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cCMP and cUMP: emerging second messengers

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The cyclic purine nucleotides cAMP and cGMP are established second messengers. By contrast, the existence of the cyclic pyrimidine nucleotides cytidine 3′,5′-cyclic monophosphate (cCMP) and uridine 3′,5′-cyclic monophosphate (cUMP) has been controversial for decades. The recent development of highly sensitive mass spectrometry (MS) methods allowed precise quantitation and unequivocal identification of cCMP and cUMP in cells. Importantly, cCMP and cUMP generators, effectors, cleaving enzymes, and transporters have now been identified. Here, I discuss evidence in support of cCMP and cUMP as bona fide second messengers, the emerging therapeutic implications of cCMP and cUMP signaling, and important unresolved questions for this field.

Early years of cCMP and cUMP research

Second messengers are intracellular signaling molecules that transfer the extracellular signal inherent to a first messenger (hormone or neurotransmitter) to the cytosol. cAMP and cGMP are the classic second messengers. A second messenger is generated by a first messenger-regulated enzyme, activates specific effector proteins, exerts specific biological effects, is eliminated via defined inactivation mechanisms, and is mimicked by membrane-permeable second-messenger analogs and bacterial toxins (Table 1). All second messenger criteria are fulfilled for cAMP and cGMP.

In 1965, Hardman and Sutherland [1] reported on high activity of a cUMP-degrading phosphodiesterase (PDE) in dog heart. In 1971, the Stock group identified a cUMP-degrading PDE in fat cells [2], but these findings were not followed up on. Development of the young field was compromised by claims of the existence of a specific mammalian cytidylyl cyclase (CC) [3] (see Glossary) that turned out to be a methodological artifact [4]. Antibodies against cCMP showed cross-reactivities with other nucleoside 3′,5′-cyclic monophosphates (cNMPs) [5], and early MS methods were insufficiently sensitive and specific to unequivocally quantitate and identify cCMP and cUMP [6]. As a result of nonreproducible publications and methodological difficulties, skepticism in the cNMP research community regarding the relevance of cCMP and cUMP as second messengers was large and little research activity occurred during the subsequent 40 years. By marked contrast, cAMP and cGMP research exploded and important drugs modulating cAMP and cGMP signaling were developed [7,8]. Here, I develop the hypothesis that, in addition to cAMP and cGMP, cCMP and cUMP are bona fide second messengers.

Revival of cCMP and cUMP research

During the early 2000s, a research program aimed at the development of potent and selective inhibitors of membranous adenylyl cyclases (mACs), soluble guanylyl cyclase (sGC) and bacterial adenylyl cyclase (AC) toxins serendipitously revealed that the UTP analog uridine 5′-[γ-thio]-triphosphate (UTPγS) inhibited sGC potently [9]. These data raised the question whether UTP is a sGC substrate. Using a classic radiometric method, evidence for cUMP formation by sGC was obtained, but definitive structural proof was not possible with this approach [9]. A few years later, 2′,3′-*O*-(*N*-methylanthraniloyl)-CTP (MANT-CTP)

Glossary

Adenylate cyclase toxin (CyaA): an NC toxin that is released from *Bordetella pertussis* and inserts into the plasma membrane of host cells. The massive cAMP production by activated CyaA paralyzes immune cell function and facilitates survival of the bacteria. Purified CyaA also has low CC and UC activity.

Adenylyl cyclase (AC): ACs convert ATP into cAMP and PP_i. Classic ACs are the membranous ACs (AC1-9), soluble AC (sAC or AC10), and the bacterial AC toxins CyaA, EF and ExoY.

Cytidylyl cyclase (CC): CCs convert CTP into cCMP and PP_i. There is no evidence for a specific CC in mammalian systems. Rather, sGC and sAC have more or less pronounced CC activity, depending on the experimental conditions.

Cytidine 3′,5′-cyclic monophosphate (cCMP): a cyclic pyrimidine nucleotide that now fulfills all criteria of a bona fide second messenger.

Edema factor (EF): an NC toxin from *Bacillus anthracis* that is taken up into host cells in complex with protective antigen. The massive cAMP production of EF paralyzes immune cell function and facilitates survival of the bacteria. Purified EF also has low CC and UC activity.

Guanylyl cyclase (GC): GCs convert GTP into cGMP and PP_i. Classic GCs are the NO-stimulated sGC and the particulate guanylyl cyclases (pGCs A-G).

Nucleoside 3′,5′-cyclic monophosphate (cNMP): general term for a cyclic nucleotide with a purine or pyrimidine base.

Nucleotidyl cyclase (NC): general designation for an enzyme that catalyzes cyclization of not only cognate NTP, but also of other NTPs. The substrate specificity of NCs may vary depending on whether the NC is studied *in vitro*, where substrate availability can be precisely controlled, or *in vivo* where substrate availability cannot be controlled.

Uridylyl cyclase (UC): UCs convert UTP into cUMP and PP_i. There is no evidence for a specific UC in mammalian cells. Rather, sGC and sAC have more or less pronounced UC activity, depending on the experimental conditions. Leaves of *Arabidopsis thaliana* contain cUMP but not cCMP; hence, there may be a specific UC in plants.

Uridine 3′,5′-cyclic monophosphate (cUMP): a cyclic pyrimidine nucleotide that now fulfills all criteria of a bona fide second messenger.

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Table 1. Fulfillment of second-messenger criteria by cAMP, cGMP, cCMP, and cUMP

Criterion	cAMP	cGMP	cCMP	cUMP
Generator	mACs, sAC (important for high basal cAMP), sGC, pGC-A	sGC, pGCs A-G, sAC (important for high basal cGMP)	sGC, sAC (important for high basal cCMP), not mACs or pGCs	sGC, sAC (important for high basal cUMP), not mACs or pGCs
Effector	PKA, PKG (cross-talk), Epac, various cNMP-regulated ion channels, ineffective in DMR (in marked contrast to cCMP and cUMP)	PKG, PKA (cross-talk), PDEs, various cNMP-regulated ion channels, poorly effective in DMR (in marked contrast to cCMP and cUMP)	PKA, PKG, HCN channels 2 and 4, not HCN channels 1 and 3, not Epac; effective in DMR (different kinetics and PKA/PKG inhibitor sensitivity compared with cUMP)	PKA, PKG, HCN channels 2 and 4, not HCN channels 1 and 3, not Epac; effective in DMR (different kinetics and PKA/PKG inhibitor sensitivity compared with cCMP)
Biological function	Broad, including function of cardiovascular system, brain, kidney, gastrointestinal tract, metabolism, and immune system	Broad, including cardiovascular system, brain, visual system, gastrointestinal system, metabolism, and immune system	Vasodilation, inhibition of platelet aggregation (PKG), accelerated heart depolarization (HCN channels); modulation of immune cell function and early embryonic development	Cell death (in combination with cGMP); functions in heart, adipocytes, secretory cells, and astrocytes likely based on cUMP occurrence and PDE expression
Inactivation	Numerous PDEs (PDE4 is an important drug target); MRPs 4 and 5	Numerous PDEs (PDE5 is an important drug target), MRPs 4 and 5, OAT2	PDE7A1 (rather exclusive), MRP5; not MPRs 1, 2,3, 4 and 8 or OAT2	PDEs 3A, 3B and 9A, MRPs 4 and 5; not MRPs 1, 2, and 3 or OAT2
Mimicry by membrane-permeable cNMP analogs	DB-cAMP (yielding 6-MB-cAMP), cAMP-AM (yielding cAMP)	DB-cGMP (yielding 2-MB-cGMP), cGMP-AM (yielding cGMP)	DB-cCMP (yielding 4-MB-cCMP), cCMP-AM (yielding cCMP)	cUMP-AM (yielding cUMP), DB-cUMP cannot be prepared for chemical reasons
Mimicry by bacterial NC toxins (intact cells)	EF (very effective), CyaA (very effective), ExoY (rather ineffective)	Heat-stable enterotoxin acting on pGC-C, ExoY (very effective), CyaA and EF are ineffective	ExoY (effective), CyaA (moderately effective), EF (poorly effective)	ExoY (very effective), CyaA (poorly effective), EF does not generate cUMP in cells

was shown to be a potent inhibitor of purified AC toxin (CyaA) from *Bordetella pertussis* [10]. Given that CyaA and the structurally related edema factor (EF) from *Bacillus anthracis* have extremely high AC activities, a combination of radiometric and high-performance liquid chromatography (HPLC)-based methods was then used to unequivocally demonstrate that the purified toxins produce not only cAMP, but also cCMP and cUMP, although at low rates [11]. That bacterial AC toxins exploit endogenous cAMP signaling for their survival [12,13], suggested that cCMP and cUMP are second messengers.

Revival of cCMP and cUMP research depended on the development of several key technologies and availability of experimental tools (Box 1). A major advance in the field was the development of highly sensitive and specific HPLC-tandem MS (HPLC-MS/MS) methods for quantitation [14,15], and HPLC-quadrupole time of flight MS (HPLC-MS/TOF) methods for unequivocal cCMP and

Box 1. Key technologies and key experimental tools for the analysis of cCMP and cUMP as second messengers

Key technologies

HPLC-MS/MS

HPLC-MS/MS is well suited for quantitation of individual cNMPs in *in vitro* and *in vivo* studies. Fragmented cNMPs are analyzed with specific qualifiers and quantifiers. Studies with organ extracts and body fluids are more difficult than with purified enzymes and cell extracts because matrix effects can lead to signal suppression. The lower limit of quantitation in the HPLC-MS/MS analysis is different for the various cNMPs and is particularly high for cUMP. Accordingly, low cUMP levels may go undetected [17]. An advantage of this technique is that it allows simultaneous determination of multiple cNMPs [14,15].

HPLC-MS/TOF

HPLC-MS/TOF is suitable for unequivocal cNMP identification via extremely precise determination of the masses of molecules and molecule fragments. A disadvantage of this method is its lower sensitivity compared with HPLC-MS/MS. Therefore, it is best to combine HPLC-MS/MS and HPLC-MS/TOF in cUMP/cCMP research, particularly when cell and organ extracts are analyzed [16].

DMR

DMR constitutes a label-free holistic cell assay in which changes in distribution of cellular proteins are analyzed. Often, this technology is used in the pharmaceutical industry for drug development and receptor deorphanization [52]. This technique is also well suited to determine whether a given cNMP-AM exhibits a cellular effect. Although this assay does not provide information about what exactly is happening in the cells, with the use of appropriate control compounds and inhibitors, valuable information about the signal transduction pathways activated by a given cNMP-AM can be obtained [24].

Key experimental tools

cNMP-AMs

In cNMP-AM compounds, the cyclic phosphate is protected by an AM group [33]. cNMP-AMs are used as experimental tools to mimic the effects of endogenous cNMPs. A major advantage of the cNMP-AM technique compared with the classic DB-cNMP technique is that it can be applied to a cNMP with any given base, whereas the DB-cNMP technique cannot be applied to uracil base-containing cNMPs [24,32,33]. As a potential limitation, cNMP-AMs cannot mimic the compartmentation of endogenous cellular cNMPs.

ExoY

ExoY is type III secretion protein from *Pseudomonas aeruginosa*. Originally, ExoY was assumed to be an AC with no pathogenic relevance for the infection [36]. Later studies showed that ExoY is in fact a cell- and tissue-damaging NC toxin with preferential cUMP- and cGMP-forming activity [35,37,39]. The toxic effects of ExoY are mimicked by a combination of membrane-permeable cUMP and cGMP analogs [24]. Experimentally, ExoY can be delivered into cells via the type III secretion system (infection of mammalian cells with bacteria) or transfection of cells with ExoY-encoding plasmid [35].