

Is cIMP a second messenger with functions opposite to those of cGMP?

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There is renewed interest in the potential role of noncanonical 3',5'-cyclic nucleoside monophosphates (cNMPs) as second messengers (Akimoto et al. 2014; Chen et al. 2014; Bähre et al. 2014). Inosine 3',5'-cyclic monophosphate (cIMP) consists of the base hypoxanthine, the ribosyl moiety and a cyclic phosphate ester linking the 3'- and 5'-*O*-ribosyl groups (Fig. 1). cIMP differs chemically from the well-established second messenger guanosine 3',5'-cyclic monophosphate (cGMP) (Schlossmann and Schinner 2012; Levy 2013) by the absence of the amino group at the 2'-position of the nucleobase (Fig. 1). The loss of the amino group results in a reduction in affinity of cIMP for the cGMP effector cGMP-dependent protein kinase I α (PKGI α) by more than two orders of magnitude (Wolter et al. 2011) (Table 1).

cIMP is generated by highly purified soluble guanylyl cyclase (sGC) in an NO-dependent manner (Beste et al. 2012) and by recombinant particulate guanylyl cyclase (pGC-A) in an atrial natriuretic peptide-dependent manner (Beste et al. 2013), inosine 5'-triphosphate (ITP) being the substrate. The properties of ITP as sGC and pGC substrate compared to the cognate substrate guanosine 5'-triphosphate (GTP) critically depend on the specific experimental conditions (Table 2). At sGC, ITP reaches a higher V_{\max} than GTP in the presence of Mg^{2+} , whereas the opposite is true for the presence of Mn^{2+} . The K_M values of sGC for ITP and GTP are also differentially regulated by Mg^{2+} and Mn^{2+} . In the presence of Mn^{2+} , sGC exhibits biphasic kinetics for both GTP and ITP (Beste et al. 2012), indicative for the presence of an allosteric regulatory site for ITP and GTP in this enzyme (Seifert and Beste 2012). In contrast to sGC, GTP reaches a higher V_{\max} at pGC-A than ITP with Mg^{2+} , whereas the

opposite is true for Mn^{2+} (Beste et al. 2013) (Table 2). The cation has little impact on K_M of GTP for pGC-A, but for ITP, Mn^{2+} is preferred. These biochemical activities of sGC and pGC-A were unequivocally documented using highly purified substrates and highly specific and sensitive high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) methods (Beste et al. 2012, 2013).

However, the crucial question is whether the cIMP-forming activities of GCs observed *in vitro* also occur in intact cells. A recent review expressed skepticism that cIMP functions as an independent second messenger, and that it has yet to be unequivocally demonstrated that cIMP exists in intact cells and/or tissues (Beste and Seifert 2013). Previous claims regarding the assumed existence of cIMP in organs are most likely unsubstantiated due to insufficient sensitivity of the primordial MS methods employed (Newton et al. 1986). There were no follow-up studies addressing the claims of the Newton group until very recently. In subsequent studies with the most advanced HPLC-MS/MS techniques currently available, we have shown that HEK293 cells possess very high basal concentrations of adenosine 3',5'-cyclic monophosphate (cAMP), cytidine 3',5'-cyclic monophosphate (cCMP), and uridine 3',5'-cyclic monophosphate (cUMP) that are controlled by the soluble adenylyl cyclase (sAC) (Hasan et al. 2014). However, cIMP levels were far below detection limit (Beste et al. 2013). A straightforward explanation for this negative finding is that cellular ITP concentrations are very low so that there is no ITP substrate for nucleotidyl cyclase to act upon. In accordance with this hypothesis, sophisticated HPLC-MS/MS analysis of neuronal cells revealed that only inosine 5'-monophosphate (IMP) is present at low levels, but no ITP (Göttle et al. 2013). The most important review in the field of cellular purine nucleotide concentrations (Traut 1994) also mentions just IMP as physiological cell constituent. Traut (1994) does not even depict ITP in his scheme of purine metabolism. Of course, it cannot be excluded that

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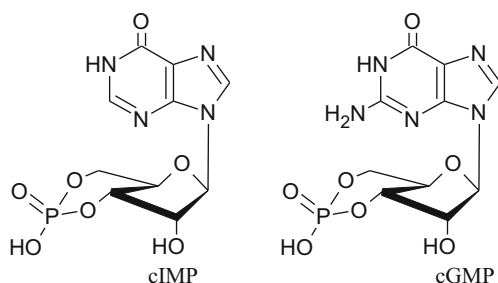


Fig. 1 Structures of cIMP and cGMP

pathophysiological conditions such as hypoxia increase cellular ITP levels (Chen et al. 2014), but common sense would rather expect a decrease of the levels of all nucleoside 5'-triphosphates. We did not explicitly analyze the effect of specific pathophysiological conditions on cNMP levels in intact cells, but another type of experimental cell stress, i.e. the process of cell transfection with exogenous plasmid DNA, substantially decreases basal cNMP levels (Bähre et al. 2014).

On this biochemical background, a recent study by Chen et al. (2014) merits discussion. These authors suggest that under conditions of hypoxia, sGC produces cIMP, but not cGMP in coronary arteries and by such a mechanism, cIMP supposedly induces vasoconstriction instead of the canonical cGMP-dependent vasodilation. The authors also suggest that at least part of the vasoconstriction is mediated via activation of rho kinase. This proposal explicitly implies that cIMP must activate a different effector than PKG. The reduction of affinity of PKG for cIMP compared to cGMP by two orders of magnitude could be compatible with the assumption that cIMP bypasses this kinase (Wolter et al. 2011) (Table 1), but what could then be the target of cIMP? Another straightforward candidate for cIMP could be cAMP-dependent protein kinase (PKA). In fact, cIMP activates two isoforms of PKA (RI α and RII α) with about tenfold higher potency than cGMP (Wolter et al. 2011) (Table 1). However, an activation of PKA would also be expected to result in vasodilation, but not vasoconstriction (Pelligrino and Wang 1998).

Therefore, one would have to postulate a novel cIMP-specific effector protein. Such a protein would have to exhibit much higher affinity for cIMP than for cGMP, i.e., at least three orders of magnitude, in order to account for the opposing actions of cGMP and cIMP and eliminate any cross-talk of

Table 1 pEC₅₀ values of cIMP and cGMP for activation of PKGI α , PKA RI α , and PKA RII α

pEC ₅₀	cIMP	cGMP
PKGI α	4.72	6.98
PKA RI α	5.89	4.91
PKA RII α	5.37	4.51

Data were taken from Wolter et al. (2011)

cIMP with PKG and PKA. To this end, such an effector protein has not yet been identified to the best of my knowledge. Purified human phosphodiesterases are very promiscuous with respect to hydrolysis of cGMP and cIMP (Reinecke et al. 2011). However, it is noteworthy that mammalian membranous ACs exhibit ~25- to 80-fold higher affinity for certain 2',3'-*O*-ribose-substituted hypoxanthine nucleoside 5'-triphosphates as compared to the corresponding adenine nucleotides (Seifert et al. 2011; Seifert and Dove 2012). Thus, there is precedence for the existence of proteins with a preference for the base hypoxanthine relative to adenine, but nonetheless, base-specificity is not exceedingly high. The application of cIMP agarose affinity chromatography may be a feasible approach to identify putative cIMP-specific effector proteins (Hammerschmidt et al. 2012).

One should also keep in mind alternative mechanisms of action of cIMP. Specifically, cIMP may be exported from the cytosol into the extracellular space by organic anion transporter 2 or multidrug resistance proteins 4 and 5 (Cropp et al. 2008; Russel et al. 2008; Keppler 2011). By analogy to the cAMP-adenosine-adenine system (Jackson and Raghvendra 2004), extracellular cIMP may then be hydrolyzed to inosine and hypoxanthine. Inosine and hypoxanthine, in turn, could exert biological effects via adenosine or adenine receptors, respectively (Schiedel et al. 2007; da Rocha Lapa et al. 2012). These two mechanisms of action of cIMP were not addressed in the study by Chen et al. (2014). Taken together, based on the available data, it is premature to conclude that cIMP by itself functions as an independent second messenger with functions opposite to those of cGMP.

Table 2 Kinetic parameters of sGC and pGC-A with ITP and GTP as substrates

Parameter	ITP	GTP
sGC, Mg ²⁺		
K_M (μ M)	796	119
V_{max} (μ mol/mg/min)	4.9	2.2
sGC, Mn ²⁺		
K_{M1} (μ M)	6.6	14.2
K_{M2} (μ M)	13,200	4,820
V_{max1} (μ mol/mg/min)	1.4	4.1
V_{max2} (μ mol/mg/min)	3.4	13.5
pGC-A, Mg ²⁺		
K_M (μ M)	1,260	272
V_{max} (nmol/mg/min)	1.0	7.3
pGC-A, Mn ²⁺		
K_M (μ M)	232	231
V_{max} (nmol/mg/min)	14.4	9.8

Data for sGC were taken from Beste et al. (2012). Data for pGC-A were taken from Beste et al. (2013)

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