5'-CMP stimulates phospholipase A-mediated hydrolysis of phosphatidylinositol in permeabilized pituitary GH₃ cells

Andrew B. CUBITT, Colette N. THAW and Marvin C. GERSHENGORN*

Division of Endocrinology and Metabolism, Department of Medicine,
Cornell University Medical College and The New York Hospital, New York, NY 10021, U.S.A.

We showed previously that 5'-CMP activates PtdIns-Ins base exchange and reversal of PtdIns synthase in permeabilized rat pituitary GH₃ cells. Here we report another effect of 5'-CMP on PtdIns metabolism in these cells. In permeabilized GH₃ cells prelabelled with [³H]Ins and incubated in buffer with LiCl and a free Ca²⁺ concentration of 0.1 μM but without added Ins, 5'-CMP stimulated formation of glycerophospho[³H]inositol (GroP[³H]Ins) after a lag period of at least 5 min. This effect was concentration-dependent; the apparent *K_m* was 0.30 ± 0.02 mM. CDP and CTP stimulated GroPIns formation less effectively than did 5'-CMP, but cytidine, 2'-CMP, 3'-CMP, 5'-AMP and 5'-GMP had no effect. 5'-CMP stimulated formation of lysoPtdIns also. In permeabilized GH₃ cells prelabelled with [³H]arachidonic acid, 5'-CMP stimulated release of [³H]arachidonic acid without a measurable lag period. These data show that 5'-CMP stimulates a phospholipase A activity in permeabilized GH₃ cells that hydrolyses PtdIns.

INTRODUCTION

5'-CMP appears to be involved in at least two ways in the metabolism of PtdIns in cells. It is formed as a secondary product in the reaction catalysed by PtdIns synthase (CDP-diacylglycerol:Ins phosphatidyltransferase, EC 2.7.8.11) when CDP-diacylglycerol reacts with Ins to form PtdIns [1]. 5'-CMP may also have a role as a cofactor in PtdIns metabolism, because it appears to stimulate the exchange of unesterified Ins with the Ins head group in PtdIns ('PtdIns-Ins base exchange') in several mammalian cell systems [2–10]. We have studied base exchange [11] and reversal of PtdIns synthase in rat pituitary GH₃ cells [12]. While performing those experiments, we observed that CMP stimulated the formation of a [³H]Ins phosphate(s) from prelabelled PtdIns in permeabilized GH₃ cells. In the present paper we characterize this effect of CMP. We demonstrate that CMP stimulates the formation of arachidonic acid, lysoPtdIns and glycerophosphoinositol (GroPIns).

METHODS

GH₃ cells were grown as reported previously [13]. Cells were prelabelled to isotopic steady state by growing them in medium containing [³H]Ins (0.1 μCi/ml) for 60 h [14]. Cells were prelabelled with [³H]arachidonic acid (1 μCi/ml) by incubation for 16 h [15]. Cells were harvested and permeabilized as described previously [11]. In brief, permeabilization was accomplished by resuspending cells on ice in 0.03 ml of buffer/10⁶ cells, containing 3 mM-ATP, 3 mM-Hepes, pH 7.4, 1 mM-EGTA and 0.1 mM-dithiothreitol. After incubation of the cells for 3 min, recovery solution (10 ×) was added to restore normal ionic strength to the following final concentrations: 140 mM-KCl, 10 mM-Hepes, pH 7.4, 5 mM-MgCl₂, 10 mM-LiCl and free Ca²⁺ concentration buffered to 0.1 μM with EGTA/Ca²⁺ buffer. Factors were added after permeabilization as 10 × stock solutions in incubation buffer. Reactions were carried out in 0.11 ml with approx. 3 × 10⁶

cells per point. Reactions were terminated by addition of 1 ml of chloroform/methanol/HCl (100:100:1, by vol.). The phases were separated by addition of 0.2 ml of 10 mM-EDTA, and the lower phase was washed with pre-equilibrated upper phase (1 ml). Radioactivity in Ins lipid was quantified by counting the lower phase or after separation by t.l.c. T.l.c. to separate PtdIns and lysoPtdIns was performed on silica gel 60 plates with chloroform/methanol/4 M-NH₃ (9:7:2, by vol.) containing 2 mM-cyclohexylenedinitrilotetra-acetic acid. [³H]Arachidonic acid-labelled PtdIns was separated by chromatography in chloroform/methanol/acetic acid/water (25:15:4:2, by vol.). Labelled lipids were sprayed with Enhance before localization by autoradiography. Standards were localized by iodine staining. Ins and Ins phosphates from the washed upper phases were concentrated under air and separated by anion-exchange column chromatography, or by h.p.l.c. based on the method of Morgan and co-workers [16]. Separations were carried out on a 5 μm Absorbosphere SAX column (Alltech; 250 mm × 4.6 mm) at a flow rate of 1 ml/min. Samples were injected in water, and this was maintained for 10 min after injection. The concentration of ammonium phosphate, pH 3.8, was then increased linearly over the next 30 min to 0.03 M and then to 1 M over the next 60 min. This concentration was maintained for 10 min before the column was returned to water over the next 15 min. Water was then run through the column for at least 10 min before application of the next sample. Fractions (0.5 ml) were collected and the radioactivity was determined by liquid-scintillation counting. Retention times of GroPIns, formed by deacylation of PtdIns, and of standard Ins phosphates were determined.

Materials

Tissue-culture supplies were from Gibco. [³H]Ins (19 Ci/mmol) was from Amersham, and [³H]arachidonic acid (240 Ci/mmol) was from New England Nuclear-Dupont. Cytidine nucleotides were from Sigma or Pharmacia. Silica-gel 60 plates were from Whatman. [³H]Ins phosphate standards and Enhance were from New England Nuclear. H.p.l.c.-grade reagents were from J. T. Baker, and reagent-grade chemicals were from Sigma.

Abbreviation used: GroPIns, glycerophosphoinositol.

* To whom correspondence and reprint requests should be addressed.

Fig. 1. H.p.l.c. analysis of the effect of 5'-CMP on GroPIns formation in permeabilized GH₃ cells

Cells were prelabelled with [³H]Ins and permeabilized as described in the text. Permeabilized cells were incubated without or with 2 mM-CMP for the times indicated at 37 °C.

Fig. 2. Time course of the effect of 5'-CMP on GroPIns formation in permeabilized GH₃ cells

Cells were prelabelled with [³H]Ins, permeabilized and incubated as described in the legend to Fig. 1. Analysis was by Dowex column chromatography. Points represent means $\pm$ S.D. of triplicate determinations in a representative experiment repeated three times. The S.D. was within the limits of the symbols where no S.D. bars are shown.

Fig. 3. Dose-dependencies of the effects of 5'-CMP, CDP and CTP to stimulate GroPIns formation

The experiment was performed as described in the legend to Fig. 1. Incubations were for 30 min with the indicated concentrations of 5'-CMP (●), CDP (□), CTP (▼) and cytidine (△). Points represent means $\pm$ S.D. of triplicate determinations in a representative experiment repeated three times. The S.D. was within the limits of the symbols where no S.D. bars are shown.

RESULTS AND DISCUSSION

GroP[³H]Ins was formed during incubation of permeabilized GH₃ cells, which were prelabelled with [³H]Ins, in buffer containing 0.1 μ M-free Ca²⁺ and 10 mM-LiCl (Figs. 1 and 2). 5'-CMP (2 mM), after a lag period of at least 5 min, stimulated GroP[³H]Ins formation (Fig. 2) without affecting the levels of ³H-labelled inositol monophosphates, biphosphates, triphosphates or higher phosphates (results not shown). The pattern of Ins polyphosphates was similar to that reported previously in GH₃ cells [17,18]. The effect of 5'-CMP to stimulate GroP[³H]Ins formation was dose-dependent (Fig. 3). Stimulation was maximal at a concentration of 2 mM-5'-CMP, and an apparent K_m of 0.30 ± 0.02 mM was determined. 5'-CMP from different sources gave identical results. CDP and CTP also stimulated GroPIns formation (Fig. 3). 5'-CMP was more effective than CDP, which was more effective than CTP; the effect of CTP appeared to be biphasic. We measured the effects of these cytidine nucleotides on Ptd[³H]Ins and on release of [³H]Ins from Ptd[³H]Ins (results not shown) and confirmed our

previously reported findings [11,12]. 5'-CMP, CDP and CTP caused a much smaller stimulation of GroPIns formation than of release of [³H]Ins from Ptd[³H]Ins. The amount of GroP[³H]Ins generated during 5'-CMP stimulation for 30 min accounted for 1–3% of the initial Ptd[³H]Ins content, whereas the amount of [³H]Ins was 20–25%. Cytidine, 2'-CMP, 3'-CMP, 5'-AMP and 5'-GMP had no effect on GroPIns formation up to concentrations of 5 mM (results not shown). These results are similar to our previous findings that these other factors did not stimulate base exchange or reversal of PtdIns synthase activity in permeabilized GH₃ cells [11,12]. GroPIns formation stimulated by 5'-CMP was observed in washed membranes prepared from GH₃ cells (results not shown), and therefore it appears that the enzyme(s) involved was membrane-bound. These results demonstrate that 5'-CMP-induced generation of GroPIns is not mimicked by other nucleoside monophosphates or isomers of CMP. The order of efficacy of the cytidine nucleotides is

Fig. 4. Effects of 5'-CMP on unesterified [^3H]arachidonic acid (a) in permeabilized cells prelabelled with [^3H]arachidonic acid and on lysoPtdIns (b) in permeabilized cells prelabelled with [^3H]Ins

The experiment was performed as described in the legend to Fig. 1 in cells labelled with either [^3H]Ins or [^3H]arachidonic acid. After incubation, lipids were separated by t.l.c. as described in the text. Points represent means $\pm$ S.D. of triplicate determinations in a representative experiment repeated three times. The S.D. was within the limits of the symbols where no S.D. bars are shown.

consistent with the possibility that CDP and CTP stimulate GroPIns formation via their metabolism to CMP; however, further study is needed to determine whether this is the mechanism of CDP and CTP action.

The formation of GroPIns is consistent with the idea that 5'-CMP stimulates a phospholipase A activity(ies) that hydrolyses PtdIns. Because both fatty acid chains would have to be cleaved from PtdIns to produce GroPIns by this mechanism, this sequence of reactions is consistent with the delay in the accumulation of GroPIns stimulated by CMP (Fig. 2). Indeed, the production of GroPIns must be preceded by the production of lysoPtdIns if this is the mechanism of its generation. To test this possibility, we measured the effect of 5'-CMP on lysoPtdIns. 5'-CMP (5 mM) stimulated a time-dependent increase in lysoPtdIns (Fig. 4). LysoPtdIns was increased after 10 min, and thereafter continued to increase at a constant rate for at least 80 min. These results are consistent with the relatively slow kinetics of GroPIns formation stimulated by 5'-CMP. The delay in the accumulation of lysoPtdIns could have been due to an intrinsic delay in activation of a phospholipase A, to other intermediate steps, or to rapid re-acylation of lysoPtdIns to PtdIns that during the early times of stimulation compensated for the activation of a phospholipase A.

To distinguish among these possibilities, GH $_3$ cells were prelabelled with [^3H]arachidonic acid. CMP stimulated a time-dependent increase in unesterified [^3H]arachidonic acid (Fig. 4). The time course of loss of [^3H]arachidonic acid-labelled PtdIns and unesterified [^3H]arachidonic acid accumulation, in contrast with the accumulation of lysoPtdIns or GroPIns, was rapid and without a measurable delay, and continued for the duration of the experiment. The absence of a delay in the release of [^3H]arachidonic acid suggests that there is no delay in activation of phospholipase A activity. The dose-dependency of this effect (results not shown) was indistinguishable from that for formation of GroPIns (Fig. 3). The magnitude of the increase in unesterified [^3H]arachidonic acid was greater than the decrease in [^3H]arachidonic acid-labelled PtdIns (results not shown), consistent with the idea that arachidonic acid may have been derived

from other lipids in addition to PtdIns. Further experiments must be performed to determine the specificity of the phospholipase(s) A activated by CMP.

CMP has been shown to affect several aspects of Ins lipid metabolism in permeabilized GH $_3$ cells. In addition to stimulating phospholipase A activity shown here, CMP stimulates exchange of Ins for the head group in PtdIns [11] and reversal of PtdIns synthase [12]. It is unclear how these effects may be related and which if any of them is important *in situ*. We found that the dose-dependencies of CMP activation of GroPIns formation ($K_m = 0.3$ mM), base exchange ($K_m = 0.037$ mM) and reversal of PtdIns synthase ($K_m = 1.7$ mM) measured in these cells under identical conditions were different. We have attempted to measure cytidine nucleotide levels in GH $_3$ cells, using ^3H -labelled cytidine; however, cytidine is rapidly metabolized to a number of products (results not shown), making such an analysis difficult. The concentrations of cytidine nucleotides measured in other cells, in the range of 0.4 mM [19,20], make reversal of PtdIns synthase unlikely as an important mechanism of PtdIns degradation *in situ*. However, it appears that regulation of base exchange and phospholipase A-mediated hydrolysis of PtdIns could be affected by cellular CMP concentrations.

In permeabilized GH $_3$ cells, phospholipase A activation appeared to be followed by re-acylation, which delayed the accumulation of lysoPtdIns and GroPIns. It is likely that lysoPtdIns formation *in vivo* would be more efficiently compensated for by re-acylation [21] and would thereby inhibit the accumulation of potentially toxic lysophospholipids. 5'-CMP could stimulate the selective removal, followed by replacement, of fatty acids in intact cells, which could be an important mechanism of regulating the acyl chain content of PtdIns and, perhaps, other phospholipids.

In summary, we have shown that 5'-CMP stimulates formation of arachidonic acid, lysoPtdIns and GroPIns in permeabilized GH $_3$ pituitary cells. The kinetics that we measured are consistent with hydrolysis of PtdIns to arachidonic acid and lysoPtdIns, which is in part re-acylated, and then of lysoPtdIns to GroPIns. As far as we are aware, this is the first report that CMP stimulates a phospholipase A activity.

This work was supported by National Institutes of Health Grant DK33468.

REFERENCES

1. Paulus, H. & Kennedy, E. P. (1960) *J. Biol. Chem.* **235**, 1303–1311
2. Berry, G., Yandrasitz, J. R. & Segal, S. (1983) *Biochem. Biophys. Res. Commun.* **112**, 817–821
3. Berry, G., Yandrasitz, J. R., Victoria, M., Gipriano, S. M., Hwang, S. M. & Segal, S. (1986) *J. Neurochem.* **46**, 1073–1080
4. Schoepp, D. D. (1985) *J. Neurochem.* **45**, 1481–1486
5. Labarca, R., Janowsky, A. & Paul, S. M. (1985) *Biochem. Biophys. Res. Commun.* **132**, 540–547
6. Tennes, K. A. & Roberts, M. L. (1984) *Aust. J. Exp. Biol. Med. Sci.* **62**, 303–308
7. Bleasdale, J. E. & Wallis, P. (1981) *Biochim. Biophys. Acta* **664**, 428–440
8. Shukla, S. D., Sergeant, S. & Kim, H. D. (1985) *Biochim. Biophys. Acta* **821**, 305–309
9. Holub, B. J. (1974) *Lipids* **10**, 483–490
10. Zborowski, J. & Szymanska, G. (1984) *Int. J. Biochem.* **16**, 1367–1371
11. Cubitt, A. B., Zhang, B. & Gershengorn, M. C. (1990) *J. Biol. Chem.* **265**, 9707–9714
12. Cubitt, A. B. & Gershengorn, M. C. (1990) *Biochem. J.* **272**, 813–816
13. Imai, A. & Gershengorn, M. C. (1987) *Nature (London)* **325**, 726–728
14. Rebecchi, M. J., Kolesnick, R. N. & Gershengorn, M. C. (1983) *J. Biol. Chem.* **258**, 227–234
15. Kolesnick, R. N. & Gershengorn, M. C. (1984) *J. Biol. Chem.* **259**, 9514–9519

16. Morgan, R. O., Chang, J. P. & Catt, K. J. (1987) *J. Biol. Chem.* **262**, 1166–1171
 17. Dean, N. M. & Moyer, J. D. (1988) *Biochem. J.* **250**, 493–500
 18. Dean, N. M. & Moyer, J. D. (1987) *Biochem. J.* **242**, 361–366
 19. Weber, M. J. & Edlin, G. (1971) *J. Biol. Chem.* **246**, 1828–1834
 20. Plagemann, P. G. W. (1972) *J. Cell Biol.* **52**, 131–141
 21. Thompson, W. & MacDonald, G. (1975) *J. Biol. Chem.* **250**, 6779–6785
-

Received 10 April 1991/13 May 1991; accepted 16 May 1991