CMP activates reversal of phosphatidylinositol synthase and base exchange by distinct mechanisms in rat pituitary GH₃ cells

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CMP is known to activate phosphatidylinositol (PtdIns)/inositol (Ins) base exchange and has been reported to activate reversal of PtdIns synthase also. Because it is possible that PtdIns synthase acting in the reverse direction, followed by re-incorporation of ambient Ins, could be responsible for base-exchange activity, we characterized these processes in rat pituitary GH₃ cells. In permeabilized GH₃ cells prelabelled with [³H]Ins and incubated in buffer with LiCl but without added Ins, CMP stimulated rapid accumulation of [³H]Ins and decreases in [³H]PtdIns; the *K_m* for CMP was 1.7 mM. CDP and CTP were less effective, whereas 2'-CMP, 3'-CMP, other nucleoside monophosphates and cytidine did not influence this process. In permeabilized cells prelabelled to isotopic equilibrium with [³H]Ins and [³²P]P_i, CMP stimulated decreases in both the ³²P and ³H labelling of PtdIns, but did not increase that of [³²P]phosphatidic acid. These findings demonstrate that in the absence of added Ins the effect of CMP is not via activation of base-exchange nor via a phospholipase D, but by reversal of PtdIns synthase. In permeabilized cells prelabelled with [³H]Ins and [³²P]P_i, unlabelled Ins inhibited loss of ³²P labelling of PtdIns caused by CMP while markedly stimulating loss of ³H labelling of PtdIns and release of [³H]Ins. These data demonstrate that Ins inhibits reversal of PtdIns synthase, but stimulates base exchange. We conclude that in GH₃ cells reversal of PtdIns synthase and PtdIns/Ins base exchange are both stimulated by CMP, but are distinct processes.

INTRODUCTION

Phosphatidylinositol (PtdIns) synthase [CDP-diacylglycerol:inositol (Ins) phosphatidyltransferase, EC 2.7.8.11] catalyses the reaction in which CDP-diacylglycerol condenses with Ins to form PtdIns and CMP [1]. CMP has been shown to stimulate exchange of the Ins head group in PtdIns with ambient Ins ('PtdIns/Ins base exchange') in several mammalian cell systems [2–10]. The significance of base exchange in cellular PtdIns metabolism is poorly understood, because it apparently leads to no net change in PtdIns or Ins. An enzyme that specifically catalyses PtdIns/Ins base exchange has not been identified. It is possible that PtdIns synthase could be responsible for base-exchange activity. This would occur if PtdIns synthase acts in the reverse direction to catalyse the reaction of CMP with PtdIns to form Ins and CDP-diacylglycerol, which then reacts with ambient Ins to re-form PtdIns. This process is possible thermodynamically because the energy in the pyrophosphate bond of CDP-diacylglycerol is equivalent to that in the phosphodiester bond of PtdIns. Indeed, reversal of PtdIns synthesis to form Ins and CDP-diacylglycerol has been shown in microsomal preparations from mouse pancreas and is dependent on CMP [11], and purified yeast PtdIns synthase has been shown to catalyse a base-exchange reaction [12,13].

We have shown that CMP stimulates PtdIns/Ins exchange in permeabilized pituitary GH₃ cells incubated in buffer containing added Ins [14]. While performing these experiments in GH₃ cells prelabelled to isotopic equilibrium with [³H]Ins, we observed that CMP stimulated accumulation of [³H]Ins while decreasing [³H]PtdIns in the absence of added unlabelled Ins. In the present paper we further characterize these effects of CMP. We demonstrate that CMP can specifically stimulate reversal of PtdIns synthase, and that this reaction is inhibited by Ins and is distinct from PtdIns/Ins base exchange.

METHODS

GH₃ cells were grown as reported previously [15]. Cells were prelabelled to isotopic steady state by growing them in medium containing [³H]Ins (1 μCi/ml) and/or [³²P]P_i (5–10 μCi/ml) for 60 h [16]. Cells were harvested and permeabilized as described previously [14]. In brief, this involved the addition on ice of 0.03 ml of buffer per 1 × 10⁶ cells containing ATP (3 mM), Hepes (3 mM), pH 7.4, EGTA (1 mM) and dithiothreitol (0.1 mM). After incubation of the cells for 3 min, recovery solution (10-fold conc.) was added to restore normal ionic strength with the following final concentrations: KCl 140 mM, Hepes 10 mM, pH 7.4, MgCl₂ 5 mM, LiCl 10 mM, and with the free Ca²⁺ concentration buffered to 0.1 μM with EGTA/Ca²⁺ buffer. Factors were added after permeabilization as 10 × stocks in incubation buffer. Reactions were carried out in 0.11 ml with approx. 3 × 10⁶ cells per point. Reactions were terminated by the addition of 1 ml of chloroform/methanol/HCl (100:100:1, by vol.). The phases were split by the addition of 0.2 ml of 10 mM-EDTA, and the lower phases were washed with pre-equilibrated upper phase (1 ml). Ins lipid radioactivity was quantified by counting the lower phase and that in PtdIns alone after separation by t.l.c. The t.l.c. was performed on silica-gel 60 plates eluted with either chloroform/methanol/4 M-NH₃ (9:7:2, by vol.) containing 2 mM-cyclohexylenedinitrilotetra-acetic acid to separate ³H-labelled lipids, or with chloroform/methanol/acetic acid/water (25:15:4:2, by vol.) to separate ³²P-labelled lipids. ³H-labelled lipids were sprayed with Enhance[®] before localization by autoradiography. Authentic 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. by the method of Morgan and co-workers [17] with minor modification.

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Materials

Tissue-culture supplies were from Gibco. [^3H]Ins (19 Ci/mmol) was from Amersham, and [^{32}P]P_i (carrier-free) was from ICN. Cytidine nucleotides were from Sigma or Pharmacia. Silica-gel 60 plates were from Whatman. Enhance[®] was from New England Nuclear. All other reagents were from Sigma.

RESULTS AND DISCUSSION

To measure accumulation of [^3H]Ins in the absence of base exchange, experiments were performed in permeabilized GH₃ cells that were prelabelled to isotopic equilibrium with [^3H]Ins and incubated in buffer without added Ins (Ins remaining in permeabilized cells would be predicted to be of the same specific radioactivity as that in the lipid, so it would not affect the net formation of [^3H]Ins). Under these conditions, the addition of 5'-CMP stimulated the formation of unesterified [^3H]Ins in a dose-dependent manner (Fig. 1a). Kinetic analysis of this response revealed an apparent K_m for CMP of 1.7 mM. This effect was specific for 5'-CMP, whereas 5'-AMP, 5'-GMP, 2'-CMP and 3'-CMP had no effect up to concentrations of 5 mM (results not shown). 5'-CMP from different sources gave similar results. The addition of other cytidine nucleotides stimulated Ins formation also. The order of efficacy was CMP > CDP > CTP; cytidine was without effect (Fig. 1a). These effects on Ins accumulation were matched by corresponding decreases in ^3H labelling of Ins lipids (Fig. 1b). Similar effects were found with washed membranes isolated from prelabelled GH₃ cells (data not shown). These results demonstrate that CMP-stimulated accumulation of Ins is specific for 5'-CMP and is not mimicked by other nucleoside

Fig. 1. Effects of CMP, CDP, CTP and cytidine on Ins accumulation (a) and Ins lipid levels (b) in permeabilized GH₃ cells

Cells were prelabelled with [^3H]Ins and permeabilized as described in the text. Permeabilized cells were incubated with various concentrations of CMP (●), CDP (□), CTP (■) or cytidine (○) as shown in the Figure for 30 min at 37 °C. 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. 2. Effect of Mg^{2+} on CMP-stimulated Ins accumulation

Cells were prelabelled with [^3H]Ins and permeabilized as described in the text. Permeabilized cells were incubated with 5 mM-CMP (●) or with 5 mM-AMP (○) as controls and with various concentrations of MgCl_2 as shown in the Figure. Incubations were for 30 min at 37 °C. 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. Inset: kinetic analysis of these data.

monophosphates or isomers of CMP. The order of efficacy of the cytidine nucleotides (CMP > CDP > CTP) is consistent with the idea that CDP and CTP stimulate Ins formation via their metabolism to CMP. The finding that these effects were observed in washed membranes isolated from GH₃ cells shows that this effect is not due to the presence of a pool of Ins within the cytosol which is of a different specific radioactivity from that of the lipid but which is capable of exchanging with the Ins present in the lipid, and that the enzyme activity(ies) that mediates this effect is membrane-bound.

[^3H]Ins accumulation stimulated by CMP was characterized with respect to Mg^{2+} -dependency (Fig. 2). The addition of Mg^{2+} stimulated CMP-mediated [^3H]Ins accumulation in a dose-dependent manner that was maximal between 10 and 20 mM. The apparent K_m for this effect of Mg^{2+} was 0.69 mM (Fig. 2, inset). Because Mg^{2+} was effective at concentrations below those of ATP (3 mM), it appears that the active species was MgATP^{2-} . Ins accumulation in the absence of CMP was not influenced by Mg^{2+} .

To determine the mechanism of Ins accumulation in the absence of added Ins, studies were carried out with cells prelabelled to isotopic steady state with both [^3H]Ins and [^{32}P]P_i. This protocol enables Ins generation caused by phospholipase D, base exchange or reversal of PtdIns synthase to be distinguished. Cells were permeabilized in the presence of unlabelled ATP to ensure that [^{32}P]phosphatidic acid could arise only through phospholipase D action and not through phospholipase C activity (followed by phosphorylation of diacylglycerol). Under these conditions CMP (5 mM) caused a time-dependent increase in [^3H]Ins (Fig. 3a, inset) and decreases in both ^3H and ^{32}P labelling of PtdIns (Fig. 3a). There were similar decreases in both radioisotopes in PtdIns, so that the ratio of $^3\text{H}/^{32}\text{P}$ radioactivity in PtdIns did not change in the presence of CMP. These results demonstrate that CMP-induced accumulation of Ins is not due to base exchange, because base exchange does not influence the ^{32}P labelling of PtdIns [18,19]. CMP did not increase the level of [^{32}P]phosphatidic acid, which would have been the product of a

Fig. 3. Time course of the effect of CMP on ^3H and ^{32}P labelling of PtdIns, ^3H labelling of Ins and ^{32}P labelling of phosphatidic acid (PtdOH).

Cells were prelabelled with $[^3\text{H}]\text{Ins}$ and $[^{32}\text{P}]\text{P}_i$ and permeabilized as described in the text. Permeabilized cells were incubated with 5 mM-CMP or with 5 mM-AMP as controls for the times indicated at 37 °C. After incubation, PtdIns ($\square$, $\bullet$) and PtdOH ($\triangle$, $\blacktriangle$) were separated by t.l.c., and Ins ($\circ$, $\bullet$) was isolated as described in the text. Symbols: $\blacksquare$, ^{32}P labelling of PtdIns; $\square$, ^3H labelling of PtdIns; $\bullet$, Ins with CMP; $\circ$, Ins in controls; $\blacktriangle$, ^{32}P labelling of PtdOH with CMP; $\triangle$, ^{32}P labelling of PtdOH in controls. 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.

phospholipase D activity, at any time point, but appeared to cause a small decrease (Fig. 3b). These results demonstrate that CMP stimulates Ins formation via reversal of PtdIns synthase and not via activation of a phospholipase D or base exchange.

To distinguish further between reversal of PtdIns synthase and base exchange, we incubated permeabilized cells prelabelled to isotopic equilibrium with $[^3\text{H}]\text{Ins}$ and $[^{32}\text{P}]\text{P}_i$ in buffer containing unlabelled Ins. Because the addition of Ins stimulates base exchange, formation of unesterified $[^3\text{H}]\text{Ins}$ could result from both processes under these conditions. However, decreases in ^{32}P labelling of PtdIns will still specifically reflect reversal of PtdIns synthase. As we reported previously [14], unlabelled Ins caused marked decreases in ^3H labelling of PtdIns and increases in $[^3\text{H}]\text{Ins}$ (Fig. 4). Importantly, unlabelled Ins inhibited the decrease in ^{32}P labelling of PtdIns caused by CMP in the absence of Ins. These results demonstrate that Ins inhibits the reversal of PtdIns synthase, presumably by promoting the forward reaction to form PtdIns, essentially redirecting reversal of PtdIns synthase to base exchange.

CMP stimulation of reversal of PtdIns synthase was mediated

Fig. 4. Effect of Ins on CMP-stimulated changes in ^3H and ^{32}P labelling of PtdIns.

Cells were prelabelled with $[^3\text{H}]\text{Ins}$ and $[^{32}\text{P}]\text{P}_i$ and permeabilized as described in the text. Permeabilized cells were incubated with 5 mM-CMP and various concentrations of unlabelled Ins for 30 min at 37 °C. After incubation, PtdIns was separated by t.l.c., and ^3H labelling ($\square$) and ^{32}P labelling ($\blacksquare$) were measured. Points represent means $\pm$ S.D. of triplicate determinations in a representative experiment repeated three times. At zero time (before incubation), ^3H and ^{32}P radioactivities in PtdIns were 94656 ± 4047 d.p.m. and 33513 ± 948 d.p.m. respectively.

over a greater concentration range than those that have been found for CMP stimulation of base exchange. For example, base-exchange activity was maximally stimulated in rat liver microsomes at 0.5 mM [4], in intact nerve endings at between 2 and 10 μM [8,9], in rabbit lung at 0.4 mM [7] and in GH₃ cells at 0.5 mM [14]. In fact kinetic analysis revealed an apparent K_m for reversal of PtdIns synthase of 1.7 mM-CMP, a value considerably higher than that for base exchange measured in the same cells under identical conditions (0.037 mM) [14]. This difference probably accounts for our observation that base exchange predominates over reversal of PtdIns synthase in permeabilized GH₃ cells when both reactions are ongoing (Fig. 4). Because estimates of total cytidine nucleotide concentrations in intact cells are approx. 0.4 mM [20,21] and Ins concentrations are approx. 0.5 mM in GH₃ cells (A. B. Cubitt & M. C. Gershengorn, unpublished work), it is likely that base exchange is more important than reversal of PtdIns synthase *in vivo* also. In fact, base exchange is readily observed in intact cells [22,23].

In summary, we have shown that 5'-CMP can stimulate reversal of PtdIns synthase to form Ins from PtdIns in permeabilized GH₃ cells incubated in buffer without Ins. We showed previously that 5'-CMP stimulates base exchange in these cells [14]. Unlabelled Ins inhibited reversal of PtdIns synthase caused by CMP while stimulating PtdIns/Ins base exchange. These data show that CMP-stimulated reversal of PtdIns synthase and PtdIns/Ins base exchange are distinct reactions that are influenced oppositely by Ins.

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