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New Insights into the Epithelial Sodium Channel Using Directed Mutagenesis

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1. Introduction

Directed mutagenesis is a fundamentally important DNA technology that seeks to change the base sequence of DNA and test the effect of the change on gene or DNA function. It can be accomplished using the polymerase chain reaction (PCR). For more than 20 years, many applications in both basic and clinical research have been revolutionized by PCR. The development of this technique allowed the substitution, addition or deletion of single or multiple nucleotides in DNA (Mullis and Faloona, 1987). Because of redundancy in the genetic code, such mutations do not always alter the primary structure of proteins. In this chapter, we will review the contribution of PCR-directed mutagenesis in the determination of the structure-function relationship of the epithelial sodium channel (ENaC), particularly with respect to the domains involved in proteolytic activation and ligand-induced stimulation of the channel.

2. Physiological role and structure of ENaC

ENaC is a key component of the transepithelial sodium transport. It is expressed at the apical membrane of a variety of tissues, such as the distal nephron of the kidney, lungs, exocrine glands (e.g., sweat and salivary glands) (Brouard et al., 1999; Duc et al., 1994; Perucca et al., 2008; Roudier-Pujol et al., 1996) and distal colon (Kunzelmann and Mall, 2002). In aldosterone-sensitive distal nephron (ASDN) and distal colon, this channel plays a major role in the control of sodium balance and blood pressure (Frindt and Palmer 2003; Garty and Palmer 1997). In lungs, ENaC regulates mucus secretion and aids in the protection of the airway surface (Randell and Boucher, 2006). Its role was clearly demonstrated in mice in which the ENaC gene was inactivated by homologous recombination (Hummler et al., 1996). ENaC belongs to a gene family with members found throughout the animal kingdom, the so-called ENaC/degenerin family, including the acid sensing ion channel (ACIC) and the Phe-Arg-Met-Phe amide-gated ion channel (FaNaCh), (Kellenberger and Schild. L, 2002). Using the *Xenopus* oocyte expression system and a distal colon cDNA library, the primary structure of ENaC was identified; and electrophysiologic characteristics of ENaC channel were determined (Canessa et al., 1993; Canessa et al., 1994; Lingueglia et al., 1993). ENaC is a heteromeric channel made of three subunits (α, β and γ) encoded by 3 different genes SCNN1a, SCNN1b and SCNN1g, respectively. Each subunit exhibits ~30% identity at the amino acid level and shares highly conserved domains. The

membrane topology of each subunit predicts the presence of two transmembrane domains (M1 and M2), a large extracellular loop (~70% of the size of the channel) and relatively short amino and carboxyl termini. The stoichiometry of ENaC was much discussed: several examples of biochemical and functional evidence are consistent with a heterotetrameric structure (2α, 1β, 1γ) (Anantharam A, 2007; Dijkink et al., 2002; Firsov et al., 1998), but octameric or nonameric structures have also been suggested (Eskandari et al., 1999; Snyder et al., 1998). Recent crystallographic data obtained on the related ASIC1 channel suggest ENaC most likely exists functionally as an αβγ heterotrimer complex (Jasti et al., 2007; Stockand et al., 2008). ENaC is characterized by high sodium selectivity ($P_{Na+}/P_{K+} > 100$), a low single-channel conductance (4-5 pS), gating kinetics characterized by long opening and closing times, and a specific block by amiloride (*Ki*: 100 -200 nM).

Sodium homeostasis requires that the entry of sodium through the apical membrane of epithelial cells is tightly controlled. This control may be realized by regulation of ENaC activity and expression. The role of different domains involved in this regulation has been determined by directed mutagenesis.

3. Mutations in ENaC subunits cause hereditary human disease

The role of ENaC in the regulation of blood pressure and regulation of extracellular fluid volume has been highlighted by the discovery of two severe human diseases. The diseases are due to loss or gain of function of ENaC. Homozygous inactivating mutations in the α, β or γ ENaC subunits cause pseudohypoaldosteronism type 1 (PHA-1), characterized by hypotension and severe hyperkalemic acidosis (Chang et al., 1996). Activating mutations in the genes for the β or γ ENaC subunits lead to Liddle's syndrome, characterized by autosomal-dominant hypertension accompanied by hypokalemic Alkalosis and volume expansion (Shimkets et al., 1994).

The mutations causing PHA-1 have been identified, and the mechanisms by which they led to a hypofunction of ENaC have been addressed. See (Kellenberger and Schild. L, 2002) for review.

In particular Chang et al. (1996) showed that a single point mutation (G37S) in the coding region for a highly conserved motif in the amino-terminal domain of the β subunit induces PHA-1. Grunder and co-authors (1997) showed that this domain is involved in the gating of ENaC. They identified that the mutation G37S in the gene for the β subunit and homologous mutations in the other subunit genes reduce channel function by changing the open probability.

Liddle syndrome has been linked genetically to mutations that delete or alter a conserved PY (proline-tyrosine) motif located in the carboxy-terminal domain of either β or γENaC (Hansson et al., 1995; Hansson et al., 1995; Shimkets et al., 1994; Tamura et al., 1996). Such deletions or point mutations lead to elevated channel function after expression in Xenopus oocytes, suggesting that the PY motif is involved in the regulation of activity and the density of ENaC channels at the cell surface (Firsov et al., 1996; Kellenberger et al., 1998; Schild et al., 1995; Schild et al., 1996; Shimkets et al., 1997). Mutations within the coding region for the PY motif were generated *in vitro* by directed mutagenesis. They have been widely studied to investigate the role of Nedd4-2 in the regulation of the number of ENaCs at the cell surface (Abriel and Horisberger, 1999; Debonneville et al., 2001; Kamynina and Staub, 2002;

Renauld et al., 2010; Staub et al., 1997). Thus, this has allowed the identification of tyrosine-carrying ubiquitin residues involved in Nedd4-2 dependent-internalization of the channel.

Intracellular C termini also harbor multiple phosphorylation sites and participate in the activity of the channel, suggesting that aldosterone, insulin, SGK1, PKA and PKC modulate the activity of ENaC by phosphorylation (Renauld et al., 2010; Shimkets et al., 1997).

4. Directed mutagenesis and regulation of ENaC by extracellular factors

Several members of the ENaC/degenerin family are clearly extracellular-ligand-gated channels. Numerous studies suggest that ENaC may also be a ligand-gated channel (Horisberger and Chraibi, 2004). A number of extracellular factors of various types have been shown to activate or inhibit ENaC. Amongst these factors, there are serine proteases, sodium itself, other inorganic cations, organic cations, and small molecules.

4.1 Activation by Serine proteases

In 1997 we cloned a serine protease that acts as a channel-activating protease, called CAP1 (Vallet et al., 1997); and we explored the mechanism by which it stimulates ENaC (Chraibi et al., 1998). We showed that the effect of CAP1 is done on the extracellular part of the channel, and it can be mimicked by trypsin or chymotrypsin. Ion selectivity, single channel conductance and channel density are not modified, which suggests that the serine proteases increase the open probability. During the last ten years, many studies showed that ENaC can be activated by other proteases, such as prostasin or furin (Hughey et al., 2004; Vuagniaux et al., 2000). Further progress in the understanding of the mechanism by which serine proteases activate ENaC has been made by functional investigation in heterologous expression systems combined with directed mutagenesis. Mutation of the CAP1 GPI-anchored consensus motif completely abolishes ENaC activation. However, catalytic mutants of CAP1 do not fully stimulate ENaC, suggesting that a noncatalytic mechanism is partly involved in this regulation pathway (Vallet et al., 2002). Thus, a putative site for CAP1 and trypsin action has been identified. However, there is no clear evidence of their role in the proteolytic activation of ENaC. Masilamani and co-authors (1999) first provided evidence for a possible cleavage of the γENaC subunit. These authors were able to show that the aldosterone infusion, or salt restriction, induced a shift in molecular weight of the gamma subunit from 85 to 70 KDa. Subsequently, it was shown that the serine proteases, including prostasin, plasmin, elastase and furin, cleave the extracellular domain of the α and γ subunits (Bruns et al., 2007; Caldwell et al., 2005; Hughey et al., 2004; Passero et al., 2008; Rossier, 2004; Vuagniaux et al., 2002). A basic motif ($RKRK^{186}$) has been identified as a cleavage site for CAP1/Prostasin in the extracellular loop of γENaC (Bruns et al., 2007; Diakov et al., 2008). Additional cleavage sites within extracellular loop of α and γ subunits have been described (Garcia-Caballero et al., 2008; Myerburg et al., 2006). However, no site for furin was described in βENaC (see Figure 1).

4.2. Effects of extracellular sodium and other small molecules

4.2.1 Self-inhibition

We have shown that the external sodium exerts a fast inhibitory effect on ENaC activity, a phenomenon called sodium self-inhibition (Chraibi and Horisberger, 2002). We observed

that the apparent affinity constant for the site responsible for self-inhibition was significantly lower, with a K½ of 100-200 mM. The kinetics of this phenomenon strongly depended on temperature and the extent of proteolytic processing of the ENaC subunits. We demonstrated that the effect of temperature was due to a large decrease in the probability of channel opening at high temperatures, while the unitary current increased with temperature (Chraibi and Horisberger, 2003). Later Sheng et al. (2004, 2002) showed that the mutation of His282 in the α subunit or His239 in the γ subunit (these amino acids reside in close proximity to the defined sites for furin cleavage) enhanced and eliminated the sodium self-inhibition response, respectively.

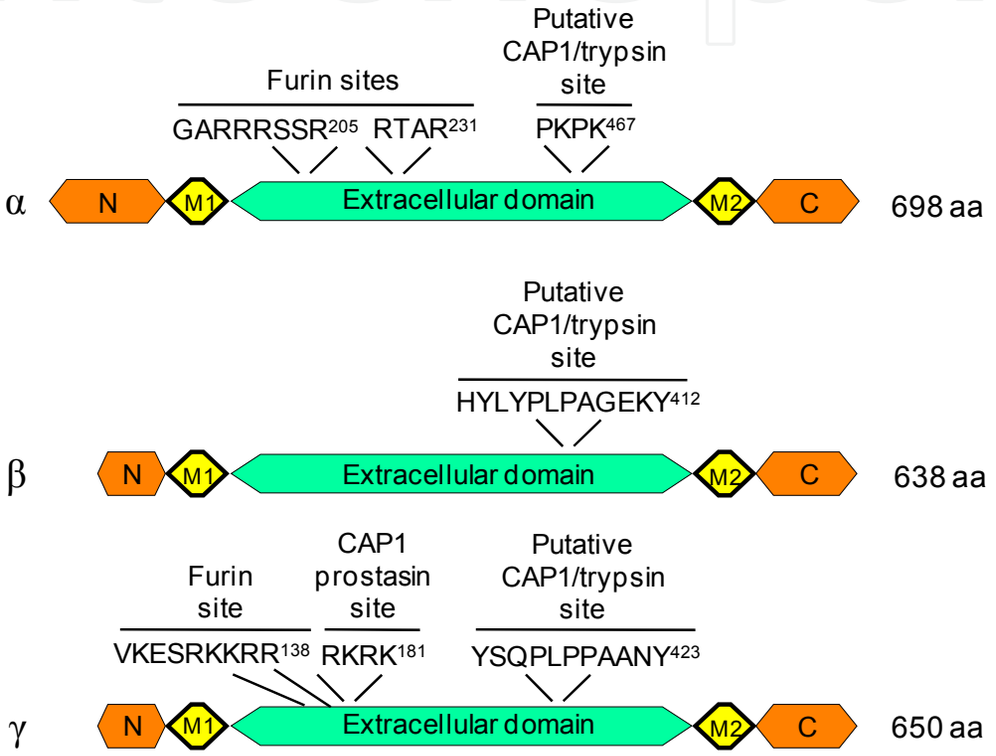

Fig. 1. Schematic representation of the rat ENaC subunits and their identified and putative sites for furin, CAP1, trypsin and prostasin. M1, M2: transmembrane domains; N, C: intracellular amino- and carboxy-termini, respectively.

4.2.2 Effects of cpt-cAMP and cpt-cGMP

cpt-cAMP, a membrane permeant cAMP analogue, has been described to be a species-dependent extracellular activator of ENaC. Rat and *Xenopus laevis* ENaC expressed in *Xenopus* oocytes are not sensitive to cpt-cAMP (Awayda et al., 1996). However, guinea pig (gp) channels could be activated by cpt-cAMP perfusion in the oocyte expression system (Liebold et al., 1996). The gp αENaC has been shown to be essential for this stimulation (Schnizler et al., 2000). However, the mechanism leading to ENaC stimulation did not exclude the possibility of an intracellular pathway involving protein kinase A (PKA). Further experiments demonstrated that PKA inhibitor PKI 6-22 did not prevent cpt-cAMP stimulation of gpENaC expressed in *Xenopus* oocytes. Furthermore, the α subunit containing the gp extracellular loop with rat intracellular C and/or N termini expressed in *Xenopus* oocytes together with rat βγ ENaC were

sensitive to cpt-cAMP (Chraibi et al., 2001). This chimeric channel demonstrated that the extracellular domain of the gp α subunit was the determinant for ENaC stimulation by cpt-cAMP. Thus, the molecule can be considered to be a ligand for the channel. Moreover, the outside-out configuration of the patch clamp showed an increase of the open probability and the number of open channels (N.Po) exposed to cpt-cAMP, confirming a direct interaction with the extracellular domain of the gpα, ratβγ chimera expressed in *Xenopus* oocytes. To determine which part of the extracellular domain of αENaC is involved in this regulation, we made four chimeric constructions of that subunit (Figure 2).

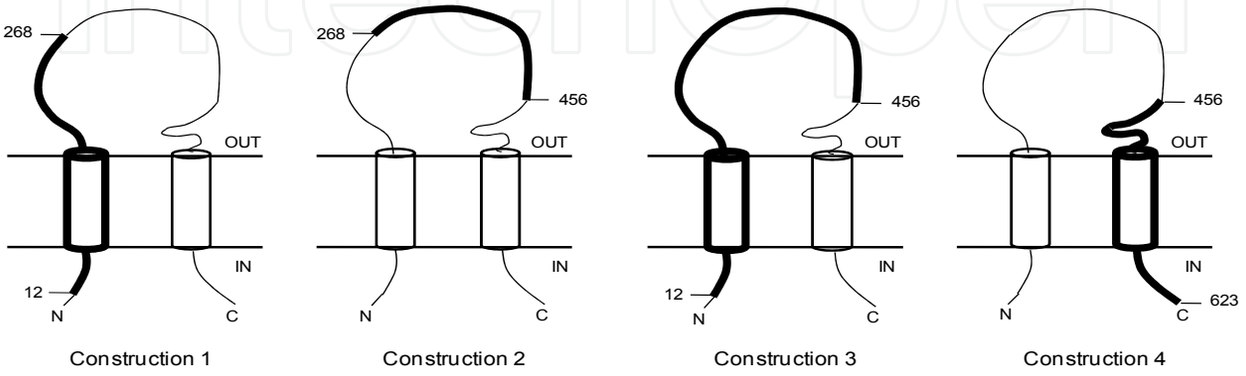

Fig. 2. Schematic representation of the ENaC subunits. Chimeric constructions of α subunits by fusion of the coding region for the αgp part (bold line) with the αrat part (thin line). Numbers indicate residues at corresponding positions on the αgp sequence.

To do so, two restriction sites were generated in guinea pig and rat αENaC cDNAs at homologous positions using a PCR technique. Then the appropriate fragment of gp cDNA was inserted into the rat cDNA between the restriction sites. Amiloride-sensitive current was measured in the presence and absence of 10 µM cpt-cAMP. We generated eleven swapping mutants of rat and gp αENaC using PCR-directed mutagenesis and expressed each of these mutants with the rat β and γ subunits in *Xenopus* oocytes. Among the eleven substitutions, Ile481 in the gp αENaC extracellular domain plays a major role in cpt-cAMP-induced ENaC activation. The *I481N* mutation in the gene for the αgp subunit completely abolished stimulation of ENaC. The *N510I* mutation in the gene for the αrat subunit caused intermediate sensitivity to cpt-cAMP. All other mutations or combination of mutations, including *N510I* in the αrat gene, did not increase the cpt-cAMP effect (Renauld et al., 2008).

Similarly to what we described with cpt-cAMP, Hong-Guan and coworkers (Nie et al., 2009) suggested that cpt-cGMP stimulates human, rat and mouse ENaC through direct interaction and not through the intracellular pathway. Indeed directed mutagenesis of the coding regions for potential phosphorylation sites for the cGMP-dependent kinases on ENaC did not affect cpt-cGMP-induced activation in *Xenopus* oocytes. Furthermore, knockdown of PKG isoforms did not prevent cpt-cGMP-dependent activation. Han and colleagues (2011) confirmed that cpt-cGMP-induced ENaC activation was mediated through direct interaction and an increases of N.Po. By directed mutagenesis, these authors were able to show that the mutations abolishing self-inhibition (βΔ*V348* and γ*H233R*) lost their responses to cpt-cGMP. The mutations augmenting this phenomenon (α*Y458A* and γ*M432G*) facilitated the stimulatory effects of this compound. Thus, these data suggest that the elimination of self-inhibition may be a novel mechanism for cpt-cGMP to stimulate ENaC.

α subunit	Icpt/Ictl	SEM	unpaired t-Test VS α rat wt
α gp wt	2.28	0.05	P<0.001
α rat wt	1.13	0.01	
construction 1	1.14	0.01	NS
construction 2	1.28	0.03	P<0.001
construction 3	1.34	0.03	P<0.001
construction 4	1.85	0.06	P<0.001
αr L493S	1.06	0.02	NS
αr S500N	1.08	0.03	NS
αr S507N	1.06	0.03	NS
αr I509T	1.05	0.02	NS
αr N510I	1.45	0.03	NS
αr K524T	1.02	0.03	NS
αr E531Q	1.12	0.02	NS
αr N542S	1.05	0.04	NS
αr K550N	1.12	0.02	NS
αr F554Y	1.16	0.02	NS
αr K561R	1.12	0.02	NS
αgp I481N	1.58	0.07	P<0.001

Table 1. Effect of cpt-cAMP on different constructions and mutants of the αENaC subunit expressed in *Xenopus* oocytes together with the β and γrat subunits. Results are presented as a ratio of amiloride-sensitive current measured after and before cpt-cAMP perfusion (Icpt/Ictl). gp, guinea pig; r, rat; wt, wild type; NS, not significant relative to αrat wt

4.2.3 Effects of glibenclamide

The same experimental approach was used to study the stimulation of ENaC by glibenclamide (Renauld and Chraibi, 2009). Glibenclamide, a high affinity-blocker of the K_{ATP} channel, has been shown to stimulate *Xenopus* ENaC (but not rat ENaC) expressed in *Xenopus* oocytes. The α subunit has been shown to be critical for this activation (Chraibi and Horisberger, 1999). As described with cpt-cAMP, patch clamp recordings in the outside-out configuration showed an increase of N.Po when *Xenopus* ENaC was exposed to glibenclamide. Another study has demonstrated that the αgp subunit, but not the αrat subunit, conferred sensitivity of ENaC to glibenclamide (Schnizler et al., 2003). Using mutagenesis, these authors were able to produce other chimeric rat/gp α subunits; and they suggested that the extracellular loop or the transmembrane domain of the αgp subunit is involved in the activation of the ENaC channel by glibenclamide. Thus, similarly to cpt-cAMP activation, channels composed of the αgp subunit and the β and γ subunits from rat are sensitive to glibenclamide, while channels composed of the α, β, and γ subunits from rat are resistant. We used the chimeras of the α subunit previously generated and found that construction 4 was also important for glibenclamide stimulation of the channel. Unlike cpt-cAMP, glibenclamide had no effect on the other constructions expressed with the β and γ subunits from rat. Moreover, directed mutagenesis did not reveal particular residues involved in this regulation.

α subunit	Iglib/Ictrl	SEM	unpaired t-Test VS α rat wt
α gp wt	1.63	0.02	P<0.001
α rat wt	0.96	0.01	
construction 1	1.03	0.04	NS
construction 2	1.04	0.05	NS
construction 3	1.03	0.01	NS
construction 4	1.27	0.04	P<0.001
αr L493S	0.92	0.03	NS
αr S500N	1.01	0.03	NS
αr S507N	0.98	0.02	NS
αr I509T	0.99	0.02	NS
αr N510I	1.00	0.01	NS
αr K524T	0.97	0.02	NS
αr E531Q	0.99	0.05	NS
αr N542S	0.98	0.02	NS
αr K550N	0.91	0.01	NS
αr F554Y	0.99	0.02	NS
αr K561R	0.96	0.02	NS

Table 2. Effect of glibenclamide on different constructions and mutants of αENaC expressed in *Xenopus* oocytes together with the β and γ subunits from rat. Results are presented as a ratio of amiloride-sensitive current measured after and before glibenclamide perfusion (Iglib/Ictl). gp, guinea pig; r, rat; wt, wild type; NS, not significant relative to αrat wt

4.2.4 Effects of other molecules

Capsazepine has been described as the first selective active activator for the δENaC subunit (Yamamura et al., 2004). Indeed capsazepine specifically stimulates human-made δ subunit, but not the α subunit expressed in *Xenopus* oocytes. Moreover, this molecule can stimulate the δENaC monomer, whereas no other vanilloid compound can produce changes in sodium amiloride-sensitive current. However, the authors did not determine any amino acids involved in this activation. Directed mutagenesis could be a powerful tool to understand differences between the α and δ subunits and resolve the structure-function relationships of both proteins.

S3969 is a small molecule described as a reversible activator of human, but not mouse, αβγ ENaC through direct interaction with the extracellular side of the channel by increasing N.Po (Lu et al., 2008). Interestingly, S3969 stimulates amiloride-sensitive current in oocytes expressing the δ subunit instead of α. The authors showed that βENaC was critical for this activation. Mouse-human chimeras of the β subunit confirmed the implication of the extracellular domain. More specifically, deletion of Val348 in βENaC completely abolished S3969 activation of ENaC. Maturation and optimal transport of ENaC to the plasma membrane requires furin cleavage of the β and γ subunits at a specific Arg. Mutations of the furin cleavage site in which Arg was replaced by Ala did not prevent ENaC activation by S3969, suggesting that proteolytic activation prior to S3969 stimulation is not necessary. Mutations producing pseudohypoaldosteronism type 1 (PHA1), resulting in salt-wasting, a genetic disease, have been generated in the α ($R508_{STOP}$) and β (*G37S*) subunits. These

mutants decreased amiloride-sensitive current, but the S3969 compound was still able to stimulate ENaC activity.

5. Conclusion

The epithelial sodium channel (ENaC) has been used for decades as a therapeutic target against type 1 hypertension and Liddle syndrome. More recently, several studies pointed to ENaC as a potential target for cystic fibrosis (Zhou et al., 2011), a pathology characterized by an impaired Cl^- secretion through the cystic fibrosis transmembrane conductance regulator (CFTR) and an increase of Na^+ reabsorption through ENaC. The studies of mutations involved in these diseases have been extremely helpful in determining the molecular mechanisms by which they lead to a dysfunction of ENaC. Furthermore, the experiments carried out on this topic have shown the contribution of the PCR-directed mutagenesis technique in the determination of the structure-function relationships of ENaC. These studies have led to a better understanding of the domains involved in ion selectivity, gating and expression of the channel at the cell membrane. Additional studies are needed to define other key domains of ENaC. They may provide a new strategy for the treatment of pathologies linked to dysfunction of this channel.

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