Cum Grano Salis: The Epithelial Sodium Channel and the Control of Blood Pressure

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In the early fifties, when I started my secondary school, Latin was mandatory if you wanted to study medicine. For 8 years I had no choice but to learn the language. I found it terribly boring and, most of the time, useless. I therefore thought it was appropriate to select this title for this lecture so that, at least once, I could use my knowledge of the Roman culture. I am going to describe the role of the epithelial sodium channel in controlling sodium homeostasis and blood pressure. I will describe recent scientific evidence that suggests that salt-sensitivity and salt-resistance in the human population may be linked to the expression of specific genes in the kidney. However, as always in science, there is no absolute truth, and new concepts are constantly challenged by experimental data. From this point of view, the Latin expression cum grano salis (“with a grain of salt”) is appropriate, but on the other hand, I will also take this expression literally, to try to convince you that a grain of salt can indeed make a difference.

Claude Bernard, the famous French physiologist, was the first to define the importance of the milieu intérieur as a critical factor to ensure proper cell function (1). Homer Smith pioneered the idea of the constancy of our milieu intérieur throughout the evolution. This was beautifully described in his famous book, From Fish to Philosopher (2). I would like to quote a few words of his introduction: “Recognizing that we have the kind of internal environment we have, because we have the kind of kidneys that we have, we must acknowledge that our kidneys constitute the major foundation of our physiological freedom. Only because they work the way they do, has it become possible for us to have bones, muscles, glands and brains. Superficially, it might be said that the function of the kidney is to make urine, but in a more considered view, one can say that the kidney makes the stuff of philosophy itself...”

Woody Allen, in his famous movie Everything You Always Wanted to Know About Sex (But Were Afraid to Ask), stated that the second most important organ of your body is the brain; one can debate which is first, but for today’s lecture it is clear that the kidney is first.

The kidney plays a most significant role in controlling the homeostasis of the extracellular space (3). Sodium and water balances are regulated to a great extent by renal mechanisms, resulting in the constancy of plasma osmolality. In mammals, plasma osmolality is set at approximately 290 mosmol/L, which is primarily accounted for by the presence of the sodium ion. The plasma osmolality is controlled within a small range (plus or minus 2%) despite large variations in salt and water intake. The relationship between sodium balance and the control of blood pressure was recognized by clinicians as early as 1855. At that time, a loss of function of the adrenal renal axis was shown by Addison to cause hypotension and proved to be lethal. It took another 80 years to demonstrate that a gain of function of the adrenal renal axis could cause the opposite phenotype, namely high blood pressure, as established by Cushing in 1932 and later by Conn in 1955. Hypercorticoidism and aldosteronism are, however, responsible for only a small percentage of patients suffering from hypertension. Hypertension is one of the most common diseases in Western countries (4). Beyond the age of 60, up to 20% of the population has an abnormal high blood pressure. This trait carries risks for cardiovascular diseases such as stroke, kidney failure, and myocardial infarction. It also represents the highest single expense in public health for Western countries. Unfortunately, in 95% of hypertensive patients, the primary cause remains unknown. Hypertension is a multifactorial disease. Genetic and nongenetic factors play a significant role. It has been proposed that genetic factors may account for as much as 30% of the trait variation in the general population (5). Among the nongenetic factors, excess of weight, smoking, drugs (like the contraceptive pill), lack of physical training, and salt intake have been most often proposed as risk factors, based on a number of epidemiological and experimental studies. The role of salt intake in causing hypertension, or, alternatively, the role of a low salt intake in decreasing normal or high blood pressure (6) has been a very controversial issue. Two recent studies published in 1996 clearly illustrate this point.

In the first study, published in the British Medical Journal in May 1996, Elliot et al. (7) reported a strong positive association between 24-hour urinary sodium excretion and systolic pressure was demonstrated. One major conclusion of this study was to support recommendations for reduction of excessive salt intake in the human population to prevent and control adverse blood pressure levels.

In a second study, Midgley et al. (8) conducted a meta-analysis of randomized control trials on the effects of reduced dietary sodium intake on blood pressure. The conclusions of
the authors were not strong. They stated that dietary sodium restriction for older hypertensive individuals might be considered, but they concluded that the evidence in the normotensive population did not support current recommendations for universal dietary sodium restriction. I will not discuss in detail the reasons for the different findings and all of the limitations of large clinical epidemiological human studies. I would like, however, to stress two factors that may have been overlooked. The first is the fact that genetic factors, if important in determining salt-sensitivity or salt-resistance, are probably widely distributed in the human population. The data interpretation of large human samples may be difficult. Another difficulty of these controversial studies is the fact that determining the effect of salt intake as a single variable on blood pressure on a long-term basis is clearly not possible in human populations. Recent experiments addressing this question have been performed on our closest relative, the chimpanzee (9). In the wilderness chimpanzees feed on a low-salt diet, eating almost exclusively vegetarian food with a high potassium content. This diet is probably very similar to that used by our ancestor primates living in eastern Africa 4 to 5 million years ago. This experimental model gives us the unique opportunity to test whether the chimpanzee is sensitive to a high salt intake and to demonstrate whether this is a factor in determining high blood pressure. A colony of chimpanzees given a vegetarian diet of very-low-sodium and high-potassium intake was maintained in long-standing, socially stable small groups for 3 years. Half of the colony had salt added progressively to their diet over 20 months. The study was conducted in a single blinded fashion, and the results are striking. Eight of 13 animals (60%) were sensitive to increased salt intake, which was progressively increased to up to 15 g/d. This group developed hypertension, which was, however, reversible upon return to a low-salt, high-potassium diet. Five of 13 animals (40%) remained resistant to the high-salt intake. The control group was left on a low-salt diet for the same period of time, and their blood pressure did not change. This experimental study is reminiscent of the conclusions of an epidemiological human study that demonstrated that up to 40% of Japanese living in the Akita region in northern Japan and eating up to 26 g of sodium per day developed severe hypertension and cardiovascular diseases (10).

The two main issues I would like to address today are the following:

1. Are there genes that are directly involved in sodium homeostasis and thus in the control of blood pressure?
2. Are there genes that may confer salt-sensitivity or, conversely, salt-resistance to high salt intake in the human population?

According to the classic hypothesis of Guyton (11), the best candidate genes should be expressed in the nephron. These genes may encode proteins involved in sodium reabsorption from the proximal tubule to the medullary ducts. Sodium reabsorption along the nephron is mediated by ion transporters (exchangers, cotransporters, and ionic channels) located in the apical membrane of the tubule cells and by the sodium pump located in the basolateral membrane of the same cells. For Na,K-ATPase (12), which is expressed along the entire length of the nephron (13), no monogenic or polygenic disease with a hypertensive phenotype has been mapped to Na,K-ATPase genes until now.

In the proximal tubule, NHE3, a sodium/proton exchanger (see reference 14 for review), is responsible for a large fraction of iso-osmotic sodium reabsorption in this part of the tubule. There is, however, no evidence that NHE3 is involved in human hypertension.

In the loop of Henle, the furosemide receptor (the Na,K 2Cl cotransporter) that has recently been cloned (15,16) plays a critical role in Na,K balance. Interestingly, recent studies by Lifton and his colleagues show that a loss of function of these genes in human monogenic diseases (Bartter’s syndrome) is characterized by a salt-losing phenotype (17). ROMK-1, a potassium channel that has also recently been cloned (18,19), is expressed in the apical membrane of the same cell and is required for proper function (potassium recycling of the Na,K 2Cl cotransporter). A loss of function mutation in ROMK-1 has recently been shown to also cause a Bartter’s syndrome (20). In the distal convoluted tubule, the receptor for the thiazide diuretic, the NaCl cotransporter—a member of the same gene family as the Na,K 2Cl cotransporter—has also been linked recently to Gitelman’s syndrome (21), which is also characterized by a salt-losing phenotype. The Na,K 2Cl cotransporter, ROMK-1, and NaCl cotransporter are therefore qualified as candidate genes for salt-resistance in man. So far, however, no gain of function mutation has been identified in these genes, which could lead to salt-sensitivity and, therefore, high blood pressure. In the most distal part of the renal tubule, namely in the collecting ducts (cortical and medullary), sodium reabsorption is mediated by an amiloride-sensitive electronic sodium transport through the amiloride-sensitive epithelial sodium channel (ENaC) (22). Until now, ENaC is the only sodium transport protein for which we have genetic evidence for its involvement in the genesis of either hypertension or hypotension (4). I will therefore describe the structure and the function of the ENaC molecule, then I will discuss how ENaC is regulated by aldosterone in the kidney, and finally I will discuss monogenic diseases (Liddle’s syndrome and pseudohypoaldosteronism, type 1[PHA-1]) in man, which clearly indicate the physiological importance of ENaC in vivo. I will also describe animal models that have recently been produced in the laboratory to demonstrate an unexpected role of ENaC in lung fluid clearance.

Structure and Function of the Epithelial Sodium Channel

Biophysical Properties

Four properties are important for this discussion (see references 23 through 25 for recent reviews in detail).

First, the epithelial sodium channel is highly sodium-selective. This means that the permeability to sodium is greater than the permeability to potassium. The PNa/PK ratio is very high, in most cases over 20 if not over 100. This means that this
channel does not allow the entry or the exit of any potassium ions. In fact, only sodium, lithium, or protons can cross the membrane through this channel.

The second important property is that, as characterized by patch-clamp analysis, the open-state conductance of the single channel is low. Typically, in the presence of sodium, the conductance is around 5 picoSiemens (pS) and, in the presence of lithium, 7 to 8 pS, indicating that lithium is twice as permeant as sodium through the channel.

The third property is the gating kinetics of ENaC. The times that the channel spends in the open state or in the closed state are typically long, between 3 and 4 s. Large variations, however, are observed. The open probability (P₀) varies, depending on the studies, between 0.05 and 0.9. It is not yet well understood how P₀ is regulated, but the exquisite sensitivity of ENaC to regulatory factors changing P₀ is probably of great importance for sodium balance in vivo.

Finally, the epithelial sodium channel has a high affinity for amiloride, typically in the nM range, with a pharmacological profile that makes it possible to distinguish the epithelial sodium channel from other sodium or non-selective cation channels (22).

**Primary Structure of ENaC**

ENaC is a heteromultimeric protein composed of three homologous subunits called α, β, and γ, varying in length from 650 to 700 amino acids (26–34). The protein level for each subunit shares 35% identity with the others, indicating replication of an ancestor gene a long time ago. Indeed, the gene family is growing rapidly, and different subfamilies have been identified in the nervous system, in the heart, and in epithelial cells (see the recent review in reference 23).

**Membrane Topology**

Three different laboratories have proposed the same model of membrane topology for ENaC, using different experimental approaches (35–37). This model is shown in Figure 1. Only two transmembrane domains are predicted, leaving a large ectodomain protruding outside the cell with two short amino and carboxy termini in the cytoplasm.

The exact number of subunits required for channel function is not known, and the stoichiometry remains to be elucidated. Recent preliminary data presented at this meeting (38) indicate that more than three subunits may be involved.

The role of each subunit in determining channel activity has recently been reassessed by a new method, combining a functional and a binding assay performed on the same oocyte (39). This new method has been useful to characterize the functional domains of the protein, particularly the ones that have been identified by studying monogenic diseases such as Liddle's syndrome or PHA.

The sodium-transport assay measures the activity of all channels expressed at the cell surface of *Xenopus* oocytes injected with the cRNA coding for each subunit of ENaC. An amiloride-sensitive sodium inward current is measured by a two-voltage clamp assay. The assay is specific for ENaC activity.

The second assay, the binding assay, allows quantitation of the total number of both active and inactive channels, expressed at the surface of the same oocyte that was used for the sodium-transport measurement.

An eight-amino-acid epitope flag was inserted into the ectodomain of each subunit of ENaC, allowing the specific and selective detection at the cell surface for each subunit by the use of an iodinated monoclonal antibody directed against the flag epitope. The assay is highly selective, very sensitive, and, importantly, does not disturb the physiological activity of the channel. In other words, the main biophysical properties of ENaC (conductance, ion selectivity, gating), are not modified by the presence of antibodies binding to the flag epitope. We were also able to demonstrate that the binding stoichiometry is one antibody for one subunit. Without going into too many technical details, we can say that the ability to measure the amiloride sodium-sensitive transport and the total number of active and inactive channels in the same cell gave us recent insights into important properties of ENaC. One is the possibility of measuring the average P₀ at the single-cell level. The data indicate that no truly silent channels are expressed at the cell surface, but rather channels with large variability in their P₀, ranging from 0.005 to 0.8 (39).
A second outcome of these recent studies has been to establish that hetero-oligomerization in the endothelial reticulum (ER) compartment is a prerequisite for maximal targeting to the plasma membrane. Indeed, it is only when the three subunits are coexpressed that maximal activity is observed (Figure 2). This is an important experiment because it demonstrates that monomeric α subunits or heterodimeric α/β or α/γ subunits can be transported to the plasma membrane only with very low efficiency (0.5 to 2% of the heterotrimeric complex). The α subunit therefore plays a specific role in the targeting of the channel protein at the surface. On the other hand, the β and the γ subunits are not merely chaperone proteins, and, as shown below, they participate in amiloride binding and ion permeation. As shown on this figure, which depicts only two of the subunits involved in the formation of the channel, five functional domains have been identified thus far.

Domain 1, at the amino terminus, is characterized by a gating domain involved in channel opening and closing, according to the data I will discuss later in this presentation by studying one of the mutations causing PHA-1. Domain 2 has been proposed by Kleyman and collaborators as an amiloride-binding domain (40). Domain 3, is a second amiloride-binding domain found in α, β, and γ subunits, as described recently by Schild and collaborators (41). Domain 4 is a putative ion-selectivity filter domain located on the α helix of transmembrane 2 (M2), as proposed by Lazdunski and colleagues (42), and finally, Domain 5 is a repressor domain located on the carboxy terminus of β and γ subunits that will be discussed in the context of the Liddle syndrome.

**Physiology: Cell- and Tissue-Specific Expression**

ENaC is a low copy number protein; typically, only a few hundred molecules are expressed per cell. Its tissue distribution is highly restricted to a few organs, and in situ and immunocytochemistry (43–47) have shown that ENaC is expressed in the apical membrane of aldosterone-responsive tissues, such as those found in the distal part of the nephron in the kidney (from distal convoluted tubule [DCT] to inner medullary collecting duct [IMCD]), in the distal part of the colon (restricted to the surface epithelial cells), and in the ducts of exocrine glands (sialyv and sweat glands). In these target cells, the mineralocorticoid receptor is coexpressed together with 11β-HSD, an enzyme that ensures mineralocorticoid specificity (48).

Recently, ENaC has also been shown to be expressed in the epidermis of the skin and in hair follicles (47). No physiological explanation for that specific expression is known.

ENaC is also expressed in the lung, which is not an aldosterone- but typically a glucocorticoid-responsive tissue. Recent data demonstrate an important heterogeneity in the cell expression of ENaC in the various parts of the lung airways (45,46).

In the nephron, all segments that express mineralocorticoid-dependent electrogenic sodium reabsorption—distal convoluted tubule, connecting tubule (CNT), cortical collecting duct (CCD), outer medullary collecting duct (OMCD), and IMCD—mineralocorticoid receptors, and 11β-HSD type 2 enzyme possess the three subunits of ENaC (43,49).

The CCD has been most extensively studied and is under the tight control of hormones such as aldosterone and vasopressin (3,50). In the CCD, only the principal cells appear to be involved in amiloride-electrogenic-sensitive sodium reabsorption. A model of a principal cell of the CCD is shown in Figure 3.

This model of a principal cell of the CCD indicates the main pathway for sodium reabsorption and potassium secretion across this epithelial layer. The urinary sodium crosses the apical membrane through the highly selective amiloride-sensitive sodium channel, following a favorable downhill electrochemical gradient. Intracellular sodium is then pumped out of
Figure 3. Schematic diagram shows molecular mechanisms of action of aldosterone. Aldosterone (Aldo) binds to an intracellular receptor (R), and the active hormone-receptor complex interacts with hormone-responsive elements of the DNA to modulate the transcription of specific genes, leading to the expression of specific proteins, the aldosterone-induced proteins (AIP). Candidate targets for the action of these AIP are the amiloride-sensitive sodium channels in the apical membrane (ap m) and the Na,K-ATPase in the basolateral membrane (bl m). Because of the low intracellular sodium concentration maintained by the Na,K pump, sodium ions from the luminal fluid enter the cell passively through the sodium channels. Sodium ions are transported across the cell from the lumen to the blood, and potassium ions accumulated in the cell by the Na,K pump recirculate across the basolateral membrane through K channels, and—in K-secreting epithelium—also across the apical membrane. The tight junctions prevent the back-leak of transported ions.

Pathophysiology of the Epithelial Sodium Channel

Loss of Function of ENaC

Pseudohypoaldosteronism type I is divided in two clinically distinct entities. The first disease is autosomal dominant. It is not clinically very severe and is restricted to the kidney. The genes implicated in this disease have not yet been identified. The second form is autosomal recessive. It is a severe multiorgan disease, characterized by failure to thrive, hypovolemia, hyponatremia, hyperkalemia, and metabolic acidosis (which can be lethal). It responds poorly to sodium chloride therapy and potassium chelation. During the last two years, a number of mutations have been identified by Lifton’s group at Yale (54) and Gardiner’s group in London (58). As shown in Figure 4, the mutations are distributed on the three subunits. Some mutations predictably produce either deletions of large portions of the α subunit and the γ subunit or, as in the case of the β subunit, a missense mutation of a glycine residue (G375) in a highly conserved domain of the protein (55). From a physiological standpoint, a missense mutation such as G375 may be highly informative in defining a functional domain of the sodium transporters at either the apical or the basolateral membranes or indirectly regulate the activity of these transporters. When an animal is put on a low-salt diet, plasma aldosterone increases and ENaC activity is detected in the apical membrane of principal cells (52). Upon resalination, and within a short period of time (about 12 hours), ENaC activity decreases rapidly as plasma aldosterone declines. In mammals, it therefore appears that ENaC activity in the apical membrane varies dramatically in function depending on the level of plasma aldosterone. In a normal-salt diet (or in the presence of a high-salt intake), ENaC activity is not detected significantly by the current biophysical methods. Indeed, the amiloride-sensitive electrogenic sodium transport pathway appears to be activated only when an animal is put on a low-salt diet, the physiological trigger for aldosterone secretion. Hyperaldosteronism (Conn’s syndrome) therefore leads to an increased sodium reabsorption, increased blood volume, and hypertension. On the other hand, a loss of aldosterone production—as shown in Addison’s disease—will cause the opposite phenotype, with hypovolemia, hyperkalemia, and a severe salt-losing syndrome. In two monogenic diseases in humans (53), the target cell does not respond properly to the physiological stimuli triggered by hyper- or hypovolemia. In the autosomal recessive severe form of pseudohypoaldosteronism (PHA-1), the effector, the sodium channel, or a regulator of the channel is deficient (54,55). ENaC can no longer respond properly to the aldosterone stimulus. Plasma aldosterone increases secondarily to the salt-losing syndrome and hypovolemia. In pseudohypoaldosteronism (Liddle’s syndrome) (56,57), the channel becomes constitutively active independent of the aldosterone level, which is typically low, thus escaping normal hormonal control.

In the second part of this lecture, I will describe the pathophysiology of ENaC in human and in animal models.
Pseudohypoaldosteronism type 1 (PHA-1): loss or partial loss of function mutations detected in the autosomal recessive form of PHA-1 (for details, see text and references 54 and 58).

The corresponding mutation gives approximately the same degree of partial inactivation, whereas the corresponding mutation in the α subunit leads to an almost complete inactivation of channel activity. Interestingly, cell surface expression of this mutant was not different from the wild-type, suggesting a drastic decrease in the average of the open channel probability. This was confirmed by the direct measurement of $P_o$ in cell attached patches. We therefore propose that the highly conserved domain of the protein, around glycine-37, is indeed involved in the gating of the channel. With the exception of the I68L mutation, which predicts the deletion of M1 and M2 transmembrane domains and leads to almost no detectable activity in the Xenopus oocyte system, all the other PHA-1 mutations were characterized by a partial loss of ENaC function in the heterologous expression system. A total loss of ENaC function can also be produced in animal models by inactivating ENaC genes; for instance, in mice. A new mutation in mice was recently produced in our laboratory by a classic gene-targeting method (59). In this model, the entire coding sequence of α ENaC is deleted, leaving only a few amino acids at the amino terminus stretch, which is not conserved among different species.

According to our knowledge about the physiological role of ENaC in various tissues, namely the critical role of the α subunit as a limiting factor in channel activity (see above), the cell-specific expression of ENaC in aldosterone-responsive tissues and in the lung, and the physiological role of ENaC in mediating an amiloride-sensitive electrogenic sodium transport, a number of predictions were made about the possible phenotypes of such a null mutant.

First of all, no phenotype mutant would favor the existence of gene redundancy, a possibility that must always be considered.

An embryonic lethal phenotype was also possible during early or late development. An amiloride-sensitive pathway has been described during blastulation and organogenesis. Another possible phenotype would be a classical PHA type I, ENaC activity being suppressed in all aldosterone-responsive cells—namely the colon, salivary ducts, and the kidney—leading to a salt-losing syndrome and mimicking the human disease. Another possibility was an unexpected phenotype linked to the expression of ENaC in the skin or hair follicle.

Germline transmission was obtained. Heterozygote animals were bred, and a large number of offspring were examined. At birth (Figure 5A), the neonates looked perfectly healthy and could not be distinguished from each other. However, a few hours after birth, 25% of the animals became ill and died with RDS. As shown on Figure 5B, one can observe just before death this very cyanotic neonate (“blue mouse”), compared with its pink wild-type littermate. The lethality is severe because no survival among $(-/-)$ animals beyond 40 hours after birth was observed. The histology of the lung of the knockout animals is normal, both at the light microscopic and ultrastructural levels, but the lungs of $(-/-)$ animals were filled with water, so-called “water-logged” lungs, a parameter that can be quantified by measuring the wet over dry weight ratios, which were dramatically increased in these animals. A similar RDS is observed in human premature neonates, especially in the clinical category that is resistant to surfactant therapy (64,65). The role of ENaC in the human RDS remains to be studied and elucidated.

**Figure 4.** Pseudohypoaldosteronism, type 1 (PHA-1): loss or partial loss of function mutations detected in the autosomal recessive form of PHA-1 (for details, see text and references 54 and 58).
PHA syndrome, evidenced by normal plasma electrolytes but high plasma aldosterone and normal glucocorticoid levels.

To summarize the data concerning the loss of function of ENaC in various pathophysiological conditions, one can state that the majority of pedigrees suffering from the recessive form of PHA have mutations in ENaC. There is good functional evidence that these mutations actually cause the disease. Some of these mutations identify highly conserved glycine residues in the amino terminus of each subunit of ENaC that is involved in the gating properties of the channel.

Finally, the mouse model provides strong evidence for the limiting role of α ENaC in lung fluid clearance at birth. The role of ENaC in human RDS, both in premature babies and in adult lung edema must be evaluated in the future. The genetic rescue of α knockout mice is possible and generates animals that express a severe PHA phenotype during the first two weeks of life. Genetic rescue, using specific lung promoters, should refine in the future our understanding of ENaC functions in various organs. Because ENaC can be directly linked to a salt-wasting syndrome in the kidney, it follows that ENaC is an excellent candidate gene for salt-resistance in the human population. Gene polymorphism, for instance, in the amino terminus of α, β, γ subunits, could lead to a relatively “hyporesponsive” channel, which in turn could confer salt-resistance to those who have inherited this polymorphism.

**Gain of Function: Liddle’s Syndrome**

Two years ago at this meeting, we reported the linkage of the syndrome to mutations on the β subunit of ENaC (66). Since that time, our laboratory and others have made significant progress in understanding the mechanisms of the disease and the possible role of ENaC in salt-sensitive hypertension (67–70).

In 1993, David Warnock and his colleagues published a study (57) that was an extensive follow-up of the original description made by Liddle 30 years ago (56). Liddle’s syndrome is an autosomal dominant disease characterized by the early onset of severe hypertension, metabolic alkalosis, renal potassium wasting, and hypokalemia, which is, however, not a consistent finding and is present in only 50% of the cases. Despite the signs of hyperaldosteronism, plasma renin and plasma aldosterone levels are typically low. The hypertension is spironolactone-resistant but sensitive to amiloride or triamterene, providing that the patient is on a low-salt diet. In 1994, Shimkets et al. (66) studied four pedigrees of Liddle’s syndrome in the United States and established linkage with the β subunit of ENaC. Meanwhile, a number of interesting new pedigrees have been studied, and a summary of all mutations reported so far is shown in Figure 6. Unlike the PHA mutation, all of the mutations in Liddle’s syndrome map to a very specific part of the protein, namely the carboxy terminus of either the β or the γ subunit. No mutations have so far been mapped to the α subunit. The most informative mutations were described last year; one missense mutation, a proline to leucine-P616L, has been described as a de novo mutation in an American family (69). A tyrosine to histidine-Y618H has been recently observed in a Japanese family (70). These point mu-
3. Change in the open probability of the channel.

In a recent article, Snyder et al. (72) proposed that the Liddle's mutation causes an increase in cell-surface expression of the channel, which in turn would increase the rate of sodium reabsorption in the kidney cell. Using our novel functional binding assay, it has been possible to test this proposal directly.

As shown in Figure 8, we found that the Liddle's mutation not only increases the number of channels at the cell surface by a factor of 2 \((P = 0.001)\) but also, and even more significantly, increases the sodium current with respect to the binding. One can deduce that not only is the number of channels increased, but the gating property must change, i.e., the \(P_0\) must be increased in the Liddle's mutation. We therefore propose that the Liddle's mutation has a dual effect on channel activity (39).

We have no clue about the mechanism by which the Liddle's mutation increases the \(P_0\) of the channel. However, the molecular mechanism by which this mutation leads to an increased cell-surface expression has recently been provided by studies conducted by Olivier Staub and Daniela Rotin in Toronto (73). Their data show that the PXY highly conserved motif of the protein is a region of protein/protein interaction (74). Using a double-hybrid screening system in yeast, they identified a novel protein (Nedd-4) that specifically binds to the Liddle's motif, the PXY sequence. Nedd-4 is a protein that had previously been identified in the brain during development and for which no function was yet known (75). It is a large protein of 115 kD in length, which expresses 3 WW domains that are stretches of approximately 40 amino acids boxed by two highly conserved tryptophanes (74,76). These WW domains can bind with high affinity to the PXY motif expressed on ENaC subunits. At the carboxy terminus (C-terminus) of the Nedd-4 protein, one can find a so-called (hect) domain (77), which is involved in protein degradation by ubiquitination (78). The following working model (Figure 9) (73) has therefore been proposed: Nedd-4 (or a specific analogue) normally binds through its 3 WW domains to the proline-rich region of the C-terminus of each subunit of ENaC. Normally this interaction would promote a rapid internalization and degradation of ENaC molecules. In Liddle's syndrome, Nedd-4 cannot bind with high affinity to ENaC. The turnover of ENaC at the plasma membrane is slowed down, and the number of channels at the cell surface increases. This model is well supported by experimental data (Staub et al., personal communication). This model also predicts that the turnover of the channel protein must be rapid. This is indeed the case, as shown recently in the A6 kidney cell (May and Rossier, unpublished observations), in which the model ENaC subunits turnover half-life is less than one hour.

Some Perspectives

To summarize, one can say that ENaC mutations can explain the Liddle's phenotype and, although not yet formally demonstrated in the human kidney, a hyperactive channel is probably expressed in CCD, causing an increased sodium reabsorption, increased blood volume, and, thus, hypertension. ENaC mutations identify a physiological repressor domain at the C-termi-
Figure 7. Effects of four different Liddle mutations on amiloride-sensitive sodium current (\(I_{Na}\)) in Xenopus oocytes. Deletion mutants (\(\alpha\beta\gamma\), \(\beta_{R564}\gamma\), \(\alpha\beta_{W574}\gamma\)) and missense mutations (\(\alpha\beta_{P610}\gamma\) and \(\alpha\beta_{Y618}\gamma\)) induced a significant increase in \(I_{Na}\) compared with the wild-type (\(\alpha\beta\gamma\)) (data taken from references 67, 68, 69, and 70).

Figure 8. Effect of \(\beta_{R564}\) stop-mutation on \(I_{Na}\) and channel expression at the cell surface. Five independent experiments were performed in which ten to 12 oocytes were injected with either \(\beta\) subunit or \(\beta_{R564}\) stop mutants together with \(\alpha\) and \(\gamma\) ENaC wild-type. Open bar, mean \(I_{Na}\) (0.94 ± 0.27 pAmp versus 5.31 ± 1.01 pAmp, \(P < 0.05\)); shaded bar, mean monoclonal antibody binding (0.29 ± 0.006 fmol versus 0.56 ± 0.1 fmol, \(P < 0.05\)) (adapted from reference 39).

Conclusions

Before closing, I would like to stress the importance of my collaborators and my collaborations over the last 20 years in my proposing some of the concepts I have discussed today. In Lausanne, and presently working in my group, I would like to mention Edith Hummler, responsible for the gene-targeting project, Stefan Grönder, for the structure-function relationship of PHA mutants, Dmitri Firsov, who established the novel assay permitting the measurement in the same cells of a number of ENaC molecules and their transport, and Anne May, who has recently studied the effect of aldosterone in A6 kidney cells. All of the experiments are done in tight collaboration with two established investigators, Jean-Daniel Horisberger and Laurent Schild, who are both masters of electrophysiological techniques. We also entertain extensive collaborations with Yale University, specifically with Richard Lifton and Cecilia Canessa, a former postdoctoral fellow who was involved in the cloning of ENaC when she was working in Lausanne. At Chapel Hill, we are collaborating with Ric Boucher, Jack Stutts, and Pierre Barker on the characterization of the function of ENaC in the lung. In Paris, we have a long-standing collaboration with Nicolette Farman and Jean-Pierre Bonvalet at the INSERM. Also, we collaborate with Daniela Rotin, Olivier Staub, and Hugh O'Brodovich at the
normal

ENaC

β

γ

α

Hect

NEDD 4

CaLB

Liddle's syndrome

ENaC

β

γ

α

Hect

NEDD 4

CaLB

Figure 9. A postulated model for ENaC-Nedd4 interactions, and implications for Liddle's syndrome. The three WW domains of Nedd4 bind to the PY motifs in the C termini of α, β, and γ ENaC, thereby bringing the ubiquitin ligase (Hect) domain in close proximity to ENaC. This allows the ubiquitination and subsequent degradation of the channel by proteosomes, with the resultant decrease in channel numbers. In Liddle's syndrome, deletions, mutations, or both within the PY motifs of βENaC (or γ ENaC) leads to abrogation of Nedd4-WW binding, resulting in a lack of the putative suppressive effect of Nedd4, which may explain the increase in channel activity associated with the Liddle phenotype. The role of the CaLB (C2) domain is not known, but it may be associated with channel mobilization from the apical membrane (adapted from reference 73).

University of Toronto, Haim Garty at the Weizmann Institute, and Steven Ernst at Ann Arbor. I would also like to acknowledge the tremendous input of former collaborators. Among them, I would especially like to mention Käthi Geering, Jean-Pierre Kraehenbühl, François Verrey, and Frédéric Jaisser, who have been involved in the sodium, potassium, and H,K-ATPase projects. M. Girardet, A. Truscillo, I. Corthévy, O. Staub, P. Burgener-Kairuz, A. Pellanda, M.-P. Paccolat, P.M. Mathews, P.X. Chen, H. Duperrex, C.M. Canessa, and A. Puoti are former students or postdoctoral fellows in the laboratory; they have all contributed to the work I have described. Finally, among many of my mentors, I would like to mention Georges Peters, Michel Dolivo, A.F. Müller, and especially Isidore Edelman—without him, I would not be here today. In a special category, I would like to mention my wife Michelle, and Anne and Hélène, my daughters; they are indeed very special mentors.

I would like to thank Doug Fambrough. I spent eight months in his laboratory in 1989 and 1990 and learned how to master site-directed mutagenesis with the PCR technique, which was just emerging at that time. But I learned more than novel techniques. I realized how significant a sabbatical stay is, during which you can be immersed in a novel and stimulating environment. This is also what we tried to offer to a number of foreign visitors who have been tempted by the “Lausanne” experience. I had the privilege of having a number of colleagues and friends spend 6 to 12 months in our laboratory. It has always been a very successful experiment for us. I hope it has been for them too. Over the years I had the pleasure to work with Tony Lo, Darrell Fanestil, David Warnock, Larry Palmer, James Schafer, Henry Binder, Robert Chevallier, Robin Felder, Nikolai Modyanov, and Steve Ernst.

Finally, I would like to return to Homer Smith. Homer Smith was a great scientist, and his input in renal physiology is now widely recognized, as was recently shown nicely at the occasion of a symposium entitled Symposium on Homer W. Smith and the Smith Legacy (79). Besides all his scientific achievements, I would like to stress an even more important facet of Homer Smith’s personality, namely his ability to recognize that he was not always right. In a famous Harlow Brooks Memorial Lecture entitled “The Fate of Sodium and Water in the Renal Tubules” (80), Homer Smith described the countercurrent exchange system that was proposed to account for the experimental data provided by Wirz and colleagues (81), a concept that was very much against the common model proposed by Homer Smith at the time. I would suggest to everybody that they read the Harlow Brooks Memorial Lecture as an example of scientific fairness, something that tends to disappear in such a competitive scientific environment of today’s life.

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