β or γ subunits, or they substitute residues in a proline-rich motif present in the carboxyl termini of both subunits (4, 5). Expression of ENaC channels with truncated β or γ subunits in Xenopus oocytes induces amiloride-sensitive whole-cell currents 3-5-fold larger in magnitude than wild-type channels (6). Two mechanisms have been proposed to explain the increase in sodium permeability induced by Liddle's mutations: an increase in the number of channels expressed at the cell surface, and an increase in the open probability (7–9).

In this work, we sought to determine the mechanism that regulates the expression of channels at the cell surface and to investigate the role played by the carboxyl terminus in this process. As in other studies (5–8), we have used Xenopus oocytes to express ENaC and have measured whole-cell amiloride-sensitive sodium currents. We demonstrate that channels with carboxyl-terminal truncations of the β and γ subunits have significantly longer half-life at the plasma membrane than wild-type channels. This difference is due to a very slow rate of clathrin-mediated endocytosis of the truncated channels and was reproduced by single amino acid mutations βY618A and γY628A but not by αY673A. These results indicate that the tyrosine residues present in the proline-rich motifs of the β and γ subunits encode signals recognized by the endocytic machinery and that mutations or deletions of these signals result in accumulation of channels at the plasma membrane due to reduced retrieval. Since these same residues are mutated or deleted in Liddle's syndrome, the results also explain the mechanism that increases the number of cell-surface channels in Liddle's mutants.

MATERIALS AND METHODS

Measurement of Whole-cell Currents with the Two-electrode Voltage-clamp (TEVC) Technique—Oocyte whole-cell currents were measured using the two-electrode voltage clamp technique (Oocyte Clamp C-725B, Warner Instrument Corp.). Epithelial sodium channel currents were calculated as the difference in whole-cell current before and after the addition of 10 μM amiloride to the bathing solution at a holding membrane potential of –100 mV. The bath solution contained (in millimoles/liter): 100 Na as the gluconate salt, 1.8 CaCl₂, 2 MgCl₂, 4 KCl, 5 BaCl₂, 5 HEPES, final pH 7.4. Results are expressed as the mean ± standard error (S.E.); n (number of observations) was equal or greater than 10 for each given value.

Expression of ENaC and Dynamin in Oocytes—Stage V–VI oocytes were isolated, treated with collagenase, and injected with approximately 5 ng of each cRNA, either wild-type or mutant ENaC subunits alone or co-injected with wild-type or mutant dynamin, in a final volume of 50 nl. Oocytes were kept in modified Barth's solution (MBS) (in millimoles/liter: 85 NaCl, 2.4 NaHCO₃, 1 KCl, 0.8 MgSO₄, 0.7 CaCl₂, and 5 HEPES, final pH 7.4) at 19 °C until experiments were performed. To prevent sodium loading and to extend the viability of oocytes by several days, oocytes were kept in MBS solution containing 1 μM amilo-
ride prior to experiments. DNAs were propagated in the transcription competent vector pSF15 in Escherichia coli DH5α. Capped cRNAs were transcribed in vitro from linearized cDNAs with SP6 RNA polymerase according to the instructions for the mMESSAGEmACHINE kit from Ambion (Austin, TX). For the time-course experiments, whole-cell currents were measured every 12 h after cRNA injection for up to 120 h. In experiments where the effect of brefeldin A (BFA) was examined, oocytes were voltage-clamped 2 days after injection, when the current reached a steady state, and every 4 h after the addition of BFA. Oocytes co-injected with ENaC and dynamin were also measured 2 days after cRNA injection.

Construction of Mutants—The cDNAs of the carboxyl-terminal truncations of β and γ subunits were constructed by the introduction of premature stop codons at amino acid positions β564 and γ566, respectively, using polymerase chain reaction. Point mutations of tyrosine for alanine residues were generated by polymerase chain reaction as follows: αY617A, βY618A, and γY628A, according to the protocol described by Nelson et al., (10). All constructs were sequenced at the Yule facility using an ABI 373 automated DNA sequencer employing dye terminators following a standard protocol supplied by the manufacturer (Applied Biosystems).

RESULTS

Time Course of the Amiloride-sensitive Whole-cell Current of Wild-type and Truncated ENaC Channels—We have shown previously that injection of cRNA from the rat ENaC subunits into Xenopus oocytes generates amiloride-sensitive whole-cell sodium currents that exhibit all the functional properties of wild-type channels (12). We examined the time course of the expression of amiloride-sensitive whole-cell currents in oocytes injected with wild-type α, β, and γ subunits or with wild-type α in combination with truncated β and γ using the two-electrode voltage-clamp technique as described in methods. Fig. 1 shows the time course of the amiloride-sensitive current expressed by oocytes injected with all three wild-type subunits or wild-type α with truncated βR564Stop and γQ566Stop subunits. The magnitude of current of wild-type channels increased in the first 2 days, and after a plateau it started to decline. Although the length of the plateau phase was variable depending on the batch of oocytes, it was consistently followed by a decrease in current. Either inactivation of channels or a change in the ratio of delivery and retrieval to the cell surface could account for the decrease in current observed several days after injection of cRNA. Oocytes expressing wild-type α and truncated β and γ subunits exhibited 3–5-fold larger magnitude of amiloride-sensitive currents than wild-type channels as has been shown previously (6). The time course of the current of truncated channels followed a pattern different from wild-type channels; the plateau phase extended for several days with little evidence of decrement after injection. Substitution of the truncated β and γ subunits for subunits carrying point mutations βY618A and γY628A gave similar results. Mutation of the corresponding tyrosine in the α subunit Y673A did not have a significant effect in the total magnitude or in the time course of the amiloride-sensitive current (data not shown). The time-course experiments are consistent with previous reports, showing an increase in the magnitude of the amiloride-sensitive current with truncated β and γ subunits, but more importantly they show that the behavior of the current over the time of observation is significantly different between wild-type and truncated channels. These results suggest either that truncated channels are more efficiently delivered to the plasma membrane or, alternatively, that there is a process that reduces the activity of the wild-type but not of truncated channels in the plasma membrane.

The Half-life of ENaC Activity Is Prolonged in Liddle’s Mutations—To determine whether the effect of Liddle’s mutations is increased delivery or decreased removal from the cell surface, we inhibited delivery of new channels to the plasma membrane by adding BFA. BFA is a fungal metabolite that inhibits the secretory pathway by blocking anterograde vesicular transport from the endoplasmic reticulum and causes redistribution of the cisternae of the Golgi complex into the endoplasmic reticulum (13). Although BFA has multiple targets in vesicular transport, it does not affect clathrin-mediated endocytosis in the plasma membrane (14). It has been shown previously that BFA is active in Xenopus oocytes and that it effectively inhibits protein traffic in these cells (15). Prevention of insertion of new channels into the plasma membrane permits us to directly examine the decay of the amiloride-sensitive current with the TEVC. Fig. 2 (A and B) shows the effect induced by 5 μM BFA on amiloride-sensitive whole-cell currents of oocytes injected with wild-type or truncated channels. BFA was added to the incubation medium (arrow pointing down) 24–36 h after cRNA injection when the current was in the plateau phase of the time course. After the addition of BFA, the amiloride-sensitive current of oocytes expressing wild-type channels progressively decreased over 24 h, with a time course well described by a single exponential fitted with a first-order rate constant k of 0.228 that corresponds to a half-life of 3.6 h (Fig. 2C). To verify that the decrease in current was a specific effect of BFA on vesicular traffic and not simple due to a toxic effect on cells, BFA was removed after 8 h (arrow pointing up) and the incubation was continued for another 12 h in MBS alone. Oocytes recovered amiloride-sensitive current to levels similar to those of untreated cells, demonstrating that the effect of BFA was specific and fully reversible upon removal of the drug. In contrast, BFA had very little effect on the magnitude of the amiloride-sensitive current of oocytes expressing the truncated form of the channel (Fig. 2A); we observed only a 15% reduction in current after 20 h of treatment. The data did not fit well to a single exponential but better to a linear decay, which gave a calculated half-life of approximately 30 h, much longer than the value obtained for wild-type channels (p < 0.001). The results show that the activity of wild-type channels decreases much faster than that of truncated channels. This observation is independent of delivery of new channels to the plasma membrane and, thus, rules out the possibilities of increased RNA stability, increased rate of protein synthesis, or increased rate of delivery of newly synthesized channels to the cell surface.

Wild-type ENaC Channels Are Removed from the Plasma Membrane by Endocytosis—The decrease in current of oocytes expressing wild-type channels can be attributed either to progressive inactivation or to removal of channels from the cell surface. We therefore examined whether the decrease in the activity of wild-type channels is caused by internalization of
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channels from the plasma membrane via clathrin-mediated endocytosis.

Dynamin is a large molecular weight GTP-binding protein with high intrinsic GTPase activity that is required for clathrin-mediated endocytosis (16). Dynamin assembles into rings around the neck of coated pits when GTP is bound inducing fission and release of vesicles upon hydrolysis of the GTP (17). Mutations that disrupt the GTP-binding domain such as lysine 44 of dynamin (DK44) lead to inhibition of clathrin-mediated endocytosis (18), producing a dominant negative phenotype because assembly with wild-type dynamin arrests its function.

To specifically block the process of endocytosis, we co-injected cRNAs of ENaC with either wild-type dynamin or with the dynamin mutant DK44. Two days after injection, the amiloride-sensitive current was measured with the TEVC. Oocytes co-injected with wild-type ENaC and wild-type dynamin expressed currents of similar magnitude to oocytes injected only with ENaC (Fig. 3A). This result is consistent with previous reports that showed that an excess of wild-type dynamin does not make endocytosis a more efficient process (18). In contrast, oocytes injected with wild-type ENaC and DK44 had a 3-fold increase in current (p < 0.01 compared with oocytes expressing wild-type ENaC and dynamin). Neither wild-type dynamin nor DK44 co-expressed with truncated channels had an effect on the magnitude of current (Fig. 3B). These results indicate that wild-type channels are normally removed from the plasma membrane by clathrin-mediated endocytosis and that the signals recognized by the endocytic machinery are encoded in the carboxyl termini of the β and γ subunits.

To prove that the endocytic signal is encoded by the tyrosine residues in the proline-rich motifs of β and γ, dynamin wild-type or DK44 were co-injected with αβγγ channels. Channels carrying mutations of the tyrosine residues in the β and γ subunits did not increase amiloride-sensitive currents when co-injected with DK44 (Fig. 3C). In contrast, mutation of the tyrosine in the proline-rich domain of the α subunit had little effect on the magnitude of currents, and channels with this mutation responded to DK44 with a significant increase in current (Fig. 3D) (p < 0.01). These data indicate that Tyr-673 in the α subunit does not play an important role in endocytosis in contrast to the corresponding residues in β and γ.

The number of subunits and the stoichiometry of mature channels have not yet been elucidated. However, since both β and γ subunits have endocytic signals, it is expected to find at least two, if not more, of these signals per channel molecule. To investigate whether the endocytic signals produce an additive effect in the recruitment of channels into clathrin-coated pits, DK44 was co-injected with αβγγ, αβγγ, and αβγγ channels. Channels with βγ or γγ exhibited approximately 5-fold more current than wild-type channels (30.08 and 28.7 μA/oocyte, respectively compared with 5.08 μA/oocyte in wild-type), and channels with both βγ and γγ exhibited even more current (57.7 μA/oocyte) (Table I). Co-injection of DK44 induced a significant increase in current expressed by αβγγ, αβγγ, and αβγγ channels (p < 0.01), but it did not have an effect on oocytes expressing αβγγ channels. From these results, we conclude that the endocytic signals in the β and γ subunits have an additive effect in the removal of channels from the plasma membrane. Inasmuch as the number of subunits in ENaC is still unknown, we cannot say how many signals are necessary to recruit channels into clathrin-coated pits. However, the data support the notion that, at least in proteins formed by association of many subunits, more than one signal renders their endocytosis more efficient.

Fig. 2. Effect of BFA on the magnitude of the amiloride-sensitive current of oocytes injected ENaC. Two days after cRNA injection, oocytes were divided into a control group (open symbols) and BFA treated group indicated by the arrow pointing down (filled symbols). The amiloride-sensitive current was then measured at 2-h intervals. After 12 h of BFA treatment, half of the oocytes were transferred to control medium, indicated by the arrow pointing up, and the current was measured after 12 h. A, with wild-type ENaC; B, truncated form of ENaC; C, data points from wild-type channels were fitted to a single exponential term by linear least-squares regression analysis (r = 0.99). Each point represents the mean of 20 oocytes; error bars represent the S.E.
FIG. 3. Effect of wild-type and DK44 mutant dynamin on amiloride-sensitive whole-cell currents. A, oocytes injected with wild-type ENaC and wild-type or DK44 dynamin. B, oocytes injected with wild-type α, truncated β and γ subunits, and with wild-type or DK44 dynamin. C, oocytes injected with wild-type α, βY618A, and γY628A, and with wild-type or DK44 dynamin. D, oocytes injected with αY673A, wild-type β, and γ subunits in addition to wild-type or mutant dynamin. Each column represents the mean of at least 12 oocytes ± S.E. (*, p < 0.001).

**Table I**

Effect of mutations of tyrosines in the proline-rich motifs of the β and γ subunits and of DK44 in the magnitude of amiloride-sensitive currents

Oocytes were co-injected with wild-type channels or with combinations of mutant β or γ subunits carrying substitutions of the tyrosines in the proline-rich motifs for alanine residues and with or without DK44. I represents the mean of n individual oocytes. p is given for the conditions ± DK44. NS, not significant.

|          | αβγ | αβγ + DK44 | αβγ | αβγγ + DK44 | αβγγ | αβγγγ + DK44 | αβγγγ | αβγγγγ + DK44 |
|----------|-----|-----------|-----|-------------|------|--------------|-------|--------------|
| I (µA/oocyte) | 5.08 | 22.99 | 30.84 | 43.91 | 28.7 | 36.32 | 57.72 | 54.11 |
| S.E.     | 0.88 | 2.40 | 3.40 | 3.93 | 2.92 | 5.21 | 5.05 | 6.27 |
| n        | 12  | 11  | 13  | 9   | 12  | 10  | 14  | 11  |
| p        | <0.001 | <0.01 | <0.01 | NS  |

**DISCUSSION**

We have demonstrated that one of the mechanisms for the increase of sodium current seen in channels bearing Liddle’s mutations is a prolonged half-life at the cell surface due to a defect in clathrin-mediated endocytosis, and that Tyr-618 in β and Tyr-628 in γ subunits are necessary for internalization of wild-type channels. The ability of oocytes expressing truncated channels to maintain high levels of channel activity 5 days after injection in contrast to the rapid decay of the wild-type channels demonstrates that, in a time-dependent fashion, the defect of truncated channels is in the pathway leading to the removal from the cell surface. The internalization signals of many receptors that are removed from the plasma membrane via clathrin-coated pits are short linear arrays of four amino acids that contain a tyrosine residue conforming to the sequence PPRYNSL, respectively, induce turns according to the predicted secondary structure of the proteins (21). Substitutions by alanine residues induce loss of the turns in the carboxyl terminus of β and γ as is shown in Fig. 4. Although the α subunit sequence PPAYATL also has a tyrosine, the mutant αY673A neither increases the magnitude of amiloride-sensitive current nor alters the predicted secondary structure of the carboxyl terminus. This indicates that the carboxyl terminus of α does not contain an internalization signal, consistent with the absence of mutations in the α subunit in patients with Liddle’s syndrome. In all three subunits, the tyrosine residues are shared by two adjacent endocytic signals, NPXY and YXXL. Amino acids distal to the tyrosine have been examined previously by alanine mutagenesis with only minor functional effects (22), indicating that the preferred endocytic signals are the ones containing the proline residues.

The rate of decay of the amiloride-sensitive current in oocytes expressing wild-type channels corresponds to a half-life of 3.6 h. This value does not strictly represent the rate of endocytosis, as recycling of endosomes back to the plasma membrane may result in underestimation of the rate of endocytosis (16). The percentage of ENaC that is internalized from the plasma membrane and delivered directly to the lysosomes is not known. For other proteins, the percentage varies greatly; it can be as low as 3% for transferrin and low density lipoprotein and as high as 90% for the epidermal growth factor receptor, where internalization constitutes an important mechanism for down-regulation of receptors. In contrast to wild-type channels, the rate of decrease of amiloride-sensitive current in the truncated form is approximately 8–10-fold slower than for wild-type. It can be argued that truncated channels are normally endocytosed but the lack of a degradation signal allows them to evade targeting to the lysosomes and endocytic vesicles containing truncated channels could be recycled back to the plasma mem-
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brane. Such a degradation signal could be ubiquitination-mediated by Nedd4, a putative ubiquitin ligase that interacts with the PY motifs of the channel, as has been proposed recently (22–24).

The finding that wild-type channels are normally removed from the cell surface by clathrin-mediated endocytosis suggests that this may constitute a physiological mechanism used by cells to decrease the sodium permeability of the plasma membrane. In addition, it demonstrates that one of the underlying cells to decrease the sodium permeability of the plasma membrane. Such a degradation signal could be ubiquitination-mediated by Nedd4, a putative ubiquitin ligase that interacts with the endog-

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REFERENCES

1. Eaton, D. C., Becchetti, A., Ma, H., and Ling, B. N. (1995) Kidney Int. 48, 941–949
2. Shimkets, R. A., Warnock, D. G., Bostia, C. M., Nelson-Williams, C., Hansson, J. H., Schambelan, M., Gill, J. R., Jr., Ulick, S., Milerova, R. V., Fingland, J. W., Canessa, C. M., Rossier, B. C., and Lifton, R. P. (1994) Cell 79, 407–414
3. Hansson, J. H., Nelson-Williams, C., Suzuki, H., Schild, L., Shimkets, R., Lu, Y., Canessa, C., Iwasaki, T., Rossier, B., and Lifton, R. P. (1995a) Nat. Genet. 11, 76–82
4. Hansson, J. H., Schild, L., Gauetschi, I., Lu, Y., Wilso, T., Shimkets, R., Nelson-Williams, C., Canessa, C., Rossier, B. C, and Lifton, R. P. (1995b) Proc. Natl. Acad. Sci. U. S. A. 92, 11345–11349
5. Tamura, H., Schild, L., Enomoto, N., Matsu, N., Marumo, F., Rossier, B. C, and Sasai, S. (1996) J. Clin. Invest. 97, 1780–1784
6. Schild, L., Canessa, C. M., Shimkets, R. A., Gauetschi, I., Lifton, R. P., and Rossier, R. C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 56696–56703
7. Snyder, P. M., Price, M. P., McDonald, P. J., Adams, C. M., Volk, K. V., Keizer, B. G., Stokes, J. B., and Welsh, M. J. (1995) Cell 83, 969–978
8. Firsov, D., Schild, L., Gauetschi, I., Merillat, A.-M., Schneeberger, E., and Rossier, B. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 15370–15375
9. Ismailov, I. I., Berdiev, B. K., Fullen, C. M., Bradford, A. L., Lifton, R. P., Warnock, D. G., Bubien, J. K., and Benos, D. J. (1996) Am. J. Physiol. 270, C214–C233
10. Nelson, R. M., and Long, G. L. (1989) Anal. Biochem. 180, 147–151
11. Dur, C. Farman, N., Canessa, C. M., Bonvalet, J.-P., and Rossier, B. C. (1994) J. Cell Biol. 127, 1907–1921
12. Canessa, C. M., Schild, L., Buell, G., Thorens, B., Gautschi, I., Horisberger, J.-D., and Rossier, B. C. (1994) Nature 376, 463–467
13. Pelham, H. R. B. (1991) Cell 67, 449–451
14. Wood, S. A., Park, J. E., and Brown, W. J. (1991) Cell 67, 591–600
15. Mulner-Lorillon, O., Belle, R., Cormier, P., Drewing, S., Minella, O., Poulhe, R., and Schmalzing, G. (1995) Dev. Biol. 170, 223–229
16. Jun-Ping, L., and Robinson, P. J. (1995) Endocr. Rev. 16, 590–608
17. Takei, K., McPherson, P. S., Semid, S. L., and De Camilli, P. (1995) Nature 374, 186–190
18. Danke, H., Baba, T., Warnock, D. E., and Schmid, S. L. (1994) J. Cell Biol. 127, 915–934
19. Towbridge, I. S., and Collawn, J. F. (1993) Annu. Rev. Cell Biol. 9, 129–161
20. Rapoport, L., Miyazaki, M., Boll, W., Duckworth, B., Cantley, L. C., Shoelson, S., and Kirchhausen, T. (1997) EMBO J. 16, 2240–2250
21. Chou, P. Y., and Fasman, G. D. (1978) Adv. Enzymol. 47, 45–148
22. Schmid, S. L., Warnock, D. G., Schambelan, M., Moss, L. M., Bell, L., and Schmalzing, G. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11495–11499
23. Takei, K., McPherson, P. S., Semid, S. L., and De Camilli, P. (1995) Nature 374, 186–190
24. Sudol, M. (1996) Prog. Biophys. Mol. Biol. 65, 113–132