REVIEW ARTICLE

Regulation of the epithelial sodium channel by accessory proteins

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The epithelial sodium channel (ENaC) is of fundamental importance in the control of sodium fluxes in epithelial cells. Modulation of sodium reabsorption through the distal nephron ENaC is an important component in the overall control of sodium balance, blood volume and thereby of blood pressure. This is clearly demonstrated by rare genetic disorders of sodium-channel activity (Liddle's syndrome and pseudohypoaldosteronism type 1), associated with contrasting effects on blood pressure. The mineralocorticoid aldosterone is a well-established modulator of sodium-channel activity. Considerable insight has now been gained into the intracellular signalling pathways linking aldosterone-mediated changes in gene transcription with changes in ion transport. Activating pathways include aldosterone-induced proteins and especially the serum- and glucocorticoid-inducible kinase (SGK) and the small G-protein, K-Ras 2A. Targeting of the ENaC for endocytosis and degradation is now emerging as a major mechanism for the down-regulation of

channel activity. Several proteins acting in concert are an intrinsic part of this process but Nedd4 (neural precursor cell expressed developmentally down-regulated 4) is of central importance. Other mechanisms known to interact with ENaC and affect sodium transport include channel-activating protease 1 (CAP-1), a membrane-anchored protein, and the cystic fibrosis transmembrane regulator. The implications of research on accessory factors controlling ENaC activity are wide-ranging. Understanding cellular mechanisms controlling ENaC activity may provide a more detailed insight not only of ion-channel abnormalities in cystic fibrosis but also of the link between abnormal renal sodium transport and essential hypertension.

Key words: aldosterone, channel-activating protease, cystic fibrosis, hypertension, Nedd4, serum- and glucocorticoid-inducible kinase (SGK).

INTRODUCTION

Sodium is the major ion of the extracellular fluid, and total body sodium is an important determinant of the extracellular fluid volume. The kidney of land vertebrates and the mechanisms regulating salt balance tend to be geared towards retaining salt and water in the body in an attempt to preserve the extracellular fluid volume and maintain adequate blood circulation. Increased generation of the adrenal hormone aldosterone is a major mechanism in the preservation of salt and water in response to sodium depletion. However, in the presence of a sustained large salt intake, body fluid volumes do not increase indefinitely; normally, urinary sodium excretion is increased to match the higher sodium intake, thereby preventing overt and dangerous expansion of the extracellular fluid volume. Modulation of renal sodium excretion is a key process in the maintenance of sodium balance.

The epithelial sodium channel (ENaC) in the renal distal nephron is an important component in the overall control of sodium balance. Although it only accounts for a relatively small proportion of the sodium re-absorbed (< 5%), it nevertheless constitutes the rate-limiting step for renal sodium reabsorption and therefore is of fundamental importance in the control of blood volume and thereby of blood pressure. Indeed, the significance of ENaC in the control of sodium balance and blood pressure is clearly demonstrated by rare genetic disorders of sodium-channel activity such as pseudohypoaldosteronism type 1

(PHA-1) and Liddle's syndrome, with contrasting effects on blood pressure.

The ENaC is also relevant in the regulation of sodium transport in other epithelia, such as the alveolar epithelium, distal colon, salivary duct and sweat glands [1].

The activity of the sodium channel responds to a wide variety of hormones and changes in physiological conditions. Channel activity can be modulated by several hormones and in particular aldosterone, antidiuretic hormone, insulin and atrial natriuretic peptide [1,2]. A number of diverse mechanisms, including variation in channel synthesis, regulation of intracellular channel trafficking and membrane insertion, are important in controlling channel activity. Post-translational modifications that alter channel-open probability or increase channel endocytosis are also important. Much progress has now been made on the elucidation of the intracellular events that link changes in sodium ion transport to changes in hormonal and physiological conditions. This review discusses recently identified accessory proteins in the context of the established hormonal regulation of channel activity, identifying regions of overlap between the regulatory pathways and hence opportunity for cross-talk between the mechanisms.

STRUCTURAL CHARACTERISTICS OF ENaC

At the structural level, ENaC is composed of three partly homologous subunits, α , β and γ , which are inserted into the membrane with a proposed stoichiometry of $2\alpha:1\beta:1\gamma$ [2] (see

Abbreviations used: CAP-1, channel-activating protease 1; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane regulator; CNrasGEF, cyclic nucleotide ras GEF; ENaC, epithelial sodium channel; GEF, guanine nucleotide-exchange factor; Hect, homologous to E6-AP C-terminal; hNedd, human Nedd; mCAP-1, murine CAP-1; Nedd4, neural precursor cell expressed developmentally down-regulated 4; NHE, sodium/hydrogen exchanger; PHA-1, pseudohypoaldosteronism type 1; PI3-K, phosphoinositide 3-kinase; PKA, protein kinase A; PKC, protein kinase C; SGK, serum- and glucocorticoid-inducible kinase; SNARE, soluble N-ethylmaleimide-sensitive factor attachment-protein receptor; xCAP-1, *Xenopus* CAP-1.

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Figure 1 Structure of ENaC and membrane topology

ENaC is composed of three partly homologous subunits, α , β and γ , which are inserted into the membrane with a proposed stoichiometry of $2\alpha:1\beta:1\gamma$. Small amiloride-sensitive sodium currents can be produced by the α subunit alone, but co-expression with β and γ ENaC subunits greatly augments the current to a level similar to that of the native channel. Reprinted from Rossier et al. [2] with permission from the *Annual Review of Physiology*, volume 64, ©2002 by Annual Reviews; <http://www.AnnualReviews.org>

Figure 1). A nonameric structure consisting of three of each subunit has also been proposed [3]. Small amiloride-sensitive sodium currents can be produced by the α subunit alone, but co-expression with β and γ subunits of ENaC greatly augmented this current to a level similar to that of the native channel [4].

The nucleotide sequences of the subunits predict a molecular mass of 70–80 kDa although post-transcriptional modification of the α subunit results in a mass of 92 kDa [5]. Each subunit of the ENaC has four distinct domains: the cytoplasmic N-terminus, the large extracellular loop, the two short hydrophobic segments and the cytoplasmic C-terminus.

The three genes encoding the sodium-channel subunits are located on separate chromosomes. The gene encoding ENaC α subunit (GenBank accession no. SCNN1A) is located on chromosome 12p13.3 and is comprised of 13 exons spanning 17 kb [6,7]. Exon 1 and part of exon 2 constitute the 5'-untranslated region, with parts of exons 2 and 13 encoding the transmembrane regions of the ENaC α subunit; the remaining exons code for the extracellular loop [7]. The genes for the β (GenBank accession no. SCNN1B) and the γ (SCNN1G) subunits are located within 400 kb of each other on chromosome 16p12–p13. The ENaC β subunit contains nine consensus sites for N-glycosylation, and two consensus sites for phosphorylation by protein kinase C (PKC). The ENaC γ subunit is 649 amino acid residues long, and its extracellular loop contains five sites for N-glycosylation and two for phosphorylation by PKC. β and γ ENaC both contain a C-terminal Src homology 3 ('SH3')-binding domain [8]. All three subunits are arranged in a barrel-state formation and contribute to the formation of the channel pore. The β and γ subunits are also responsible for determining the half-life of the channel and the numbers of functional channels at the surface of the membrane [9].

A PY motif found within the intracellular C-termini of all three subunits is of particular interest. This sequence is conserved between rat ENaC, *Xenopus* ENaC and human ENaC, whereas amino acids downstream of this sequence differ. The PY motif consists of the amino acids Pro-Pro-Pro-Xaa-Tyr. The tyrosine residues present in the proline-rich motifs of β and γ subunits of ENaC are recognized by the endocytotic machinery [10], and this provides a mechanism for increased retrieval from the plasma membrane. PY motifs are also consensus sites for protein–protein interactions. Both of these mechanisms appear to be important in the regulation of ENaC activity.

PATHOLOGICAL SIGNIFICANCE OF MUTATIONS WITHIN THE ENaC GENES

The importance of the renal ENaC in the regulation of sodium balance is clearly demonstrated by (i) the identification of rare disorders of renal sodium handling linked with mutations in ENaC genes in humans and (ii) investigation of transgenic animals with deletion or modification of ENaC subunits *in vivo*. ENaC also plays a fundamental role in fluid exchange in the lung and, in particular, facilitates the transition of the lung from an aqueous fetal stage to an air-conducting system at birth [11]. Transgenic mouse models with dysfunction in the regulation of ENaC activity demonstrate not only the kidney salt-wasting phenotype, but also that ENaC was essential for lung-fluid clearance in newborn mice. Disruption of the α subunit resulted in a complete abolition of ENaC-mediated Na^+ transport and this was associated with neonatal death. Such an essential role of the α subunit for lung-fluid clearance is supported by the demonstration that knockout of the β or γ subunit had only minor effects on lung-fluid clearance [12].

Figure 2 Schematic representation of domain organization of human and mouse isoforms of Nedd4

WW domains are tryptophan-based motifs of 38 amino acid residues that bind to PY motifs. The interaction between the WW domains of Nedd4 and PY motifs of ENaC is an important mechanism for targeting channels for endocytosis/proteolysis.

Comparison of phenotype expression observed with inactivation of each of the three subunits suggests that the α subunit is also essential for the normal function of the renal ENaC. Indeed, transgenic mice with deletion of the three subunits displayed a salt-wasting state similar to the rare human syndrome PHA-1 [12].

Paradoxically, patients homozygous for loss-of-function, disease-causing mutations within the ENaC α subunit with PHA-1 show little evidence of apparent lung disease. It is not clear whether this is simply a reflection of differences in the relative importance of ENaC in lung-fluid clearance between species or whether the mutated ENaC α subunit could retain sufficient residual activity to produce a small Na^+ current when assembled with the β and γ subunits.

PHA-1 is an inherited disease presenting soon after birth. The presence of hyperkalaemia, hyponatraemia and metabolic acidosis suggest hypoaldosteronism, but in fact plasma aldosterone levels are unusually high. Two modes of inheritance have been described. The autosomal dominant renal form is caused by mutations in the gene coding for the mineralocorticoid receptor, whereas the autosomal recessive, systemic form is caused by mutations in all three genes encoding ENaC subunits [12]. Both forms are associated with failure to retain sodium and thereby are associated with low blood pressure.

On the other hand, Liddle's syndrome, a rare form of hypertension, usually inherited in an autosomal dominant manner, is attributed to gain-of-function mutations in the β and γ subunits of the channel, with all mutations discovered so far affecting the C-termini of the subunits [13]. However, several sporadic cases of Liddle's syndrome have also arisen from *de novo* mutations [14–16].

The original Liddle kindred was Caucasian, but causal mutations have now been identified within Japanese [15] and African-Americans [16]. All mutations identified to date affect the last 30

amino acids of the intracellular C-termini and cause the premature truncation or alteration of amino acid structure within this region, leading to a phenotype of early-onset hypertension accompanied by hypokalaemic alkalosis, suppressed plasma renin activity and low plasma aldosterone [17].

The absence of adequate down-regulation of ENaC in patients with Liddle's syndrome implicates the C-termini of the subunits. Additionally, the presence of consensus sequences for protein–protein interactions within these termini led to a search for regulatory proteins that may bind to the channel, possibly through PY motifs, and to the identification of Nedd4 (neuronal precursor cells expressed developmentally down-regulated gene 4) as an important regulator of ENaC activity [18].

REGULATION OF ENaC ACTIVITY BY Nedd4

Nedd4 is one of 10 novel genes that were identified by Kumar et al. [19] using a subtraction cloning approach. Nedd4 was present as a highly expressed transcript in the mouse embryonic brain, but this expression is down-regulated during development. It was subsequently established that lower levels of Nedd4 are also expressed in adult tissue and cultured cells. Within the kidney, Nedd4 has been co-localized by immunoprecipitation to the same epithelial cells as ENaC, being expressed in the principal cells (but not in intercalating cells) and outer medullary collecting duct of the distal nephron [20].

At least two Nedd4 proteins have now been identified in the mouse (mNedd4-1 and mNedd4-2), which behave differently in terms of ENaC regulation [21]. These proteins both have homologues in the human, hNedd4-1 and hNedd4-2, which are encoded by the genes KIAA0093 and KIAA0439 respectively. The human Nedd4 (hNedd4-1) is located on chromosome 15q21.1 and consists of 29 exons encoding a C2 domain, four WW domains and a ubiquitin-ligase domain (Hect, which stands for

homologous to E6-AP C-terminal; see below) Figure 2 shows a schematic representation of these domains. The deduced polypeptide sequence of Nedd4 shares 62% sequence identity with that of hNedd4-2 (also known as Nedd4-L), a second isoform located on chromosome 18q21. Multiple transcripts of Nedd4-2 have been isolated, differing in their transcription start and termination sites together with the presence or absence of an alternatively spliced exon. The human Nedd4-2, and Nedd4-La, an alternatively spliced transcript, contain four WW domains and a Hect domain; however, the hNedd4-2 protein does not appear to contain a C2 domain.

Nedd4 C2 domain

The C2 domain consists of approx. 130 residues, and was first discovered as the Ca^{2+} -binding site in PKCs. Within PKC, the C2 domain functions in the Ca^{2+} - and phospholipid-mediated membrane translocation of the protein [22], potentially providing a mechanism for the inhibition of ENaC by increased cytosolic Ca^{2+} , although there is no direct evidence supporting a role for Nedd4 in this inhibition. The exact role of the C2 domain remains unclear. The localization of Nedd4 to the plasma membrane may be mediated by the interaction between the C2 domain and annexin XIIIb, a protein found in apical rafts [23]. The presence of this domain, however, does not appear to be essential for effective inhibition of sodium-channel activity.

Patch clamp experiments using the *Xenopus laevis* expression system demonstrated that removal of the C2 domain of hNedd4-1 led to an increase in the ability of the isoform to down-regulate sodium-channel numbers at the cell surface [21], suggesting that the C2 domain appears to be detrimental in terms of the ability of Nedd4 to down-regulate ENaC. Moreover, hNedd4-2a, which naturally lacks the C2 domain, was used to demonstrate that this domain was not needed to mediate binding to ENaC in either the *Xenopus* oocyte expression system or Fischer rat thyroid epithelia [24].

Additionally, separate experiments have tested the effects of adding a C2 domain to the hNedd4-2a isoform. This did not alter the ability of Nedd4-2a to bind to and down-regulate the sodium channel [21].

Hect domain

The Hect domain is so-called because of its sequence similarity to the C-terminus of E6-AP, a 100 kDa cellular protein. This acts as a ubiquitin-protein ligase and is involved in the degradation of p53 through a ubiquitination pathway; this domain is the catalytic domain of all E3 enzymes [25].

WW DOMAINS AND BINDING OF Nedd4 TO ENaC

WW domains are tryptophan-based motifs of 38 amino acid residues that bind to PY motifs. The interaction between the WW domains of Nedd4 and PY motifs of ENaC is an essential for the subsequent inhibition of ENaC activity [26–28].

Internal fluorescence has identified interactions between the WW domains of Nedd4 and the PY motifs of human β and γ ENaC subunits. As the WW domains are crucial for recognition of binding motifs in target proteins, it is surprising to discover that the number of WW domains is not conserved throughout the species studied: there are three in mice and rats and four in humans. The variable number of WW domains within each isoform has therefore raised questions as to which domains are important for interactions with ENaC.

WW domains 2–4 each bind to α , β and γ ENaC subunits *in vitro*; these interactions can be abolished by the systematic

WW Domain 1

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RatNedd4 : SGLPPGWEERQDVLGRITYYVNHESRTTQWRPSPEDDL : 38
hNedd4-1 : SGLPPGWEERQDVLGRITYYVNHESRTTQWRPSPEDDL : 38
hNedd4-2a : LPPGWEERQDVLGRITYYVNHESRTTQWRPSL----- : 32

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WW Domain 2

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RatNedd4 : SGLPPGWEERQDGRGRSYVDHNSKTTTWSKPTMQDDPR : 39
hNedd4-1 : SGLPPGWEERQDGRGRSYVDHNSKTTTWSKPTMQDDPR : 38
hNedd4-2a : LPPGWEERQDGRGRSYVDHNSKTTTWSKPTMQDDPR : 30

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WW Domain 3

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RatNedd4 : -----SKIPAH : 6
hNedd4-1 : GPLPKGWEVRHAPNGRPFIDHNKTTWEDPRLEKIPA : 38
hNedd4-2a : LPPGWEERQDGRGRSYVDHNSKTTWEDPRLEKIPA : 30

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WW Domain 4

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RatNedd4 : SGLPPGWEERTHTDGRVFFINHNKKTQWEDPRMQNVA : 38
hNedd4-1 : SGLPPGWEERTHTDGRVFFINHNKKTQWEDPRMQNVA : 38
hNedd4-2a : LPPGWEERTHTDGRVFFINHNKKTQWEDPRMQNVA : 30

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Figure 3 Comparison of amino acid sequences of rat and human Nedd4 WW domains

Sequences were compared using GeneDoc software [85] (<http://www.psc.edu/biomed/genedoc/>). Asterisks represent every tenth residue.

mutation of two residues in each of the WW domains. WW3 is both necessary and sufficient for the binding of hNedd4 to ENaC, as supported by the finding that inhibition of the channel is nearly abolished when WW3 is mutated. However, WW domains 2 and 4 are also important for the down-regulation of ENaC, as mutation of these domains decreases inhibition [28]. Thus WW domains 2, 3 and 4 are all needed for the efficient binding to and inhibition of ENaC activity.

Despite 86.8% homology over the 38 amino acids comprising Nedd4 WW1 domains between the rat and the human forms, unlike the rat, the hNedd4 WW1 domain is unable to bind to β and γ ENaC subunits [29]. This could be due to specific differences in amino acid sequences of the WW domains affecting the physicochemical properties of the binding sites within Nedd4 (the amino acid sequences of rat and human Nedd4 WW domains are shown in Figure 3). That differences in the properties of amino acids contribute to the altered binding affinities of the WW domains is highlighted by investigation of the WW1 domain in response to specific amino acid changes. The native human WW1 domain does not appear to bind to the ENaC subunit but recent work has demonstrated that binding affinity can be substantially increased by a single amino acid change within the WW1 domain (Arg to Thr). This substitution was sufficient to enable binding to the α subunit and accounts, to some extent at least, for the differences in binding affinities between the rat and the human WW1 domains [28].

Recently, the solution structure of rat Nedd4 complexed to a peptide corresponding to the PY motif-containing region of the β subunit of ENaC (β P2; see Figure 4) was resolved, highlighting important issues regarding the interactions between these two structures [29].

Several amino acids have been identified as being crucial for the interaction, including Tyr-618 and Leu-621 of the C-terminal sequence Tyr-Ser-Asp-Leu of the ENaC β subunit (see Figure 4).

C Terminal residues of rat β ENaC involved in the interaction with Nedd4

618 621

APYTGPPPTVAELVEAHTNFGFQPD⁶¹⁸TTSCRPN⁶¹⁹AEVYPDQQT⁶²⁰**TLPIPGT⁶²¹PPPNYDSLRLQ**

PLDTMESDSEVEAI

rNedd4 WW3 residues involved in the interaction with β ENaC

TLPVLLPTSSGLPPGWEEKQDDGRGRSYVDHNSKTTTWSKPTMQDDPRSKIPAHLRGK

TPVDSNDLGLPPGWEERTHTDGRVFFINHN⁶¹⁸IKKTQWEDPRMQNVAITGPAEPYSRDY

Figure 4 Structural features and amino acid sequences of an ENaC β -subunit-derived peptide complexed to the rat WW domain 3 peptide

Top panel: the NMR-derived three-dimensional structure of the peptide β P2, derived from the rat β ENaC subunit, complexed to WW domain 3 of rat Nedd4 (from PDB code 1I5H). Bottom panel: in the upper sequence, residues within β P2 are in bold and underlined. In the lower sequence, the underlined residues are involved in the interaction between WW3 and the peptide representing the C-terminal residues of β ENaC. The rat WW3 sequence shown here, named according to Kanelis et al. [29], corresponds to the rat WW4 sequence in Figure 3.

This sequence lies immediately downstream of the recognized PY motif. Typically, proline-rich peptides complexed to WW domains bind in an extended conformation and this had previously been thought to be the case for ENaC. However, within β P2, a peptide containing the PY motif of r β ENaC (β subunit of rat ENaC), this sequence forms a helical turn (Figure 4). Thus it was proposed that the PY motif be extended to encompass the amino acids Pro-*Pro-Pro*-Xaa-Tyr-Asp-Ser-*Leu*, where proline, tyrosine and leucine (in italics) are in contact with the WW domain.

ENaC UBIQUITINATION AND CLATHRIN-MEDIATED ENDOCYTOSIS

The ubiquitination-based pathway is an important mechanism for degradation of intracellular proteins. Following ubiquitination, most cytoplasmic proteins are degraded by the 26 S proteasome, a cytosolic threonine protease complex. However,

some transmembrane proteins are also ubiquitinated, which targets them for endocytosis and degradation by the endosomal/lysosomal pathway. The key features of the ubiquitination process are summarized in Figure 5 (for comprehensive reviews, see [30–32]).

The PY motif–WW interaction brings the ubiquitin-protein ligase Hect domain into close proximity with the channel, leading to channel ubiquitination, endocytosis and lysosomal/proteosomal degradation [33]. The importance of this domain for the regulation of ENaC is demonstrated by experiments in which Nedd4 with a catalytically inactive Hect domain stimulates channel activity, probably by competing with endogenous Nedd4 in the *Xenopus* oocyte expression system [34]. Therefore ubiquitination involving the Hect domain is implicated in the regulation of numbers of channels at the cell surface. It appears, in this case, that there is an increase in both cell-surface retention or recycling of ENaC and incorporation of new channels at the plasma

Figure 5 Protein degradation by the ubiquitin system

Ubiquitin (Ub) is a small protein which, when complexed to a target protein, either singly or as part of a polyubiquitin chain, tags that protein for a variety of cellular fates, including degradation by the lysosome/proteasome. Polyubiquitinated proteins (those with a chain consisting of at least four ubiquitin molecules) are degraded by the 26 S proteasome, a complex of proteases. Monoubiquitination regulates processes as diverse as membrane transport, transcriptional regulation and virus budding (see [30–32] for reviews). The ubiquitination of target proteins involves a multi-enzyme cascade, requiring three sequentially acting enzymes.

membrane [35]. Both the proteasome and the lysosomal/endosomal degradation system could be important and it has been proposed that whereas the unassembled subunits are degraded by the proteasome, the assembled $\alpha\beta\gamma$ ENaC complex is targeted for lysosomal degradation [36,37].

Conserved lysine residues, such as those found in the N-terminal regions of all three ENaC subunits, are the target sequences for ubiquitination and polyubiquitination. Mutation of the N-terminal lysines to arginine in human α and γ ENaC subunits, both individually and in combination, results in reduced ubiquitination, slower turnover and increased sodium-channel activity due to the retention of an increased number of mutant channels at the plasma membrane. These effects were less pronounced when the N-terminal lysines of human β ENaC subunit were systematically mutated and expressed with α or γ ENaC subunits, indicating that the β ENaC subunit is not ubiquitinated *in vivo* [36].

Interestingly, the sequence Tyr-Ser-Asp-Leu within the C-terminal region of β ENaC also conforms to the consensus

sequence recognized by the endocytic machinery, particularly the AP-2 adapter complex [38]. This motif is then used to sort proteins from the plasma membrane to the endosome, via clathrin-mediated endocytosis. The AP-2 complex itself can recognize the tyrosine-based motif within the C-termini of the channel subunits. Although this interaction is weak, it could be strengthened by the involvement of clathrin. If this is the case, clathrin-mediated endocytosis could be either alternative or complementary to Nedd4-mediated inhibition of ENaC, as proposed by Shimkets et al. [39].

In support of ENaC activity being modulated, at least partly, by clathrin-mediated endocytosis, channels with C-terminal truncations of β and γ ENaC subunits have longer half-lives at the membrane than wild-type channels. By co-expression with a dominant-negative dynamin mutant, an inhibitor of endocytosis, it was demonstrated that this increase in half-life was due to defective internalization. Systematic mutagenesis revealed that β Tyr-618 and γ Tyr-628, residues within the β and γ subunits of ENaC respectively, were recognized by the endocytic machinery.

When β Tyr-618 is mutated to alanine, the interaction between Nedd4 and ENaC is abrogated [13]. However, as this mutation disturbs both the PY motif and the sequence Tyr-Ser-Asp-Leu, the sequence recognized by the endocytic machinery, it is unclear whether any effects on the sodium channel are due to defective internalization by the endocytic machinery or defective internalization of the channel via Nedd4 and ubiquitination.

The regulation of ENaC by Nedd4 and subsequent ubiquitination and clathrin-mediated endocytosis are not mutually exclusive. One possibility is that endocytic proteins containing a ubiquitin-interacting motif ('UIM') might bind to the ubiquitinated tails of membrane proteins such as ENaC [40]. The endocytic proteins may then interact with components of the clathrin coat and so function as adaptors between ubiquitinated proteins and further components downstream in the endocytotic pathway.

Shimkets et al. [39] argue that truncated channels containing Liddle's mutations are endocytosed, but the lack of a degradation signal means they are not targeted to the lysosome and the endocytic vesicles are recycled back to the plasma membrane. Degradation signals could be mediated by Nedd4.

Further evidence for the involvement of Nedd4 comes from the fact that, according to the modelling prediction by Shimkets et al. [39], the Pro-Pro-Pro-Xaa-Tyr-Xaa-Xaa-Leu sequence of ENaC forms a tight turn, necessary for recognition by the endocytic machinery. However, as discussed above, NMR has demonstrated that this secondary structure is induced only when complexed to the WW domain of Nedd4 [29]. Therefore a possible mechanism could be that Nedd4 binds to the PY motif of the β ENaC subunit and induces the tight turn that is needed for recognition and subsequent clathrin-mediated endocytosis. Ubiquitination may somehow be involved in this pathway, or could constitute an entirely separate means of ENaC degradation. Alternatively, Nedd4 could bind to the sodium channel, ubiquitinate it and then be released, thereby freeing the PY motif and allowing it to be recognized by the AP-2 complex, a component of the endocytotic machinery. Further investigation into the exact mechanism is needed, especially in light of the recent NMR resolution of the structure of ENaC in complex with Nedd4, as discussed above.

REGULATION OF ENaC ACTIVITY BY THE SERUM- AND GLUCOCORTICOID-INDUCIBLE KINASE (SGK) AND OTHER ALDOSTERONE-INDUCED PROTEINS

It has long been known that the stimulatory actions of aldosterone on sodium reabsorption in renal distal tubular cells are mediated by intracellular mineralocorticoid receptors and the subsequent changes in transcription and translation of target genes. An increase in the apical ENaC and, to some extent, an increase in basolateral Na^+/K^+ -ATPase activity, is of fundamental importance in accounting for the increase in sodium reabsorption [41]. Recent research now provides a detailed insight into the intracellular events leading to an increase in ENaC activity in response to aldosterone.

The response of the sodium channel to aldosterone treatment can be divided into three phases; the latent phase, lasting approx. 45 min, during which there is no discernible effect on channel activity, the early phase (45 min–3 h) and the late phase (from several hours to several days post-treatment) [42].

Although the late phase is associated with distinct increases in mRNA of the α ENaC subunit, during the early phase the reabsorption of salt and water precedes the accumulation of ENaC mRNA. This suggests that aldosterone induces or represses expression of genes involved in cell-surface insertion or

single-channel properties of ENaC. Several such aldosterone induced mRNAs have been identified. A promising candidate for the regulation of ENaC is SGK.

SGK1 (Genbank accession no. XM_037046) has been mapped to chromosome 6q23. The 2343 bp mRNA encodes a putative 431 amino acid protein with a predicted molecular mass of 49 kDa. Two further isoforms, SGK2 and SGK3, located on chromosomes 20 and 18, respectively, have been identified. Sequence comparisons show that SGK is conserved across species, with the predicted amino acid sequences sharing 96–98 % homology between each other. Only SGK1 appears to be relevant to the control of ENaC activity. SGK1 mRNA and protein is induced early on (within 30 min) through a direct effect of aldosterone on gene expression [43,44].

In order to test the function of SGK at the cellular level, it was co-expressed with all three ENaC subunits in the *Xenopus* oocyte expression system. After a 36 h incubation period, the oocytes co-injected with ENaC and SGK displayed a 2-fold increase in amiloride-sensitive sodium current compared with the oocytes expressing ENaC alone ($9.78 \pm 0.9 \mu\text{A}$ compared with $4.42 \pm 0.66 \mu\text{A}$) [45].

In the same study, a 3-fold increase in the abundance of ENaC in the plasma membrane was observed in the presence of SGK compared with the control, indicating that SGK probably increases the activity of the sodium channel by increasing the abundance of ENaCs in the plasma membrane rather than by an increase in the open probability or a change in the kinetics of ENaC.

SGK AS AN INTEGRATOR OF THE INSULIN-, MINERALOCORTICOID- AND Nedd4-MEDIATED REGULATION OF ENaC

SGK was originally identified in rat mammary epithelial cells as a serum- and glucocorticoid-regulated gene, its closest relative being protein kinase B. Protein kinase B is an integral part of the insulin signalling pathway, its ability to phosphorylate targets being controlled by phosphoinositide 3-kinase (PI3-K). When recruited, PI3-K is localized to the plasma membrane, where it catalyses the production of 3-phosphorylated inositide lipids such as $\text{PtdIns}(3,4,5)\text{P}_3$. $\text{PtdIns}(3,4,5)\text{P}_3$ associates with and activates 3-phosphoinositide-dependent kinases 1 and 2 (PDK1 and PDK2), which in turn activate SGK in a $\text{PtdIns}(3,4,5)\text{P}_3$ -dependent manner [46].

These observations suggest that whereas mineralocorticoids control the abundance of SGK through changes in gene expression, the activity of SGK can also be modulated by the PI3-K pathway at the enzyme level [47]. Thus both mineralocorticoids and PI3-K must be present for high levels of sodium transport to occur. A postulated scheme whereby SGK could be viewed as an integrator of the effects of insulin and aldosterone on ENaC activity is summarized in Figure 6.

This model is supported by the recent observation that insulin was able to activate SGK activity in human embryonic kidney fibroblasts [46]. Moreover, it has been demonstrated that PI3-K inhibitors inhibited the early action of aldosterone and phosphorylation of SGK1 [48]. Whether these mechanisms are physiologically relevant in the control of sodium balance during changes in sodium intake is not known. In particular, further work will be required to understand the role of insulin in the control of sodium balance under normal conditions.

Because aldosterone induces phosphorylation of serine and threonine residues within the C-termini of β and γ ENaC subunits by an unidentified kinase [49], and because the hormone

Figure 6 Hypothetical scheme for SGK as a means of integrating the insulin-, aldosterone- and Nedd4-mediated regulation of ENaC activity

See the text for further details.

also increases the expression of SGK, it seemed likely that SGK could be implicated in this control pathway. Several investigations have addressed more closely the interactions between SGK and ENaC at the molecular level. *In vitro*-translated SGK was able to bind α and β ENaC–glutathione S-transferase fusion proteins expressed in *Escherichia coli*. This occurred with both the phosphorylated (active) and non-phosphorylated forms of SGK.

Mutations of tyrosine residues within the ENaC sequences necessary for endocytosis and degradation of the sodium channel, discussed above, did not affect the response to SGK. Nor did a point mutation in the β ENaC subunit, known to impair the down-regulation of the channel by Nedd4, namely the substitution of Tyr-618 with Ala, have a significant effect on the response to SGK when expressed in *Xenopus* oocytes [50], indicating that this amino acid is not crucial in mediating the response of the channel to SGK.

Recent research does not support the view that ENaC is a direct substrate for SGK but points to the possibility that SGK may also bind to and phosphorylate ENaC regulatory proteins. The presence of a PY motif in SGK suggested that SGK may bind to Nedd4; this is supported by the recent observation that SGK phosphorylated hNedd4-2 (but not hNedd4-1), an interaction which reduced the binding of hNedd4-2 to the α ENaC subunit [50]. The subsequent reduction in ENaC ubiquitination provides a mechanism for increased ENaC density at the plasma membrane and thus for an increase in ENaC-dependent sodium reabsorption.

Although the exact downstream sequence of events remains to be fully clarified, the potential importance of SGK in the control of sodium balance is further supported by the recent observation on *sgk1*-gene-knockout mice. These showed that in *sgk1*-knock-out (*sgk1*^{-/-}) mice and wild-type (*sgk1*^{+/+}) mice, when NaCl intake was standardized, renal water and electrolyte excretion were indistinguishable. By contrast, dietary NaCl restriction

reveals an impaired ability of *sgk1*^{-/-} mice to adequately decrease Na⁺ excretion despite increases in plasma aldosterone levels. These animals also displayed a decrease in blood pressure and glomerular filtration rate suggesting the presence of a salt-wasting syndrome [51].

K-Ras 2

The Ras superfamily is a large group of small GTP-binding proteins that function as switches in numerous molecular pathways. Some of the cellular processes controlled by Ras proteins include proliferation, differentiation and apoptosis. Since Ras was first identified over 20 years ago, over 100 related proteins have been identified and studied [52].

One such protein is XK-Ras 2, the *Xenopus* orthologue of the mammalian KRas 2A. K-Ras 2A itself is a splice variant of K-Ras 2, with the C-terminus containing a lysine-rich stretch, in contrast to the palmitoylation site found in K-Ras. Through the use of differential display PCR, Spindler and colleagues [53] first identified XK-Ras 2A as one of four adrenal steroid up-regulated RNAs ('ASURs'). The biosynthesis of Ras proteins was increased approx. 6-fold in A6 epithelia 2.5 h after the addition of aldosterone. The K-Ras 2 mRNA is also regulated *in vivo*, as demonstrated using the *Xenopus* kidney, after being exposed to aldosterone for the same period of time [54].

Functional studies have demonstrated that K-Ras 2A possesses a dual effect on the activity of ENaC. First, it induces the maturation of *Xenopus* oocytes, the expression system most widely used for functional studies of ENaC. Associated with the maturation of the oocyte there is a reduction in the number of channels at the surface [54]. However, K-Ras 2 also increases the activity of those channels already present at the membrane. To overcome this problem, these studies utilized a K-Ras 2A mutant that was constitutively active (XK-Ras 2A^{G12V}), and the effects on the channel activity were distinguished from those induced by the maturation of the channel by the use of progesterone treatment, which encourages the maturation of oocytes by a separate pathway from that utilized by K-Ras 2 [54].

Small GTP-binding proteins, such as K-Ras, are activated by GTP binding, and are inactivated by the hydrolysis of GTP to GDP. These processes are influenced by several proteins, including guanine nucleotide-exchange factors (GEFs) [55]. GEFs catalyse the release of bound GDP and the capture of new nucleotides, usually GTP. One such GEF has recently been demonstrated to bind Nedd4 [56]. Cyclic nucleotide Ras GEF (CNrasGEF) was identified as a substrate for Nedd4 in an expression library screen of 16-day-old mouse embryo using WW domain 2 of Nedd4 as a probe. Like ENaC, CNrasGEF contains PY domains which are essential for the binding to Nedd4. Nedd4 binds to CNrasGEF, ubiquitinating it, thereby tagging it for subsequent degradation by the proteasome. It is therefore possible that the association of Nedd4 with CNrasGEF may provide a mechanism connecting ENaC to the Ras pathway.

INVOLVEMENT OF THE SOLUBLE N-ETHYLMALIMIDE-SENSITIVE FACTOR ATTACHMENT-PROTEIN RECEPTOR (SNARE) MACHINERY IN THE HORMONAL REGULATION OF ENaC

Components of the SNARE machinery, particularly syntaxin 1A, were initially discovered for their role in synaptic vesicle exocytosis, but syntaxins have since been shown to interact with and functionally regulate a number of ion channels, including the amiloride-sensitive sodium channel. They may be involved in the regulation of ENaC by the hormones aldosterone, vasopressin and insulin.

Interactions with SNARE proteins govern the insertion and retrieval of membrane vesicles containing secretory products or integral membrane proteins such as ENaC [57]. Intracellular vesicles transport their cargo to the cell membrane through the binding of vesicular-membrane associated SNAREs ('v-SNAREs') to target membrane-associated SNAREs ('t-SNAREs'), including members of the syntaxin family, at the cell membrane.

Syntaxins also contain several domains predicted to form α -helical structures, regions likely to be involved in protein-protein interactions. Taken together, these observations support the involvement of syntaxins in ENaC trafficking. This is further supported by the finding that several syntaxin isoforms, including syntaxin 1A, 3 and 4, have been localized to the apical membrane in cultured epithelial cells and principal cells of the rat cortical collecting duct [58], and thus could be co-localized with ENaC. Moreover, co-precipitation experiments have demonstrated that syntaxin 1A was able to co-precipitate γ ENaC, but not the α and β subunits from the A6 cell line [57], suggesting an interaction between the γ ENaC subunit and syntaxin 1A. This interaction may mediate the inhibition of ENaC by selective sequestration and degradation as a means of reducing sodium currents in a similar manner to the regulation of neuronal calcium channels and the cystic fibrosis (CF) transmembrane regulator (CFTR) Cl^- channel by syntaxin 1A [58]. But whether this is the case is unclear as no reduction in protein levels was observed during the co-expression of syntaxin and ENaC.

Alternatively, syntaxin 1A may disrupt channel exocytosis. Syntaxin 3 has also been shown to interact with the ENaC complex. Both syntaxins 1A and 3 enhance the surface expression of ENaC complexes, but have opposite effects on ENaC function. Syntaxin 1A was able to decrease Na^+ current when expressed with ENaC in *Xenopus* oocytes while syntaxin 3 was unable to reproduce this effect [57]. However, syntaxin 1A is present in only low levels in some native epithelia, being principally expressed in neurons and, because of the strong homology between the syntaxin family of proteins, it remains unclear as to which of the syntaxin isoforms may be important for the regulation of ENaC.

Channel-activating protease 1 (CAP-1)

Exposure of the apical membrane of a *Xenopus* kidney epithelial cell line (A6) to the serine protease inhibitor aprotinin was found to effectively halve trans-epithelial sodium transport. However, transport could be restored by the presence of the non-specific protease trypsin in excess over aprotinin, suggesting the involvement of an endogenous protease active at the extracellular side of the apical membrane in regulating the activity of the amiloride-sensitive sodium channel [59].

Proteins involved in this regulation were isolated by means of a functional complementation assay in *Xenopus* oocytes. An A6 cDNA library was screened by co-injection of cRNA together with cRNA for the α , β and γ subunits of xENaC. A single 1340 bp cDNA clone was isolated that was able to stimulate amiloride-sensitive sodium current 3-fold when co-injected with $\alpha\beta\gamma$ ENaC subunits. The clone contains a 987-nucleotide open reading frame which encodes a protein of 329 amino acids. This protein was named CAP-1. Sequence comparison reveals homology with several members of the serine protease family, including prostatic, a cloned serine protease found in semen, with which CAP-1 shares 53% homology. Significant similarity also exists between CAP-1 and trypsin, hepsin, kallikrein, tissue plasminogen activator and urokinase [60].

A signal peptide at the N-terminus preceded by a characteristic signal, as seen in murine CAP-1 (mCAP-1), is typical of glycosylphosphatidylinositol-anchored proteins. Glycosylphosphatidylinositol anchoring is known to target the protein to the apical membrane in polarized renal epithelial cells, and the sequence similarity indicates that CAP-1 is either membrane-anchored or secreted [59]. Therefore the activity of ENaC may be controlled by a protein anchored on the membrane of the same cell, indicating the possible involvement of a novel mechanism of autocrine regulation of ion channels.

Northern blot analyses shows the presence of CAP-1 mRNA in many *X. laevis* tissues, the main mRNA species of approx. 1.5 kb being highly expressed in kidney, intestine, stomach, skin and lung, all tissues which express ENaC mRNA. Other transcripts of a higher molecular mass are detected in skin, kidney and stomach [59].

Exposure of oocytes to CAP-1 resulted in an increased basal sodium current which could not be further stimulated by subsequent exposure to trypsin, suggesting that CAP-1 and trypsin act through a common mechanism to enhance ENaC activity. Proteases were able to increase the channel-open probability via an effect that occurs independently of the activation of G-protein-coupled receptors; it is thought that this change in channel gating is induced by proteolysis of a protein that is either a constitutive part of the channel itself or is closely associated with it [61]. However, immunoprecipitating FLAG-tagged $\alpha\beta\gamma$ ENaC bore no evidence for the proteolytic cleavage of the extracellular loop of ENaC proteins.

The two effects of CAP-1 on ENaC, namely the activation of the channel and the apparent abolition of sensitivity to trypsin, are both abolished by incubation with aprotinin; therefore CAP-1 may increase sodium current through the action of a serine protease [59].

In order to identify specific proteases which can activate the amiloride-sensitive sodium channel in a mammalian kidney epithelial cell line, Vuagniaux et al. [62] used reverse transcriptase PCR with degenerate oligonucleotides to *Xenopus* CAP-1 (xCAP-1); this revealed a novel full-length 339 amino acids serine protease (mCAP1) in murine cortical collecting duct cell line mpkCCDC14 [62]. mRNA expression was examined by Northern blotting, and strong hybridization signals were detected in kidney, lung and salivary glands. Alignment of the predicted sequence showed that the deduced protein belongs to the serine protease family, sharing 50% homology with xCAP-1 and 80% with human prostatic. Unpublished data, as cited in [60], indicate that prostatic is the human equivalent of mCAP-1 and xCAP-1.

Functional analysis of mCAP-1 in the *Xenopus* oocyte expression system demonstrated a 6-fold increase in intracellular sodium current upon co-expression of mCAP-1 mRNA with each of the subunits of *Xenopus* ENaC, rat ENaC and human ENaC. In the absence of $\alpha\beta\gamma$ ENaC subunits, mCAP-1 injected into oocytes did not generate measurable amiloride-sensitive sodium currents. Although most of the functional effects of mCAP-1 are similar to those of xCAP-1, mCAP-1 is less sensitive to aprotinin than xCAP-1, indicating that the binding site for the serine protease inhibitor may be different. Alternatively, mpkCCDC14 cells may express other proteolytic enzymes that are resistant to aprotinin [62].

The CFTR

The CFTR, a multifunctional membrane protein, was first identified by positional cloning in 1989, as a result of the search for the CF locus. The gene encoding CFTR, located on chromo-

some 7q21–31, encodes a protein of 165 kDa, comprising 1480 amino acids. The sequence is that of an ABC transport protein [63].

Under normal physiological conditions, CFTR is expressed in the apical membranes of various epithelia, including those of the renal tubules, intestine, airways, secretory glands, bile ducts, epididymis and sweat glands [63]. Throughout the body, CFTR functions as a Cl^- channel and in some epithelia (e.g. within the colon), CFTR may account for the entire apical Cl^- conductance.

The CFTR is now known to be a voltage-independent anion channel, requiring the presence of hydrolysable nucleotide triphosphates for efficient activity. The level of phosphorylation has a marked influence on its activity. Several kinases/phosphatases and, in particular, cAMP-dependent protein kinase A (PKA) and PKC, are important in modulating the phosphorylation state of the CFTR [64].

In CF, the major consequence of the gene defect is an impairment in chloride conductance. Over 1300 mutations within the CFTR have been identified in CF, the most common being $\Delta\text{Phe-508}$, which is present in 70 % of affected individuals [63]. CF mutations have been divided into five classes that define the mechanism for defective chloride conductance and this provided a useful framework for considering genotype–phenotype relationships [63]. Consequences of the molecular variants in CF range from mutations leading to premature termination of mRNA translation, and thus essentially no protein production (class 1), to those leading to marginally reduced activity (classes 4 and 5). In class 2 mutations, which include the most common CF mutation, Phe-508, the CFTR protein fails to mature properly in the biosynthetic pathway, with degradation of translated protein before it can progress past the endoplasmic reticulum [63].

In addition to impairment of chloride transport, CF is also associated with disturbances in ion transport and in particular in sodium transport. Major pathophysiological symptoms of CF probably result from an inability of the mutant CFTR to inhibit ENaC, manifesting itself in an increased rate of sodium reabsorption in the airway, pancreatic and colonic epithelia of affected individuals. The production of dehydrated secretions in pancreatic ducts induce duct occlusion and consequently lead to impairment of function and eventually to tissue destruction.

The impaired secretion of Cl^- from the serous cells of airway submucosal glands and the enhanced absorption of Na^+ and consequent hyperabsorption of electrolytes and water are crucial for the pathophysiology of the lung disease seen in CF. Usually, ion transport can be switched from a net absorption under resting conditions towards net NaCl/KCl secretion when stimulated by secretagogues. CF airways are unable to enhance their secretory transport and may even be hyperabsorbing due to enhanced activity of ENaC. This leads to a volume depletion of the airway surface liquid layer in CF airways. Consequently, mucus impactions form, resulting in an impaired mucociliary clearance and subsequent predisposition towards bacterial infections [65].

By contrast, it has long been known that CF is associated with a reduction in sodium reabsorption within the sweat gland lumen. This accounts for the presence of ‘salty sweat’ in individuals with CF and provides a characteristic diagnostic feature for CF [66]. To some extent this can be accounted for by the reduction in Cl^- conductance of the apical membrane of the epithelial cell. This is expected to decrease the electrochemical driving force for sodium to enter the cell and thereby leads to the secretion of fluid containing a much higher content of sodium. However, it has been suggested that failure to reabsorb sodium is due not only to the electrochemical effects arising from the primary loss of chloride conductance through the CFTR but also

to a secondary inability to activate sodium-channel conductance [67].

The exact mechanism linking changes in CFTR activity and sodium-channel activity remains to be resolved and several hypotheses are currently being explored. To some extent, there is evidence suggesting that the Cl^- ion flow itself may be important.

Because of the apparent enhanced activity of ENaC within the lung in CF, a number of studies using the *Xenopus* oocyte expression system were carried out to determine whether there were any interactions between the CFTR and ENaC.

In *Xenopus* oocytes co-expressing ENaC and CFTR, uptake of $^{22}\text{Na}^+$ through ENaC is modulated by activation of CFTR, the effect being dependent on extracellular Cl^- concentration. Inhibition of ENaC can also be mimicked by the co-expression of Cl^- channels other than the CFTR [68]. Work by Konig et al. [69], again in *Xenopus* oocytes, has demonstrated that the amiloride-sensitive Na^+ current is reduced by the co-expression of both CFTR and ClC-0 , a Cl^- channel, further demonstrating that inhibition of ENaC is not a unique property of CFTR.

Voltage-clamping studies have shown that when there is high Cl^- in the extracellular bath of permeabilized oocytes, there is a strong inhibition of ENaC, again suggesting that the sodium channel is inhibited by high intracellular Cl^- concentrations [70].

At the molecular level the CFTR has two nucleotide-binding domains (NBD1 and NBD2). The requirement of an intact nucleotide-binding domain (NBF1) within the CF membrane transporter for inhibition of ENaC has been demonstrated [70]. With the aim of determining amino acid sequences within NBF1 of CFTR that may be important for the regulation of ENaC, mutants were generated. However, these channels, harbouring mutations within the Walker A and B motifs, stretches that are homologous to GTP-binding proteins and consensus sequences known to activate GTP-binding proteins, lost their abilities to generate a Cl^- current [70]. This was paralleled by an impaired ability to inhibit the amiloride-sensitive sodium current, further supporting the view that the ability of CFTR to generate a Cl^- current and the inhibition of ENaC may be closely linked [71].

This aside, technical problems with functional studies in oocytes suggest that it is difficult to extrapolate the results of these studies to native tissue. Additionally, the relative expression of CFTR and ENaC is not known for any cell type of the airways *in vivo*, and the direct effect of intracellular Cl^- concentration on sodium current through ENaC in native airways epithelium remains to be resolved.

The defective CFTR in CF is associated not only with disturbances of chloride secretion and sodium transport through ENaC but also with defects in the transport of other electrolytes. In this context it is relevant to note that CFTR has been demonstrated to affect both intracellular and extracellular pH regulation by alterations in either HCO_3^- secretion via activation and increased expression of the $\text{Cl}^-/\text{HCO}_3^-$ antiporter [72,73], or by alterations in sodium/hydrogen exchanger (NHE) activity [74,75].

The mechanisms by which CFTR exerts its regulatory role over other epithelial ion transporters are still incompletely understood. This is important as disturbance in these other transport systems indirectly could also influence cellular sodium homeostasis. Suggested mechanisms include effects due to electrochemical coupling, second-messenger-related regulation, cellular trafficking and direct interaction between the transporter and the other transport systems within the membrane. One hypothesis relates to indirect regulation via release of ATP through the CFTR with a subsequent autocrine activation of luminal purinoceptors and modulation of intracellular calcium, which in turn could regulate other transporters [76].

Figure 7 Schematic representation of regulation of ENaC activity by aldosterone and accessory proteins

The activity of the sodium channel responds to a wide variety of hormones and changes in physiological conditions. Channel activity can be modulated by several hormones and in particular aldosterone, antidiuretic hormone, insulin and atrial natriuretic peptide. A number of diverse mechanisms including variation in channel synthesis, regulation of intracellular channel trafficking and membrane insertion are important in controlling channel activity. Post-translational modifications that alter channel-open probability include activation by a membrane CAP-1. The enhancement of ENaC surface expression and activity is counterbalanced by channel retrieval from the membrane through the ubiquitination pathway mediated by Nedd4-related components. Transcriptional stimulation of SGK1 leads to phosphorylation of Nedd4-2, which subsequently disrupts ENaC–Nedd4-2 interactions. The associated reduction in ubiquitination of ENaCs increases ENaC stability in the apical membrane and leads to enhanced sodium reabsorption (see text for further details). Ub, ubiquitin; GR, glucocorticoid receptor; MR, mineralocorticoid receptor.

However, more recent research also points to direct intramembrane or cytoplasmic protein–protein interaction through intermediary proteins containing PDZ-binding domains. These scaffold-based regulatory proteins are localized to specific sites in polarized epithelial cells. *In vitro* binding studies have demonstrated that CFTR can bind to the PDZ1 domain of the NHE regulatory factors with an intracellular C-terminal domain ending in Asp-(Ser/Thr)-Xaa-Leu [77–79], providing a potential mechanism for the CFTR modulation of the regulation of other membrane proteins such as the NHE type 3 (NHE3). Differential expression of these proteins could provide tissue-specific regulatory relationships which might account for the remarkable differences in cellular sodium handling in CF between airways and colon on one hand and sweat gland on the other. Evidence supporting a reciprocal interaction between CFTR and NHE3 activity via shared regulatory protein complexes has recently been reported by Bagorda et al. [80] in a renal epithelial cell model. However, the results of Boucherot et al. [70] using *Xenopus* oocyte expression system were not consistent with a role of PDZ-binding domains in the inhibition of ENaC activity by CFTR.

The impact of differential expression of intermediary proteins is clearly an area for further research. Interestingly, lung tissue from CF patients strongly expresses SGK-1, whereas this is not the case for non-CF tissue. Thus the up-regulation of lung ENaC activity in CF could in fact be due to the increased expression of SGK-1, rather than interactions between the CFTR and ENaC, or the impaired Cl^- permeability of CF tissue itself [81]. SGK-1 may interact with NHERF-2, an NHE regulatory factor, and

other scaffolding proteins within the apical membranes of epithelial cells. CFTR itself is an NHERF-2-associated transporter, providing yet another alternative mechanism for cross-talk between ENaC and CFTR. Further work is necessary to identify which, if any, of these mechanisms is physiologically and pathophysiologically relevant.

PERSPECTIVES

It has long been known that several hormones, and aldosterone in particular, are important in the regulation of the activity of the amiloride-sensitive ENaC. Over the past few years there has been considerable progress in our understanding of the corresponding intracellular mechanisms. Channel activity can be influenced by proteins/enzymes that increase the open-channel probability of channels within the plasma membrane and/or by modulation of channel trafficking and insertion/retrieval at the plasma membrane. CAP-1 seems to activate ENaC by increasing the overall open probability of existing membrane channels. This enzyme is predicted to be secreted and/or glycosylphosphatidylinositol-anchored and it may form part of a hitherto unrecognized pathway for autocrine regulation of the channel.

The up-regulation of ENaC activity by the steroid hormone aldosterone requires the induction of intermediate proteins and it is now apparent that the activity of the sodium channel in response to aldosterone is under the influence of a panoply of intracellular accessory factors. Two such transcripts are SGK and the small G-protein K-Ras 2A. The upstream regulator that

phosphorylates SGK is 3-phosphoinositide-dependent kinase 1 (PDK1), a PtdIns3P-dependent kinase which is also required for insulin-stimulated sodium transport in A6 renal epithelia.

These components do not work in isolation and there is considerable interaction and convergence. For instance, the actions of SGK on the sodium channel appear to be mediated through a Nedd4-dependent mechanism, possibly by phosphorylation of Nedd4-2 as an intermediate stage (Figure 7).

Nedd4 appears to be central in the regulation of ENaC expression at the cellular membrane as it is an important component of the ubiquitination-dependent targeting of the channel for endocytosis and degradation.

Despite the original identification of the Nedd4-1 isoform as a binding protein of ENaC, more recent work suggests that Nedd4-2 may be more functionally important in the regulation of ENaC activity *in vivo* [21]. Nedd4-2 also binds to ENaC subunits. Fotia et al. [82], using whole-cell patch-clamp techniques, found that WW3 and WW4 are required for both the binding to ENaC subunits and the regulation of Na⁺ feedback control of ENaC. Although both WW3 and WW4 individually were able to interact with all three ENaC subunits *in vitro*, both domains together were essential for *in vivo* function of Nedd4-2 in ENaC regulation.

However, a potential role of Nedd4-1 cannot be excluded entirely. Although the reduced efficiency of Nedd4-1 in down-regulation of the ENaC can be attributed partly to the presence of the C2 domain, it is still possible that alternative splicing and/or proteolytic cleavage of Nedd4-related proteins may generate the more efficient C2-lacking Nedd4-related proteins.

More importantly, however, despite the demonstration that Nedd4 and Nedd4-2 bind to and inhibit ENaC in heterologous expression systems, it is not known whether these protein factors regulate ENaC in actual native epithelia. Moreover, in these cells there could be additional Nedd4-like factors which regulate epithelial ENaC. This is highlighted by the recent identification of a distinct ubiquitin-protein ligase, WWP2, as a potential candidate for the regulation of ENaC in sodium-transporting epithelia [83]. Interestingly, alternative splicing affects the domain structure of WWP1, leading to forms that contain or lack an N-terminal C2 domain [84].

Such a multiplicity of factors begs the question of whether all of the known Nedd4 components are functionally important. Although this remains to be assessed, available evidence suggests additional layers of control through distinct mechanisms. As recently emphasized [83] this is supported by (i) the differential binding of their WW domains to ENaC subunits, (ii) differences in the presence or absence of the C2 domain in some ubiquitin-protein ligases (Nedd4-2 and some splice forms of WWP1 lack the C2 domain) which could lead to differences in their Ca²⁺-dependent regulation or localization and (iii) the differential phosphorylation of the three ubiquitin-protein ligases by SGK. SGK phosphorylates hNedd4-2 and reduces its binding to ENaC, providing a molecular pathway to explain the rapid aldosterone-mediated up-regulation of the Na⁺ channel. In contrast, SGK does not phosphorylate hNedd4 or WWP2.

Undoubtedly many questions still remain open and there is clearly a need for more research at both the structural and functional levels. The cellular localization of the interaction between ENaC and Nedd4 proteins and the intracellular pathways taken by the ubiquitinated channel (e.g. endocytosis, sorting and/or lysosomal incorporation) also remains to be clarified.

Whole-body sodium homeostasis is of crucial importance for the overall control of the extracellular fluid volume and blood pressure. Indeed, the potential role of the renal sodium channel in the control of blood pressure is highlighted by the identification of the gene defects in Liddle's syndrome and PHA-1 with

contrasting effects on renal sodium reabsorption and blood pressure.

The identification of the biochemical defects in these rare conditions and the recognition of the links with defects in the control of sodium balance has led to renewed interest in the importance of genes involved in the control of sodium excretion as candidate genes for essential hypertension. This is based on the possibility of the existence of common variants in these genes displaying more moderate effects. Such effects, although small, could be amplified in the presence of consistent, long-term exposure to a high-salt diet, thereby leading to the development of high blood pressure.

The implications of research on sodium-channel accessory factors are wide ranging. A more detailed understanding of cellular events modulating epithelial sodium transport may provide clues for understanding not only invariably fatal monogenic diseases like CF but also the link between abnormal renal sodium transport and essential hypertension. Hypertension is an increasingly important risk factor for stroke and ischaemic heart disease, two of the most common causes of human disability and death.

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