Editorial Focus – AJP-Renal

Cleavage – What’s up with prostasin and ENaC these days?

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Within the kidney, the epithelial sodium channel (ENaC) is located within the apical membrane of the epithelial cells of the late distal tubule, connecting duct and the collecting duct of the nephron with the major physiological task of ‘fine tuning’ of Na⁺ homeostasis. The channel is composed of three structurally related subunits (α, β γ) each having two membrane-spanning domains, with a large extracellular loop and intracellular N- and C-termini (2, 3). Nearly 35 years ago, Orce et al. (9) provided the first experimental evidence that serine protease inhibitors could inhibit transepithelial Na⁺ transport, thus, providing a potential role of proteases in the modulation of ENaC. Subsequently, Rossier and colleagues (12) provided convincing results that serine proteases activated ENaC, of which, one protease was channel activating protease 1 (CAP1, also known as prostasin).

Even though the involvement of serine proteases have been known for some time, the mechanism of activation of ENaC by proteases is still being unravelled (10). The α and γ subunits of ENaC have embedded inhibitory tracts (i.e., amino acid sequences) which once cleaved by proteases, activate the channel (1, 5). Proteolysis of ENaC occurs through two steps (8). It is thought the first step of proteolysis is at the level of the trans-Golgi network where Hughey et al. (6, 7) demonstrated that furin (a serine protease and a proprotein convertase) cleaves the α subunit twice at sites just following amino acids R205 and R231 (in the mouse sequence) and cleaves the γ subunit following R143 (in mouse). Further, Hughey and colleagues (7) noted that there was a population of channels lacking cleavage (nonprocessed) that were present at the cell surface, therefore, proteolytic processing of the channel subunits can also occur either along the biosynthetic pathway or once the channels arrive at the cell membrane. So, at that point, there must be a second proteolytic event of the γ subunit to further activate ENaC. Indeed, Bruns et al. (1) reported that when ENaC and prostasin were co-expressed either in MDCK cells or Xenopus oocytes a rapidly migrating second cleavage product of the γ subunit was observed. Additionally, they identified an
RKRK\textsuperscript{186} tract ~40 amino acid residues carbonyl-terminal to the furin cleavage site in the γ subunit as a potential prostasin-dependent cleavage site, but surprisingly, the catalytically-inactive prostasin (S238A, within the catalytic triad) was still able to cleave the γ subunit. Further, Bruns et al. (1) reported that when the RKRK\textsuperscript{186} site was substituted by glutamine (Q), neither prostasin nor the mutant prostasin were able to cleave the γ subunit. So, how do prostasin and mutant prostasin activate ENaC?

In this issue of *American Journal Physiology – Renal Physiology*, Marcelo Carattino and co-workers (4) provide exciting new insight regarding the mechanism of action of prostasin on the proteolytic processing of the γ subunit of ENaC. Indeed, with their studies, they demonstrate that mutant prostasin (S238A), expressed in *Xenopus* oocytes, activates ENaC by aiding the release of an embedded inhibitory tract from the γ subunit by an endogenous aprotinin-sensitive protease. Further, these authors provide *in vivo* evidence, from rats, that a restricted salt diet promotes cleavage and release of an embedded inhibitory tract from the γ subunit; which may be, in part, responsible for the observed elevated Na\textsuperscript{+} reabsorption in the animals.

Importantly, Carattino and colleagues (4) generated an antibody (anti-γ\textsuperscript{43}) raised against residues 131-187 in the γ subunit that recognizes the region 144-186 which encompasses the furin and prostasin cleavages sites spanning the inhibitory tract. Their results are summarized as follows. First, using *Xenopus* oocytes and anti-γ\textsuperscript{43} or anti-V5 (against the C-terminus of the γ subunit) antibodies, immunoprecipitation experiments were conducted using αβHA−γ−V5 channels alone or co-expressed with either prostasin or mutant prostasin (S238A). They demonstrated that the anti-γ\textsuperscript{43} antibody detected full-length (93 kDa) and furin-cleaved (83 kDa) γ subunits. The furin-cleaved polypeptide was not detected in immunoprecipitates from oocytes expressing both αβHA−γ−V5 channels and either prostasin or mutant prostasin suggesting a release of a sequence between RKRR\textsuperscript{143} and RKRK\textsuperscript{186}. 
Additionally, in the presence of prostasin or mutant prostasin, there was a shift of the 83 kDa band to a molecular weight of 77 kDa (using the V5 antibody) consistent with proteolytic cleavage at a site distal to the furin-cleavage site. Second, the authors, using HEK-293H cells, demonstrated that aprotinin, a serine protease inhibitor which prevents the activation of ENaC by prostasin (12) and whose binding site is located close to residues in the catalytic triad of prostasin (11), precipitated prostasin (via aprotinin-agarose beads) but not the mutant prostasin. Third, using two-electrode voltage clamp experiments with *Xenopus* oocytes, they reported that amiloride-sensitive currents were greater in oocytes co-expressing ENaC with either wild type or mutant prostasin compared with currents of oocytes expressing ENaC alone. Additionally, in the presence of aprotinin, the amiloride-sensitive currents were the same for oocytes expressing ENaC alone or co-expressed with either wild type or mutant prostasin. This is surprising since there was no evidence that the mutant prostasin binds with aprotinin, but, this suggests that the mutant prostasin may initiate cleavage and activation of ENaC by an endogenous protease. Fourth, the authors hypothesized that there must be a physical interaction between the mutant prostasin and ENaC which results in the proteolytic processing of ENaC by an endogenous protease. Indeed, results from co-immunoprecipitation experiments, using anti-V5 and anti-prostasin antibodies, clearly confirmed that ENaC and prostasin, wild type or mutant, were present in a protein complex. Finally, stepping from the oocytes to native tissue (kidney), Carattino and colleagues examined whether reducing the dietary Na⁺ intake of rats would result in the release of the inhibitory tract from the γ subunit. Using their anti-γ43 antibody and an anti-γ subunit C-terminal antibody, they observed a polypeptide representing a full-length γ subunit (~85 kDa) in control experiments using both antibodies. However, in animals fed a low Na⁺ diet, a 70 kDa polypeptide was immunoprecipitated by the anti-γ subunit antibody while this polypeptide was not present when immunoprecipitated with the anti-γ43 antibody. The low Na⁺ data suggest that salt restriction promotes cleavage of the γ subunit and a dissociation
of the γ subunit inhibitory tract which provides *in vivo* corroboration of the experimental data obtained from heterologous systems. Taken together, Carattino’s paper (4) provides exciting data that further defines the complexity of the protease-dependent cleavage and activation of ENaC in both an expression system and native epithelia. Overall, results by Carattino and colleagues (4) demonstrate that mutant prostasin promotes the cleavage and activation of ENaC by an endogenous aprotinin-sensitive protease.

The paper by Carattino and co-workers provides data that takes us closer to what may really be ‘happening’ with prostasin, and in fact, also catalytically-inactive prostasin. The authors used an antibody that recognizes the inhibitory tract in the γ subunit to show that wild type prostasin and mutant prostasin promote the release of the inhibitory tract. With this antibody, the authors have provided the first evidence that wild type prostasin or mutant prostasin and ENaC can form a protein complex. This is very promising and certainly provides an experimental ‘catapult’ to search for potential scaffolding partners (adaptor molecules or chaperones) that can recruit other serine proteases, which can cleave and activate ENaC. Having shown that prostasin and ENaC formed a protein complex, it will be exciting to know which residue(s) within prostasin allows it to bind to ENaC. The next step in this prostasin puzzle will be for Crattino and colleagues to identify the putative endogenous aprotinin-sensitive protease involved in the γ-subunit dependent activation of ENaC. It should be noted that readers who are interested in the present study are directed to a paper ‘in press’ from Per Svenningsen’s group (13) in which their study sheds light on the effects on protease processing of the γ subunit of ENaC of the human kidney under physiological conditions including proteinuria.

The *in vivo* rat data presented by Carattino and co-workers are compelling as this escalates their experimental data from heterologous expression systems to live animals. Using their antibody, they were able to recapitulate the enhanced cleavage and release of an inhibitory tract from the γ subunit of ENaC in the rats that were fed a low salt diet.
We are once again reminded by this paper by Carattino and co-workers, of the complexity of the mechanism by which prostasin cleaves and activates ENaC. These data will certainly fuel continued research into the role of protease cleavage and activation of ENaC.

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