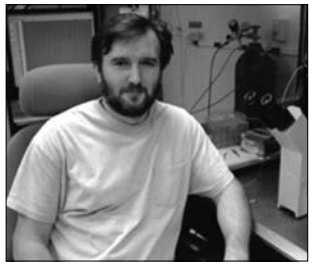
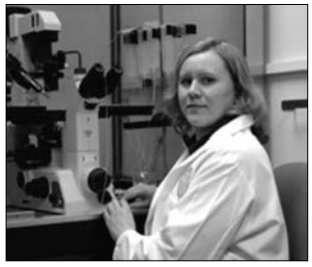
INTRINSIC CONTROL OF THE EPITHELIAL Na^+ CHANNEL (ENaC) BY PURINERGIC SIGNALING IN THE MAMMALIAN COLLECTING DUCT

V. KUCHER, N. BOIKO, J. DAVID STOCKAND, O. POCHYNIUK

University of Texas Health Science Center at San Antonio, Texas, USA
pochynyuk@uthscsa.edu



Volodymyr Kucher. *1992-1997: Shevchenko Kyiv State University, Radiophysics faculty (BSc, MSc); 1997-2001: Postgraduate student, Bohomolets Institute of Physiology NAS of Ukraine (PhD, Mentor I. Mahura), Kostyuk's dept; 2001-2007: Research scientist, Bohomolets Institute of Physiology NAS of Ukraine; 2007-2008: Postdoctoral fellow, University of Cincinnati, USA; 2008 — present: Postdoctoral fellow, University of Texas Health Science Center at San Antonio, USA.*



Nina Boiko. *1996-2001: National Technical University of Ukraine "KPI" (BSc, MSc); 2001-2005: Postgraduate student, Bohomolets Institute of Physiology, NAS of Ukraine (PhD, Mentor I. Magura), Kostyuk's dept; 2005-2007: Research scientist, Bohomolets Institute of Physiology NAS of Ukraine; 2008-present: Postdoctoral fellow, University of Texas Health Science Center at San Antonio, USA.*

James D. Stockand. *1987-1991: Texas A & M University, USA (BSc); 1992-1996: Univ. Texas HSC, Houston, USA (PhD); 1996-2000: Postdoctoral fellow, Emory Univ. Sch. Med., Atlanta, USA; 2000-present: University of Texas Health Science Center at San Antonio, Professor of Physiology.*

Oleh Pochyniuk. *1993-1999: Shevchenko Kyiv State University, Radiophysics faculty (BSc, MSc); 1999-2003: Postgraduate student, Bohomolets Institute of Physiology NAS of Ukraine (PhD, Mentors P. Kostyuk & E. Lukyanetz). 2004-2008: Postdoctoral fellow, University of Texas Health Science Center at San Antonio (Mentor J. Stockand). 2008-present: Research assistant professor, Department Physiology, UTHSC at San Antonio.*

The photos show the co-workers of P.G. Kostyuk: Kucher V., Boiko N., Pochynyuk O.

Introduction

The kidneys control reabsorption and excretion of water and electrolytes. This ability is pivotal for maintaining plasma volume and, thus, blood pressure (BP). Improper handling of a particular electrolyte, such as Na^+, results in many diseases associated with hypo- or hypervolemia. Sodium reabsorption at the distal part of the renal nephron finalizes plasma Na^+ levels. ENaC is localized at the apical plasma membrane of principal cells, where its activity is rate limiting for sodium movement across epithelial barriers. ENaC is a highly Na^+-selective, non-voltage gated, non-inactivating ion channel in the ENaC/Deg superfamily (Benos and Stanton, 1999). It is a heteromeric channel comprised of three distinct but similar subunits: α, β, and γ having stoichiometry of 1:1:1. ENaC activity is under tight control of systemic hormones via the renin-angiotensin-aldosterone system (RAAS). The physiological importance of ENaC to negative-feedback regulation of blood pressure in humans is emphasized by inheritable forms of severe hypertension resulting from gain of function mutations in the channel. In contrast, loss of function mutations leads to salt wasting and low blood pressure (Schild, 1996).

In addition to systemic control of ENaC activity by endocrine inputs via RAAS, complementary regulation of ENaC activity by paracrine factors has recently been suggested. Thus, increases in Na^+ delivery to the distal nephron upon salt loading do not cause as much increase in Na^+ reabsorption as expected. It appears that local signaling is blunting sodium reabsorption and probably ENaC activity in response to the elevated sodium input. Adonesine triphosphate (ATP) has been identified as a candidate signaling molecule possibly mediating intrinsic control of distal nephron Na^+ reabsorption. ATP is released by collecting duct cells constitutively and in response to increases in luminal flow. Moreover, ATP levels are precisely controlled by extracellular enzymes, such as ectonucleotidases.

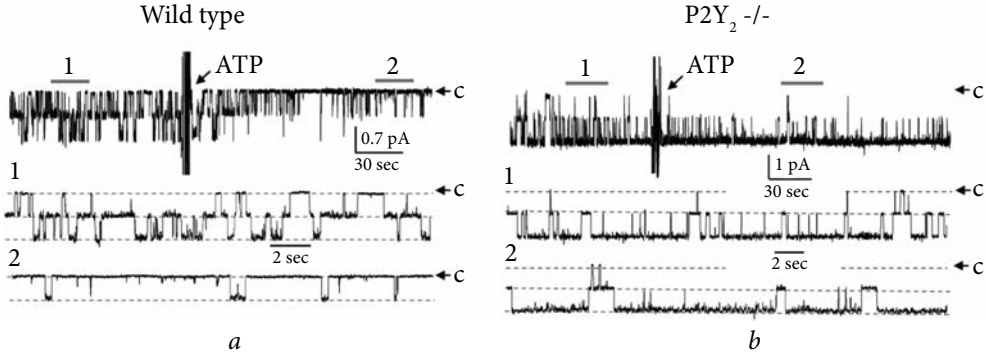


Fig. 1. ATP decreases ENaC activity in freshly isolated mice collecting duct cells; *a* — Representative continuous current trace from a cell-attached patch before and after treatment with ATP. Areas (*1*) and (*2*) are shown below at an expanded time scale. A *c* denotes the closed state; *b* — Genetic deletion of $P2Y_2$ receptors obliterates ATP actions on ENaC

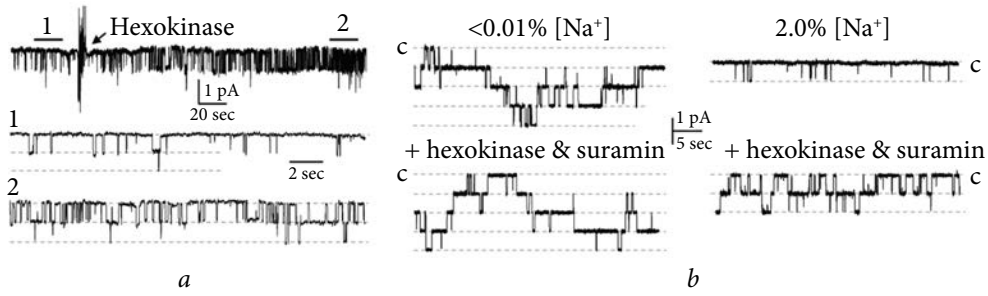


Fig. 2. Endogenous ATP released from collecting duct principal cells tonically decreases ENaC activity: *a* — representative continuous current trace for ENaC before and after addition of hexokinase. (*1*) and (*2*) are shown below at an expanded time scale; *b* — representative current traces from cell-attached patches containing ENaC from WT mice kept on low and high Na^+ diets in the absence (top) and presence of hexokinase plus suramin (bottom) to degrade local ATP and prevent nucleotide receptor activation, respectively

Together, this argues that local purinergic signaling is well designed to regulate ENaC activity in a paracrine manner.

We used patch clamp electrophysiology on freshly isolated split-opened murine collecting ducts to test the hypothesis that ENaC activity is directly regulated by local purinergic signaling. The representative current trace in Fig. 1A is a typical cell-attached experiment demonstrating that ATP acutely decreases ENaC activity by reducing open probability (P_o). ATP targets ligand-gated P2X and G-protein coupled P2Y receptors. A number of different purinergic receptors are expressed in the collecting duct, including $P2Y_{1,2,4,6,11}$ and $P2X_{1,3\text{-}6}$. Pharmacological inhibition of distinct purinergic receptors revealed that $P2Y_2$ receptors play the major role in the signaling cascade coupling ATP to ENaC (Pochynyuk et al., 2008a). Importantly, experiments on mice engineered to lack $P2Y_2$ receptors are consistent with this conclusion. Thus, ATP slightly affect ENaC activity and P_o in $P2Y_2$ -/- animals as shown in Fig. 1, *b*. This unequivocally demonstrates that $P2Y_2$ receptors are essential in the control of ENaC by local purinergic signaling.

Next, we asked: what are the patho-physiological consequences of ENaC function of inappropriate purinergic regulation? Single channel analysis revealed that disruption of local ATP signaling in $P2Y_2$ -/- mice resulted in elevated basal ENaC activity with the channel being locked in a hyperactive state with an extremely high P_o (Pochyniuk et al., 2008a). This is consistent with the recent finding that mice lacking $P2Y_2$ receptors have hypertension associated with facilitated renal sodium reabsorption (Rieg et al., 2007). This raises an intriguing probability that ENaC activity in the distal nephron is tonically inhibited by endogenous ATP and that disruption of this regulation in $P2Y_2$ -/- mice releases ENaC from the inhibition causing excessive Na^+-reabsorption and subsequently hypertension. Indeed, quenching endogenous ATP with hexokinase causes marked increases in ENaC activity above basal values as demonstrated in Fig. 2, *a*. More-

over, inhibiting purinergic receptors with suramin in the absence of any other stimuli yields similar results (Pochynyuk et al., 2008a; Pochynyuk et al., 2008b).

ENaC activity in the collecting duct is known to be controlled by sodium intake via RAAS. Thus, dietary salt restriction causes increase in ENaC activity and, conversely, salt loading markedly decreases ENaC activity. Interestingly, we found that local purinergic signaling has greater inhibitory effect on ENaC in mice loaded with Na^+ compared to salt restricted ones. Quenching endogenous ATP upon treatment with hexokinase plus suramin exerted only a minor stimulatory effect on ENaC activity in mice kept on a nominally free Na^+ diet but markedly increased ENaC P_o in animals kept on a high (2%) Na^+ regimen (Fig. 2, *b*). The salt-dependent effect of purinergic regulation of ENaC might have a beneficial role by dampening Na^+ reabsorption upon increased sodium intake. This can help avoiding excessive sodium conservation. Surprisingly, we found that ENaC P_o is always clamped to a high level and no longer sensitive to changes in dietary sodium for $P2Y_2$ -/- mice. This inability to adjust ENaC activity results in inappropriate sodium handling and, thus, hypertension which becomes more severe upon increased sodium intake.

We next delineated the signaling pathway coupling ATP with changes in ENaC activity. As discussed above, we found that, among different purinergic receptors expressed in the collecting duct cells, $P2Y_2$ receptors play the central role in transducing ATP actions. $P2Y_2$ receptors are mainly coupled to phospholipase C (PLC) via the $G_{q/11}$ cascade. Direct stimulation of PLC acutely decreases ENaC activity in similar to ATP manner and extent. Moreover, inhibition of PLC abolishes ATP-mediated decreases in ENaC activity (Pochynyuk et al., 2008b). These findings place PLC as a requirement for purinergic signaling pathway to ENaC. Activation of PLC hydrolyses the membrane phosphatidylinositide, $PI(4,5)P_2$ to yield DAG and IP_3 leading to stimulation of protein kinase C (PKC) and *raises* in intracellular Ca^{2+}. However, inhibiting PKC and the endoplasmic Ca^{2+} pump (SERCA) did not prevent ATP actions on ENaC (Pochynyuk et al., 2008b) (unpublished observations, 2009). We hypothesized that changes in membrane $PI(4,5)P_2$ levels rather than its downstream effectors may account for decreases in ENaC activity in response to ATP stimulation.

To directly monitor $PI(4,5)P_2$ metabolism during purinergic signaling, we *next* expressed a fluorescent $PI(4,5)P_2$ reporter, GFP-tagged PLC-δ1-PH, in cultured polarized principal cells. Total internal reflection fluorescent microscopy (TIRF) allows isolating a fluorescent signal from the apical plasma membrane (Axelrod, 1989). The representative TIRF micrographs in Fig. 3 show GFP emissions upon addition of vehicle (top), ATP (middle), and the PLC inhibitor, U73122 (bottom). ATP rapidly decreases apical $PI(4,5)P_2$ levels. Inhibiting PLC with U73122, in contrast, increases $PI(4,5)P_2$ levels above the basal values. ATP failed to act when co-applied with U73122. These results are important for several reasons. First, they demonstrate that rapid changes in $PI(4,5)P_2$ levels correspond to decreases in ENaC

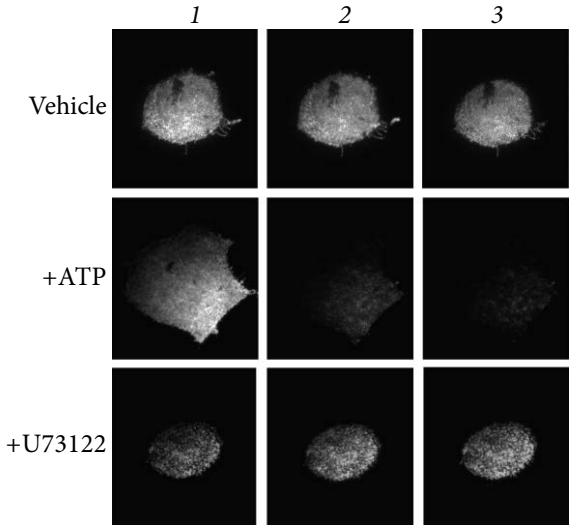


Fig. 3. Apical plasma membrane PI(4,5)P_2 levels are dynamically controlled by exogenous ATP via activation of PLC. Fluorescence micrographs of emission from the PI(4,5)P_2 reporter in the apical plasma membrane of cultured principal cells within a confluent monolayer before (*1*), and 5 (*2*), and 15 (*3*) min after treatment with vehicle (top), ATP (middle), and the PLC inhibitor U73122 (bottom). Emissions were collected in a paired manner using TIRF microscopy

activity upon purinergic stimulation. Second, they suggest that resting PI(4,5)P_2 levels are not saturated and inhibiting PLC alone is able to further increase apical PI(4,5)P_2 levels. This is consistent with ENaC activity being tonically inhibited by endogenous purinergic signaling via control of PI(4,5)P_2 levels.

Several recent findings are consistent with the idea that ENaC activity can be acutely regulated by membrane PI(4,5)P_2 levels (Pochynyuk et al., 2006; Pochynyuk et al., 2007b). In general, channel-PI(4,5)P_2 interactions are likely to be electrostatic in nature where negatively charged head groups of PI(4,5)P_2 are attracted by positive charged regions within the channel which are rich in basic residues. All three ENaC subunits contain such regions at their N-terminus and near both trans-membrane domains. Thus, we next created mutant subunits that lack positive charged residues within their intracellular domains and reconstituted ENaC into Chinese Hamster Ovary (CHO) cells. Acute depletion of the plasma membrane PI(4,5)P_2 levels markedly decreased both macroscopic ENaC current and channel P_o for wild type. However, channels lacking positive charged residues in the extreme N-termini in β- and γ- subunits (γN+βN) were resistant to manipulations of PI(4,5)P_2 (Pochyniuk et al., 2007a). Moreover, direct administration of PI(4,5)P_2 increased ENaC P_o for wild type but not for γN+βN mutant channels. Therefore, we concluded that PI(4,5)P_2 interacts and possibly *binds* to these specific regions within ENaC to control open channel probability. Dwell time analysis revealed that PI(4,5)P_2 availability directly controls ENaC gating. Decreases in the PI(4,5)P_2 levels increased ENaC closed time durations and decreased open time durations. In contrast, increases in PI(4,5)P_2 caused shortened closed time durations and prolonged open time durations (Pochynyuk et al., 2008b).

Overall, as outlined in our model of purinergic control of ENaC (Fig. 4), dietary Na^+ restriction stimulates RAAS and consequently ENaC to conserve sodium.

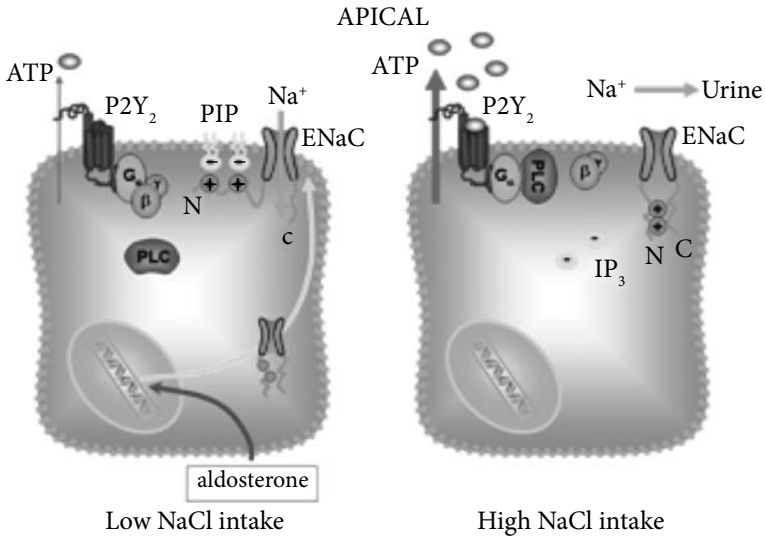


Fig. 4. A model for the regulation of ENaC open by dietary NaCl and aldosterone through altering apical ATP/$P2Y_2$ receptor tone in the aldosterone-sensitive distal nephron

Simultaneously, this leads to decrease in paracrine ATP release. Scarce ATP fails to induce $P2Y_2$ receptor activation. When $P2Y_2$ receptors are not activated, the inner leaflet of the apical plasma membrane contains high concentrations of negatively-charged $PI(4,5)P_2$, which are bound to positively charged regions of the N-termini in β- and γ-subunits of ENaC, thereby maintaining ENaC open and promoting Na^+ reabsorption. Conversely, high NaCl intake increases the apical availability of ATP most likely via increases in luminal flow. Stimulation of $P2Y_2$ receptors activates PLC, which, in turn, hydrolyzes and lowers the membrane $PI(4,5)P_2$ concentration. This induces conformation changes in the N-termini of β- and γ-ENaC and lowers ENaC P_o thereby dampening Na^+ reabsorption.

Our findings are consistent with ATP acutely affecting ENaC gating. However, the exact location and the mechanisms that control ENaC gate remain elusive. ENaC belongs to a large superfamily of ENaC/Deg channels. In contrast to other members of the family, ENaC is constitutively active with no prominent voltage sensitivity and demonstrates no time-dependent activation and inactivation. In addition, ENaC has slow gating kinetics with open and close times ranging within seconds (Fig. 5, *a*).

All members of the ENaC/Degenerin channel superfamily contain an absolutely conserved tryptophan (Trp) at the base of their first trans-membrane domain. Trp residues often reside at lipid-water interfaces in helical trans-membrane domains of many ion channels. When facing an aqueous environment, Trp may form a ring-like structure that acts as a gate, as found for M_2 proton channels. In addition, these Trp residues may play an important role in stabilizing the inner

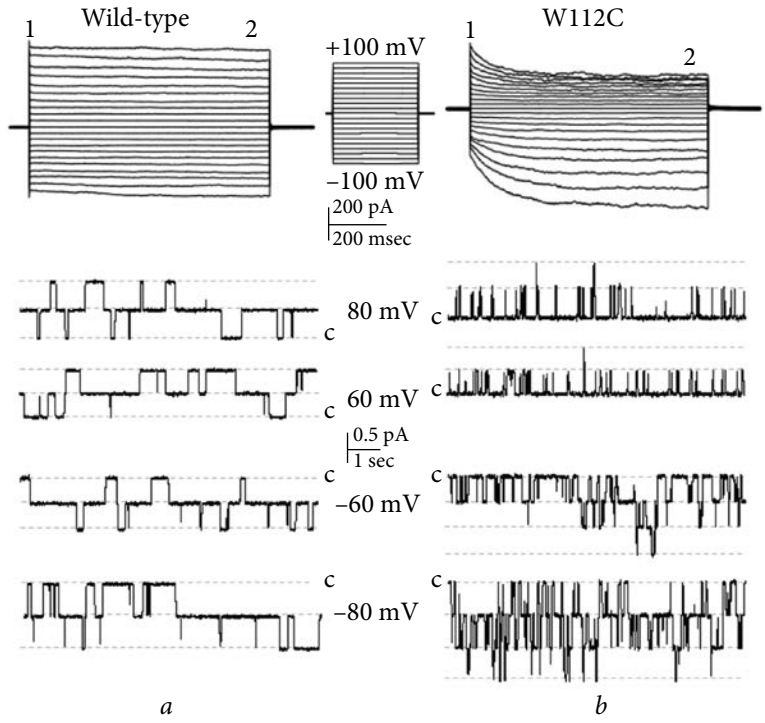


Fig. 5. Substitution of the conserved Trp at the base of TM1 confers ENaC voltage-sensitivity**.** Representative families of macroscopic currents evoked by a voltage-step protocol (inset) for wild type (*a*) and mutant ENaC (containing W112C α-subunits) (*b*) in voltage-clamped CHO cells in symmetrical solutions. Shown at the bottom are representative single channel current traces at test potentials ranging from 80 to –80 mV in excised, outside-out patches for wild type (left) and the mutant ENaC (right) expressed in CHO cells

vestibule of the pore. Thus, we next tested whether these Trp residues serve a critical role with respect to ENaC function and gating. Targeted substitution of the Trp in α- and β-ENaC markedly decreased ENaC activity and, unexpectedly, unmasked pronounced voltage-sensitivity. Mutant ENaC increased steady-state activity upon hyperpolarizing compared to hyperpolarizing voltages with transient activation and deactivation kinetics, respectively. This voltage-sensitivity reflected voltage dependent changes in ENaC P_o (Fig. 5, *b*). Voltage sensitivity was Na^+ dependent with intracellular [Na^+] playing a particularly important role in setting the voltage at which mutant ENaC has half-maximal activity. We have concluded that stabilization of the pore by Trp at the base of TM1 lessens Na^+-dependent modulation of wild type channels likely by interfering with an allosteric or pore block mechanism (Pochynyuk et al., 2009). In addition, all ENaC/Deg channels have an absolutely conserved Histidin-Glycine (HG) motif at the position that precedes the Trp by 12 amino acids. Surprisingly, targeted substitution of either residue revealed a phenotype identical to that for Trp substitution.

Importantly, mutations in the HG motif cause pseudohypoaldosteronism type 1 and associated renal salt wasting in humans. We speculate that the conserved Trp in TM1 interacts with the cytosolic HG motif to stabilize the pore and acts as an actual channel gate. Disruption of this structure then can destabilize the pore to increase sensitivity to intracellular Na^+ with voltage affecting Na^+ block or binding in the destabilized state.

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