Cpt-Cgmp Is A New Ligand Of Epithelial Sodium Channels

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Recommended Citation
Ji, Hong Long; Nie, Hong Guang; Chang, Yongchang; Lian, Qizhou; and Liu, Shan Lu, "Cpt-Cgmp Is A New Ligand Of Epithelial Sodium Channels" (2016). Translational Neuroscience. 47.
https://scholar.barrowneuro.org/neurobiology/47

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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 αβγ-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 organisms. 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 and phosphorylates protein kinase G (PKG) directly.

Amiloride-sensitive epithelial sodium channels (ENaC) behave as ligand-gated channels, similar to several other members of the ENaC/degenerin family. 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 channel. 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-expended hypertension. An autosomal recessive form of pseudohypoaldosteronism type I is caused by mutations in ENaC, with usually severe...
and persisting multiorgan symptoms. 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. 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). 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, cyclic nucleotide-dependent protein kinases, and cyclic nucleotide-gated ion channels. CPT-cGMP is a derivative analog of parental cGMP, and has long been used for studying the NO/cGMP/PKG cascade. 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. Accumulating evidence from genetically engineered animals suggests that cardiovascular phenotypes are predominant with the PKGII knockout, while PKGII deficiency leads to dysfunction in epithelial tissues. 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 δβγδ-ENaC activity in a dose-dependent manner, most probably through the stimulation of PKGII enzymatic activity and subsequent activation of channel function (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.

Table 1. Regulation of sodium channel activity by nucleotides.

| Nucleotides | Concentration | Models | Effects | Ref. |
|-------------|--------------|--------|---------|-----|
| CPT-cGMP    | 1 μM         | Xenopus 2F3 cells | Increase in Isc | 42   |
|             | 400 μM       | Rat ATII cells | No change in Isc | 65   |
|             | 2 mM         | Rat bronchioloalveolar epithelial cells | Increase in Isc/ Na influx | 64, 65 |
|             | 100 μM       | Rat ATII cells | Increase in whole cell activity | 66   |
|             | 4 mM         | Human A549 cells | Increase in whole cell activity | 67   |
|             | 10 μM        | Frog urinary bladder epithelium | Increase in single channel activity | 68, 69 |
|             | 0.1 - 1000 μM| Heterologous human δβγ 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 Isc | 15   |
|             | 1 mM         | Rat ATII cells | Decrease in single channel activity | 56   |
|             | 100 μM       | Human A549 cells | Decrease in whole cell activity | 70   |
|             | 100 μM       | MDCK cells | Increase in Isc | 71   |
|             | 40 μM        | Liddle’s disease lymphocytes | Increase in whole cell activity | 14, 21, 72 |
|             | 200 μM       | Heterologous human δβγ ENaC in oocytes | Increase in whole cell activity | 23   |
|             | 100 μM       | BeWo cells | Increase in whole cell activity | 73   |

*Abbreviations: ATII, alveolar type II epithelial; MDCK, Madin-Darby canine kidney; Isc, 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 EC50 value were derived by fitting the raw data with the Hill equation. n=9. C: Average amiloride-sensitive (AS) αβγδ 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 100 mM 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.

Our previous studies showed that external CPT-cAMP stimulated human, but not rat and murine, αβγ-ENaC in a dose-dependent and external sodium concentration-dependent fashion. ENaC mutations that abolished self-inhibition (βΔV348 and γH233R) 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. 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 αβγ-ENaC may be a novel mechanism for CPT-cGMP to stimulate salt reabsorption in the human epithelium (Figure 3).

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. 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 ENaC 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 αβγ 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 αβγ and δβγ 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 αβγ-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); 8-p-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 (βV348 and γH233R), or augmenting (αY458A and γM432G), ENaC self-inhibition. 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. βV348 is located in the center of the palm region of the subunit, and γH233 is located in the vicinity of the putative binding site for protons. These domains potentially directly or allosterically interact with CPT-cGMP.
Self-Inhibition as a Therapeutic Mechanism

Reagents able to release self-inhibition

Previous results suggested that external Zn\textsuperscript{2+} rapidly and reversibly activates ENaC in a dose-dependent manner by relieving the channel from sodium self-inhibition\textsuperscript{25}. In addition to Zn, extracellular chloride\textsuperscript{26}, temperature\textsuperscript{27, 28}, halogenated gases\textsuperscript{27-29}, sulfhydryl group-modifying reagents (e.g., p-chloromercuriphenylsulfonate, pCMPS and p-chloromercuribenzoate, pCMB)\textsuperscript{30}, p-chloromercuribenzoate benzimidazolylguanidine\textsuperscript{30-33}, protons\textsuperscript{26}, copper\textsuperscript{34}, and proteases\textsuperscript{28, 35, 36} are also proposed to modulate ENaC-gating by relieving sodium self-inhibition. A classic earlier review summarized most of these reagents\textsuperscript{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 fragments\textsuperscript{39}. 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 twice\textsuperscript{35}.

Specific domains/sites changing self-inhibition

His(88) and Asp(516) of the γ subunit play a role in the Zn\textsuperscript{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 channels\textsuperscript{40}. ENaC mutants with the mutations γC33A, γC41A, or γC33A/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 gating\textsuperscript{41}.

In contrast to the other mutations, γL511Q largely eliminated the sodium self-inhibition response, reflecting a down-regulation of ENaC open probability by extracellular sodium\textsuperscript{42}. γL511Q 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 proteases\textsuperscript{42}.

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 ENaC\textsuperscript{43}. 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 pressure\textsuperscript{44}. 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 thrive\textsuperscript{45}. 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 pathway. In fact, the clinical relevance of the abnormal regulation of ENaC by cGMP-sensitive pathway for lung fluid removal from the least for the phenotype of direct ALI. ENaC is a promising target for developing new therapeutical strategies to alleviate lung edema, at application, and increased cGMP may augment the greater in human bronchoalveolar lavage. It appears of cAMP could be a potent pharmaceutical treatment for edematous lung injury, and cGMP may have similar potential. cGMP increased in murine and rat lungs both in vivo and in vitro following NO application, and increased cGMP may augment the cGMP-sensitive pathway for lung fluid removal from alveolar sacs. Our previous study demonstrated for the first time that PKGII is an ENaC activator in non-ciliated bronchial secretory cells. Accordingly, upregulation of the rate-limiting ENaCs in respiratory epithelial cells by specific PKGII activators may be a potent clinico pharmaceutical 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.

Acknowledgements

The authors are grateful for helpful discussions with Dr. Run-Zhen Zhao (University of Texas Health Science Center at Tyler), and Dr. Zai-Xing Chen (China Medical University). This work was partially supported by NIH grants HL87017 and HL095435, the American Heart Association (AHA 14GRNT20130034 to HLJ), and the National Science Foundation of China (grants 81270098 to HGN, 31270967 and 31571407 to QL).

Abbreviations

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