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Hong Long Ji

Hong Guang Nie

Yongchang Chang

Barrow Neurological Institute, yongchang.chang@dignityhealth.org

Qizhou Lian

Shan Lu Liu

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Review

CPT-cGMP Is A New Ligand of Epithelial Sodium Channels

Hong-Long Ji¹✉, Hong-Guang Nie², Yongchang Chang³, Qizhou Lian⁴, Shan-Lu Liu⁵

1. Department of Cellular and Molecular Biology, University of Texas Health Science Center at Tyler, Tyler, Texas 75708, USA.
2. Institute of Metabolic Disease Research and Drug Development, China Medical University, Shenyang 110001, China.
3. Barrow Neurological Institute, St Joseph's Hospital and Medical Center, Phoenix, Arizona, 85013, USA.
4. Department of Medicine, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong, China.
5. Department of Molecular Microbiology and Immunology, Bond Life Sciences Center, University of Missouri, Columbia, MO 65211, USA.

✉ Corresponding author: Hong-Long (James) Ji, 11937 US Highway 271, 604 BMR, Tyler, TX 75708-3154. E-mail: james.ji@uthct.edu. Telephone: 903-877-7418. Fax: 903-877-5438.

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Abstract

Epithelial sodium channels (ENaC) are localized at the apical membrane of the epithelium, and are responsible for salt and fluid reabsorption. Renal ENaC takes up salt, thereby controlling salt content in serum. Loss-of-function ENaC mutations lead to low blood pressure due to salt-wasting, while gain-of-function mutations cause impaired sodium excretion and subsequent hypertension as well as hypokalemia. ENaC activity is regulated by intracellular and extracellular signals, including hormones, neurotransmitters, protein kinases, and small compounds. Cyclic nucleotides are broadly involved in stimulating protein kinase A and protein kinase G signaling pathways, and, surprisingly, also appear to have a role in regulating ENaC. Increasing evidence suggests that the cGMP analog, CPT-cGMP, activates $\alpha\beta\gamma$ -ENaC activity reversibly through an extracellular pathway in a dose-dependent manner. Furthermore, the parachlorophenylthio moiety and ribose 2'-hydroxy group of CPT-cGMP are essential for facilitating the opening of ENaC channels by this compound. Serving as an extracellular ligand, CPT-cGMP eliminates sodium self-inhibition, which is a novel mechanism for stimulating salt reabsorption in parallel to the traditional NO/cGMP/PKG signal pathway. In conclusion, ENaC may be a druggable target for CPT-cGMP, leading to treatments for kidney malfunctions in salt reabsorption.

Key words: amiloride-sensitive sodium channel; cyclic guanosine nucleotides; molecular docking; lung edema.

Introduction

Cyclic guanosine monophosphates (cGMP) are important cyclic nucleotides, playing a critical role in signal transduction in many different organisms¹. Signal transduction in eukaryotic cells is essential for moderating the transmission of information through the cell membrane, and as a second messenger, cGMP regulates numerous essential processes in cells by amplifying external signals, for example, from hormones and neurotransmitters. Cytosolic cGMP binds to cyclic nucleotide-gated ion channels^{1, 2} and phosphorylates protein kinase G (PKG) directly³⁻⁵.

Amiloride-sensitive epithelial sodium channels (ENaC) behave as ligand-gated channels, similar to several other members of the ENaC/degenerin family⁶. In the late part of the renal distal convoluted

tubule, sodium is predominately reabsorbed via the electrogenic amiloride-sensitive ENaC. In the connecting tube, and the collecting duct, ENaC is the only pathway for retaining salt, and ENaC controls renal sodium excretion and provides the driving force for potassium secretion through the renal outer medullary potassium channel⁷. Loss-of-function ENaC genetic variants lead to low blood pressure, while gain-of-function mutants have been identified in Liddle's syndrome, which is characterized by increased ENaC abundance, augmented opening time, and consequent salt retention, and eventually volume-expanded hypertension⁸. An autosomal recessive form of pseudohypoaldosteronism type 1 is caused by mutations in ENaC, with usually severe

and persisting multiorgan symptoms ⁹.

Five ENaC subunits have been cloned to date, namely α -, β -, γ -, δ -, and ϵ -ENaC. Among them, the β - and γ -subunits regulate the channel activity of the 'self-conducting' α -, δ -, and ϵ -ENaC subunits when heterologously expressed in oocytes and cell lines ¹⁰⁻¹². Luminal impermeable reagents and hormones have been confirmed to regulate ENaC in lung, kidney, and colon, and ENaC is regulated by a spectrum of protein kinases, such as PKG and protein kinase A (PKA) ¹³⁻¹⁵. However, our understanding of the cellular and molecular mechanisms by which cGMP regulates ENaC is incomplete.

Regulation of Epithelial Sodium Channels by CPT-cGMP

Cyclic GMP is one of the most prominent nucleotides in eukaryotic cells. Guanylyl cyclases elevate cell cGMP levels, which regulate complex signaling cascades through immediate downstream effectors, cGMP-dependent protein kinases, and cyclic nucleotide-gated ion channels ¹. CPT-cGMP is a derivative analog of parental cGMP, and has long been used for studying the NO/cGMP/PKG cascade ^{16, 17}. Regulation of epithelial sodium channels by CPT-cGMP and its cAMP analog is summarized in Table 1. Species- and culture-dependence of ENaC properties, and the strategies used for elevating cell cGMP content, including nitric oxide (NO) donors and PKG isoform-specific cGMP analogs, may contribute to these divergent observations.

Cyclic GMP/PKGII pathway

To date, three PKG isoforms have been isolated, namely, PKGI- α , PKGI- β , and PKGII ¹⁸. Accumulating evidence from genetically engineered animals suggests that cardiovascular phenotypes are

predominant with the PKGI knockout, while PKGII deficiency leads to dysfunction in epithelial tissues [19, 20]. Our results using Ussing chamber and voltage clamp techniques showed that 8-pCPT-cGMP increased the amiloride-sensitive short-circuit current across H441 cell monolayers and also increased heterologously expressed $\alpha\beta\gamma\delta$ -ENaC activity in a dose-dependent manner, most probably through the stimulation of PKGII enzymatic activity and subsequent activation of channel function ¹⁵ (Figure 1). A cartoon briefly showed the interaction of cGMP and ENaC (Figure 2). Recent studies showed that PKG-interacting proteins mediate cellular targeting of PKG isoforms by interacting with their leucine zipper domains, and that protein recognition is mediated through surface charge interactions ¹⁹.

Extracellular ligand pathway

In sharp contrast to the native ENaC, heterologously expressed $\alpha\beta\gamma$ -ENaC in oocytes has been considered to be cAMP/PKA-independent, particularly for acute regulation. The cell-permeable PKGII isoform-specific activator, 8-pCPT-cGMP, acutely stimulates human, rat, and mouse $\alpha\beta\gamma$ -ENaC reversibly. Furthermore, intraoocyte domains of ENaC subunits and traditional soluble protein kinases may not be targets of this compound ²⁰. A critical extracellular domain has recently been identified as the 8-CPT-cAMP binding site ²¹, indicating that 8-CPT-cAMP is an extracellular ligand for ENaC ^{6, 21}. Moreover, 8-CPT-cAMP can stimulate $\delta\beta\gamma$ -ENaC up to approximately 3-fold, and $\alpha\beta\gamma$ -ENaC by 2-fold ¹⁴. Co-expression of δ -ENaC with $\alpha\beta\gamma$ channels conferred CPT-cAMP-mediated activation with an EC₅₀ (concentration for 50% of maximal effect) value of 30 μ M, similar to that for $\alpha\beta\gamma$ channels (49 μ M) ²².

Table 1. Regulation of sodium channel activity by nucleotides.

Nucleotides	Concentration	Models	Effects	Ref.
<u>CPT-cGMP*</u>	1 μ M	Xenopus 2F3 cells	Increase in I _{sc}	62
	400 μ M	Rat ATII cells	No change in I _{sc}	63
	2 mM	Rat bronchioalveolar epithelial cells	Increase in I _{sc} / ²² Na influx	64, 65
	100 μ M	Rat ATII cells	Increase in whole cell activity	66
	4 mM	Human A549 cells	Increase in whole cell activity	67
	10 μ M	Frog urinary bladder epithelium	Increase in single channel activity	68, 69
	0.1 - 1000 μ M	Heterologous human $\alpha\beta\gamma$ ENaC in oocytes	Increase in whole cell activity	16
	0.5 mM	Human lung lobes	Increase in alveolar fluid clearance	24
	0.2 mM	Mouse pleural tissues	Increase in I _{sc}	15
	1 mM	Rat ATII cells	Decrease in single channel activity	56
	100 μ M	Human A549 cells	Decrease in whole cell activity	70
	100 μ M	MDCK cells	Increase in I _{sc}	
	40 μ M	Liddle's disease lymphocytes	Increase in whole cell activity	71
	200 μ M	Heterologous human $\alpha\beta\gamma$ ENaC in oocytes	Increase in whole cell activity	14, 21, 72
	100 μ M	BeWo cells	Increase in whole cell activity	73
<u>CPT-cAMP</u>				

*Abbreviations: ATII, alveolar type II epithelial; MDCK, Madin-Darby canine kidney; I_{sc}, short-circuit current; CPT-cAMP, 8-pCPT-cAMP-Na; CPT-cGMP, 8-pCPT-cGMP-Na.

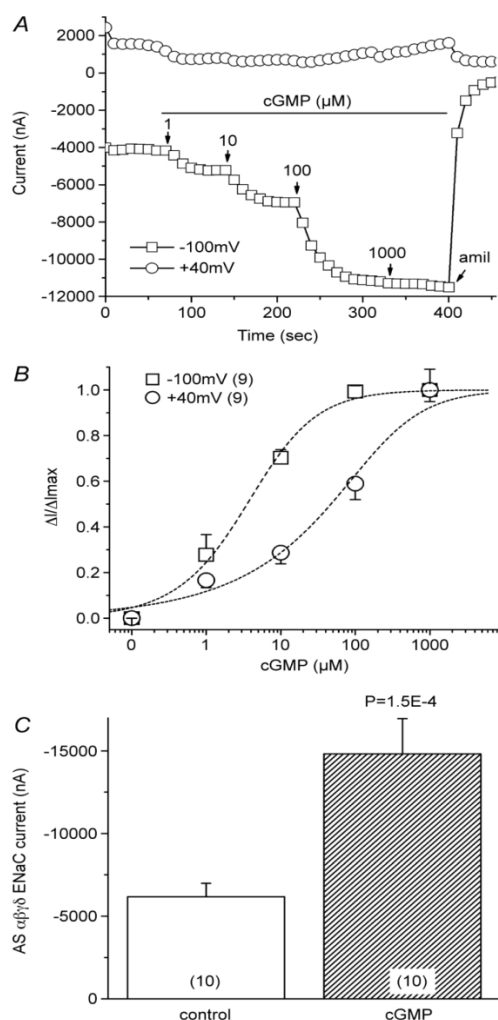


Figure 1. Stimulation of heterologously expressed human ENaCs in *Xenopus* oocytes by cGMP. A: Representative current trace recorded at +40 mV and -100 mV. Oocytes were perfused with 8-pCPT-cGMP ranging from 1 μM to 1 mM, as indicated by arrows. Amiloride was added to the bath before the recordings were terminated. B: Dose-response curve. The average cGMP-activated current fraction in the presence of cGMP (ΔI) was normalized to the maximal cGMP-elevated current (ΔI_{max}) and plotted as a function of cGMP concentration. Dashed lines and the EC_{50} value were derived by fitting the raw data with the Hill equation. $n=9$. C: Average amiloride-sensitive (AS) $\alpha\beta\gamma\delta$ ENaC currents before (control) and after cGMP perfusion. Holding potential, -100 mV. $n=10$. Figure reprinted from ¹⁵, and used with permission.

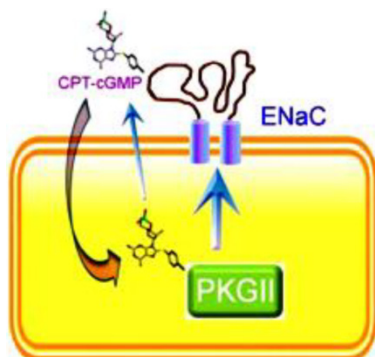


Figure 2. Extracellular and intracellular mechanisms for CPT-cGMP or CPT-cAMP to activate ENaC. These cell permeable compounds, when applied *in vivo* or *in vitro*, will stimulate ENaC activity by both acting as an external ligand to release self-inhibition and as a cytosolic signal molecule to phosphorylate cGKII that subsequently activates ENaC.

Relieving external sodium self-inhibition

External sodium self-inhibition is an intrinsic feature of ENaC. A rapidly increase in extracellular sodium ions to a physiological concentration (150 mM for mammals and 100mM for amphibians) generates a maximal peak current in seconds, and then the permeability of ENaC to Na^+ ions is gradually reduced to a relatively stable level with a current level of approximately half of the maximal value. This phenomenon is called extracellular sodium self-inhibition of ENaC activity. It differs from the down-regulation of ENaC activity by slowly accumulating intracellular Na^+ content in a feedback manner. External Na^+ self-inhibition is a crucial mechanism to limit overwhelming salt absorption to prevent a quick raise in epithelial cell volume and blood pressure. It maintains salt and fluid homeostasis at the luminal surface of the respiratory system to keep normal cilia beating in the airways, hyperpolarize apical membrane, and finely adjust fluid volume to host leukocytes and physiological regulators as the forefront battle against noxious aspirated insults ²².

Our previous studies showed that external CPT-cAMP stimulated human, but not rat and murine, $\alpha\beta\gamma$ -ENaC in a dose-dependent and external sodium concentration-dependent fashion ¹⁴. ENaC mutations that abolished self-inhibition ($\beta \Delta V348$ and $\gamma H233R$) almost completely eliminated CPT-cAMP mediated activation. In contrast, mutations that both enhanced self-inhibition and elevated CPT-cAMP sensitivity increased the stimulating effects of the compound. Our above data confirmed that CPT-cAMP acts as a ligand to regulate heterologous ENaC by relieving self-inhibition. Edelheit et al ²³ studied alanine mutations in 17 conserved charged residues of ENaC and found that these residues are involved in conformational changes that lead to channel constriction and to the sodium self-inhibition response upon sodium ion flooding. Similarly, our recent data showed that elimination of self-inhibition of $\alpha\beta\gamma$ -ENaC may be a novel mechanism for CPT-cGMP to stimulate salt reabsorption in the human epithelium (Figure 3) ²⁴.

Moiety specificity for CPT-cGMP serving as an ENaC ligand

CPT-cGMP, an analog of CPT-cAMP, was capable of activating ENaC in the identical manner in cell-free outside-out patches ¹⁶. Both point mutations of the putative consensus PKG phosphorylation sites and truncation of entire cytosolic NH₂- and COOH-terminal tails of ENaC did not alter the response to CPT-cGMP. The ENaC activity was activated to the same extent by CPT-cGMP in cells in

which PKGII expression was knocked down using small interfering RNA. As we demonstrated, the parachlorophenylthio moiety and ribose 2'-hydroxy group of CPT-cGMP are crucial for activating ENaC¹⁶, and CPT-cGMP may serve as a novel ENaC ligand

in addition to activating the PKG signal. The strict requirement for both parachlorophenylthio and the ribose 2'-hydroxy groups helps to clarify the basis for the inconsistent results observed with the regulation of ENaC by a variety of cGMP analogs (Figure 4).

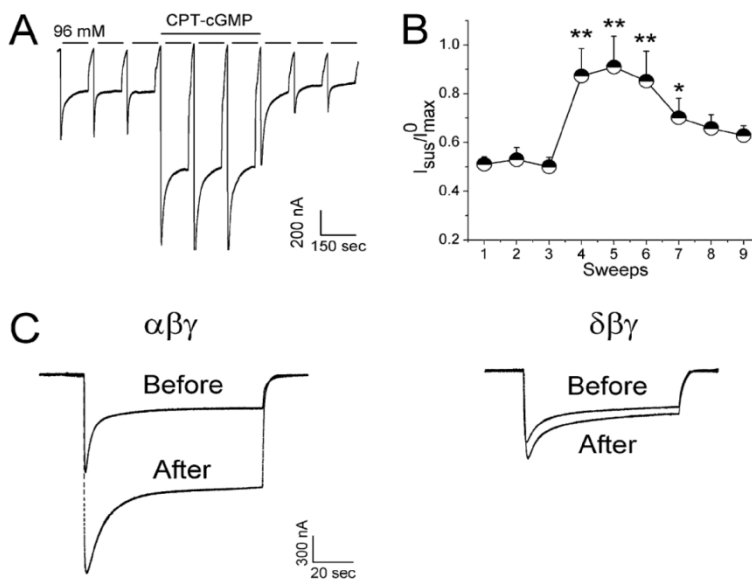


Figure 3. CPT-cGMP modifies self-inhibition of $\alpha\beta\gamma$ ENaC in oocytes. Self-inhibition was initiated by fast switching the low sodium bath solution (1 mM sodium) to regular ND96 medium (96 mM sodium). A: Whole-cell current trace digitized at -60 mV. CPT-cGMP (0.2 mM) was added after the first three sweeps and washed out for the last three sweeps. B: Normalized and averaged currents; * $P < 0.05$ and ** $P < 0.01$ versus the first sweep. C: Comparison of CPT-cGMP on self-inhibition of $\alpha\beta\gamma$ and $\delta\beta\gamma$ ENaC channels. Paired traces were recorded before and after application of CPT-cGMP, $n=9$. Figure reprinted from ²⁴, and used with permission.

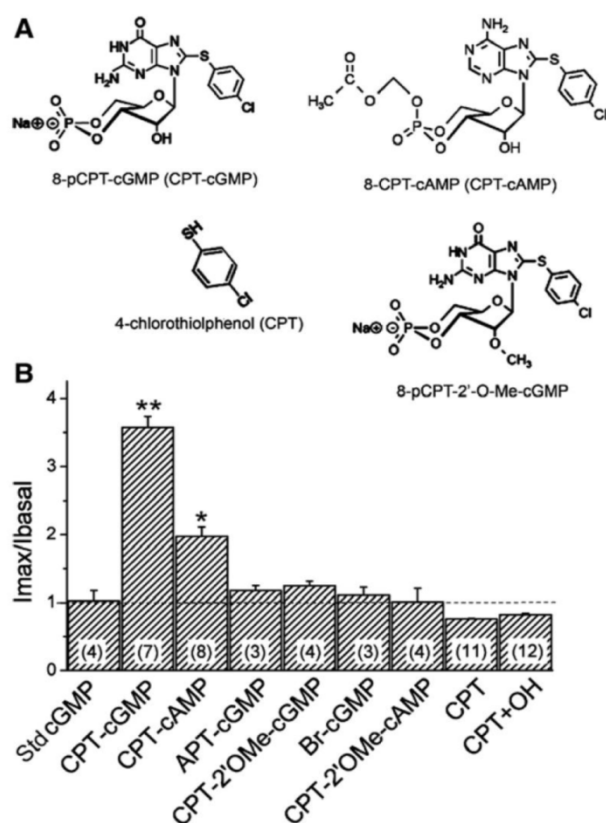


Figure 4. Domain-dependence of CPT-cGMP and CPT-cAMP analogs. A: Modified positions are shown for 8-pCPT-cGMP and 8-CPT-cAMP. B: The responses of human $\alpha\beta\gamma$ -ENaC to these compounds (0.2 mM) were compared: 8-pCPT-cGMP, (CPT-cGMP); 8-CPT-cAMP, (CPT-cAMP); 2-aminophenylthio-cGMP, (APT-cGMP); 8-pCPT-methylated ribose 2'-hydroxy (2'-O-Me)-cGMP (CPT-2'OMe-cGMP); Sp-8-Br-cGMP, (Br-cGMP); 8-CPT-2'-O-Me-cAMP, (CPT-2'OMe-cAMP); 4-chlorothiophenol, (CPT); a mixture of CPT and KOH, (CPT+OH). The normalized currents before and after application of these compounds were compared with that of the standard cGMP molecule (Std cGMP). ** $P < 0.01$, * $P < 0.05$. Numbers in parentheses are the number of oocytes examined for each group. Figure reprinted from ¹⁶, and used with permission.

CPT-cGMP ligand docking to ENaC

In our previous experiment, we constructed mutants abolishing ($\beta V348$ and $\gamma H233R$), or augmenting ($\alpha Y458A$ and $\gamma M432G$), ENaC self-inhibition^{14, 24}. The mutations eliminating self-inhibition resulted in a loss of response to CPT-cGMP, whereas those enhancing self-inhibition facilitated the stimulatory effects of this compound. Figure 5 shows the potential binding sites for the CPT-cGMP ligand in ENaC domains that are crucial for self-inhibition. $\beta V348$ is located in the center of the palm region of the subunit, and $\gamma H233$ is located in the vicinity of the putative binding site for protons. These domains potentially directly or allosterically interact with CPT-cGMP.

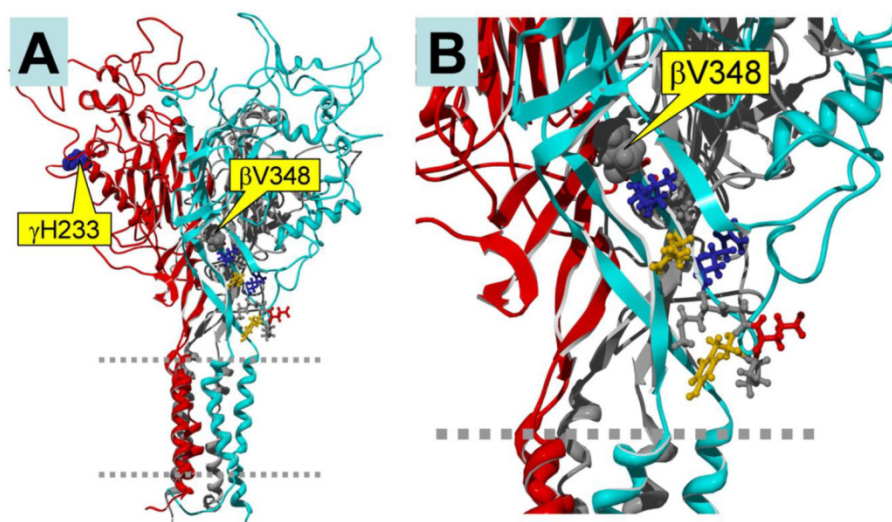


Figure 5. Homology model of trimeric human $\alpha\beta\gamma$ ENaC. The subunits are color-coded as follows: α , grey (back, center); β , cyan (front, right); γ , red (front, left). A: Side view of the entire trimer. Two dashed grey lines indicate the plasma membrane. The locations of the mutations γ H233 and β V348 are marked by yellow boxes. B: Close-up view, showing the location of β V348. Note that β V348 is located in the center of the palm region of the subunit, and its downstream residues extend to the wrist region of the subunit that seems to be important in the coupling between extracellular domains to the pore region. Thus, deletion of β V348 may change the position and orientation of the downstream residues. γ H233 is located in the vicinity of the putative binding site for protons. Since 8-CPT-cGMP is much larger than a proton, its binding site may include the H233 residue.

Self-Inhibition as a Therapeutic Mechanism

Reagents able to release self-inhibition

Previous results suggested that external Zn^{2+} rapidly and reversibly activates ENaC in a dose-dependent manner by relieving the channel from sodium self-inhibition²⁵. In addition to Zn, extracellular chloride²⁶, temperature^{27, 28}, halogenated gases²⁷⁻²⁹, sulfhydryl group-modifying reagents (e.g., p-chloromercuriphenylsulfonate, pCMPS and p-chloromercuribenzoate, pCMB)³⁰, p-chloromercuribenzoate benzimidazolylguanidine³⁰⁻³³, protons²⁶, copper³⁴, and proteases^{28, 35, 36} are also proposed to modulate ENaC-gating by relieving sodium self-inhibition. A classic earlier review summarized most of these reagents^{27,37,38}. Non-cleaved channels have a low intrinsic open probability that may reflect enhanced channel inhibition by external sodium, and cleavage at a minimum of two sites within the α - or γ -ENaC subunits is required to activate the channel, presumably by releasing inhibitory fragments³⁹. The extent of ENaC proteolysis is dependent on channel residency time at the plasma membrane, as well as on the balance between levels of expression of proteases that activate epithelial sodium channels and inhibitors of these proteases. For example, furin cleavage of ENaC subunits activates the channels by relieving sodium self-inhibition, and this activation requires that the α -ENaC subunit be cleaved twice³⁵.

Specific domains/sites changing self-inhibition

His(88) and Asp(516) of the γ subunit play a role in the Zn^{2+} -regulating sodium self-inhibition mentioned above. Recent studies showed that palmitoylation of the γ subunit activates ENaC by increasing the open probability of the channels⁴⁰. ENaC mutants with the mutations γ C33A, γ C41A, or γ C33A/C41A have significantly enhanced sodium self-inhibition and reduced open probability compared with wild type ENaCs, suggesting that ENaC palmitoylation is an important post-translational mechanism of channel regulation. Exon 11 within the human α , β , and γ ENaC genes encodes structurally homologous yet functionally diverse domains,

and exon 11 in the α -subunit encodes a module that regulates channel gating⁴¹.

In contrast to the other mutations, γ L511Q largely eliminated the sodium self-inhibition response, reflecting a down-regulation of ENaC open probability by extracellular sodium⁴². γ L511Q is a gain-of-function human ENaC variant and it enhances ENaC activity by increasing channel open probability and dampens channel regulation by extracellular sodium and proteases⁴².

Therapeutic effects of targeting self-inhibition

Renal handling of sodium and water is abnormal in chronic kidney diseases. Filtrated sodium is reabsorbed from the glomerular filtrate, and potassium is secreted through a tight epithelium in the kidney. Sodium crosses the apical membrane and enters the epithelial cell through the ion-selective ENaC⁴³. ENaC is responsible for the reabsorption of sodium through the apical membrane of the connecting tubule and the collecting duct, and plays a key role in controlling sodium balance, extracellular fluid volume, and blood pressure⁴⁴. Regulated epithelial sodium channel proteolysis has been observed in rat kidney and in human airway epithelia. Pseudohypoaldosteronism type 1 is a monogenic disorder of mineralocorticoid resistance characterized by salt-wasting, hyperkalemia, high aldosterone levels, and failure to thrive⁴⁵. An autosomal recessive form of pseudohypoaldosteronism type 1 is caused by mutations in ENaC, and is usually associated with

severe and persisting multiorgan symptoms. The relief of self-inhibition by CPT-cAMP contributes to the acute effects, in addition to the well-known cAMP-PKA signal pathway¹⁴. In fact, the clinical relevance of the abnormal regulation of ENaC by CPT-cAMP has been implicated in the upregulation of ENaC in autosomal recessive pseudohypoaldosteronism type 1⁴⁶.

Another distressed transapical sodium transport occurs in injured lungs, for example, acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) (please see classical reviews⁴⁷⁻⁵¹). Apical ENaC contributes to up to 60% of transepithelial sodium transport in mammalian lungs under physiological conditions. This critical process is sensitive to aspirated pollutants, allergens, pathogens, and bacterial endotoxins. In addition to increased leaking through alveolar microvascular endothelium (indirect ALI), lung edema usually results from reduced edema fluid resolution via ENaC (direct ALI). ENaC is a promising target for developing new therapeutic strategies to alleviate lung edema, at least for the phenotype of direct ALI^{52,53}.

CPT-cGMP and self-inhibition

Human serum cGMP level is 6 nM and 3-time greater in human bronchoalveolar lavage⁵⁴. It appears that cGMP may serve as an autocrine and paracrine to regulate ENaC function. However, the effective dose for CPT-cGMP and CPT-cAMP to blunt self-inhibition is micromolar, suggesting an uncertain physiological role for cGMP and analogs. A large dose of cGMP compound (1 mg/kg) was administered to patients as reported by two clinical trials⁵⁵, and numerous pre-clinical studies (from 100 μ M to 2 mM)⁵⁶⁻⁵⁸. It is therefore feasible to apply aerosolized nucleotides to mitigate edematous lung injury. We have demonstrated that CPT-cGMP up-regulates ENaC via two mechanisms: release self-inhibition externally and activates ENaC through cGMP/cGKII pathway intracellularly²⁴. Thus, these compounds could regulate sodium absorption via either or both mechanisms in a cell permeability-dependent manner. Administration of cAMP could be a potent pharmaceutical treatment for edematous lung injury⁵⁹, and cGMP may have similar potential. cGMP increased in murine and rat lungs both *in vivo* and *in vitro* following NO application⁶⁰, and increased cGMP may augment the cGMP-sensitive pathway for lung fluid removal from alveolar sacs⁶¹. Our previous study demonstrated for the first time that PKGII is an ENaC activator in non-ciliated bronchial secretory cells¹⁵. Accordingly, upregulation of the rate-limiting ENaCs in respiratory epithelial cells by specific PKGII activators may be a potent clinicopharmaceutical strategy for alleviating

airspace flooding in fatal edematous lung diseases. The observation of our previous study that specific moieties of 8-pCPT-cGMP are required for activating ENaC may provide pivotal information for developing potential ENaC channel openers structurally related to 8-pCPT-cGMP, which would be extremely useful for treating diseases associated with lower ENaC function. We postulate that when the tight epithelial layer is damaged, for example, in injured lungs, even though the mixture of extracellular matrix proteins, including collagens, albumin, and fibrins, will seal the epithelial cell-free alveolar surface, the potency of ENaC stimulator will be limited significantly. Therefore, the integrity of the tight alveolar epithelium should be a key factor to be considered for the usage of ENaC agonists. The anticipated restore of alveolar fluid clearance may be seen at the earlier stage of ALI and lung edema mainly caused by injured pulmonary vasculature or post regeneration of alveolar epithelium by stem cells/progenitors.

Conclusions and Perspectives

ENaC is involved in edema formation and kidney disorders. cGMP, either by serving as a ligand to regulate cyclic nucleotide-gated ion channels or by acting upstream of the PKG signaling systems, has a role in the elimination of ENaC self-inhibition, suggesting a novel mechanism for CPT-cGMP to stimulate salt reabsorption. Compounds such as specific PKGII activators and/or self-inhibition inhibitors could be the basis for novel pharmaceutical interventions for combating diseases associated with impaired ENaC function. Future directions for study include optimizing cGMP structure to potentiate its efficacy in activating ENaC, evaluating its pharmaceutical relevance in preclinical and clinical models with impaired ENaC activity, and confirming the binding sites of cGMP in ENaC.

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Abbreviations

cAMP: cyclic adenosine monophosphate; cGMP: cyclic guanosine monophosphate; EC₅₀: concentration for 50% of maximal effect; ENaC: epithelial sodium

channel; PKA: protein kinase A; PKG: protein kinase G; NO: nitric oxide

Competing Interests

The authors declare no conflicts of interest.

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