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Stimulation of Nuclear Polyphosphoinositide Synthesis by GTP-γ-S: A Potential Regulatory Role for Nuclear GTP-Binding Proteins

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The nonhydrolyzable GTP analogue GTP-γ-S was capable of stimulating in vitro phosphorylation of polyphosphoinositides in isolated nuclei prepared from mouse erythroleukemia cells. On the contrary, GDP-β-S was ineffective. The stimulation was not detectable when nuclei were prepared from erythroleukemia cells induced to differentiate by exposure to dimethyl sulfoxide. Both nuclear phosphomonoesterase and phospholipase C activities were not influenced by GTP-γ-S. Our results point to the likelihood that nuclear phosphoinositide kinases might be regulated by a GTP-binding protein.

In recent years, investigations carried out in several laboratories have shown the existence of an autonomous nuclear polyphosphoinositide metabolism [for a review see refs. 1–3]. Cocco et al. [4] first demonstrated that membrane-deprived nuclei from mouse erythroleukemia cells can synthesize in vitro PIP and PIP_2. Subsequently, several reports have indicated that other key enzymes of phosphoinositide cycle such as PLC, diacylglycerol kinase and inositol polyphosphate-1-phosphatase exist within the nucleus [5-10]. Nuclear phosphoinositide metabolism can be influenced by a variety of stimuli such as growth factors, cytokines and differentiating agents [11–14]. Nuclear inositol lipids are conceivably linked to translocation and activation of PKC to the same cell compartment, a well-documented phenomen that occurs in many cell models treated with different agonists [for a recent review see ref. 15]. Isolated nuclei also contain GTP-binding proteins [16–20] but their role in this cell compartment is still unclear. For example, a well-characterized nuclear GTP-binding protein is RAN/TC4, a 25 kDa ras-related polypeptide essential for nuclear transport and involved in mitotic control [e.g. 21]. It should be emphasized that in cell membrane preparations the activity of phosphatidylinositol-4-phosphate kinase (i.e. the enzyme responsible for PIP_2 synthesis) is stimulated by nonhydrolyzable GTP analogues, such as GTP-γ-S, thus suggesting that a GTP-binding protein could be involved in controlling enzyme activity [22–25].

With the above in mind, we have investigated whether GTP-γ-S can stimulate in vitro phosphoinositide phosphorylation in isolated mouse erythroleukemia nuclei. We have found that incorporation of radiolabel from [γ-^{32}P] ATP into PIP and PIP_2 was enhanced by addition of GTP-γ-S. On the contrary, GDP-β-S was ineffective. The stimulation was not detected when nuclei were isolated from cells terminally differentiated by exposure to DMSO.

MATERIALS AND METHODS

Cell Culture and Differentiation

Mouse erythroleukemia cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. Erythroid differentiation was induced by addition of 1.5% (v/v) DMSO to the medium for 4 days [4].

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Abbreviations: PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PKC, protein kinase C; DMSO, dimethyl sulfoxide; IP_3: inositol 1,4,5-trisphosphate.

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Isolation of Nuclei

This was accomplished as previously reported using 0.3% Triton X-100 to obtain membrane-deprived nuclei [26]. Nuclear purity was evaluated as described elsewhere, measuring glucose-6-phosphatase activity as well as recovery of the cytoskeletal marker β-tubulin by immunoblotting [4,27].

Phosphorylation of Nuclear Polyphosphoinositides

This was performed according to Cocco et al. [4] with minor modifications. Briefly, nuclei (approximately 180 μg of protein) were incubated for 10 min at 30°C in 0.25 M sucrose, 5 mM β-mercaptoethanol, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.0), 10 μM ATP and 25 μCi of [γ-^{32}P] ATP (5000 Ci/mmole). ^{32}P-labelled lipids were analyzed by thin layer chromatography on 1%-oxalate-sprayed plates developed with chloroform/methanol/water/saturated ammonia (45:35:8:2, by volume). Chromatography plates were autoradiographed before exposure to iodine, to detect internal lipid standards.

Phosphomonoesterase Activity Assay

The procedure outlined by Gilmour et al. [28] was followed with the only modification that after the 10 min incubation at 30°C, 100 μM of unlabelled ATP was added and chasing proceeded for 10 min at 30°C.

PLC Activity Assay

This was done according to Martelli et al. [14]. Briefly, nuclear protein (60 μg) was incubated for 10 min at 37°C in 100 mM (2-N-morpholino)ethanesulfonic acid (pH 6.7), 10 μM $CaCl_2$, 150 mM NaCl, 0.06% taurodeoxycholate, 3 nmol [^{3}H]PIP_2 (specific activity 30,000 dpm/nmol). Hydrolysis was stopped by adding chloroform-methanol-HCl and inositol phosphates recovered from the aqueous phase were analyzed by high pressure liquid chromatography and counted by scintillation counting.

RESULTS AND DISCUSSION

In Fig. 1 (panel A) we show the results of experiments in which different concentrations of GTP-γ-S were tested to determine whether or not a stimulation of in vitro phosphorylation of nuclear polyphosphoinositides could be detected. It is evident that maximum stimulation was achieved at 200 μM GTP-γ-S. At this concentration, incorporation of the radiolabel into PIP_2 almost doubled in comparison with the control. No further stimulation was observed with concentrations higher than 200 μM (data not shown). On the other hand, at the same concentration, enhancement of PIP synthesis was less pronounced, as the increase was only 50% over the control value.

On the contrary, GDP-β-S, in the same concentration range, was ineffective, as presented in Fig. 1, panel B.

It should be stressed that enhanced phosphorylation of PIP and PIP_2 by GTP-γ-S was not seen in nuclei prepared from DMSO-treated cells (Fig. 1, panel C).

To rule out any effect of GTP-γ-S on other enzymes involved in nuclear polyphosphoinositide metabolism, we investigated the effect of the nonhydrolyzable GTP analogue on phosphomonoesterase activity. However, as demonstrated in Fig. 2 such an activity was not affected at all by the nucleotide.

Last, we measured nuclear PLC activity both in the absence and in the presence of 200 μM GTP-γ-S (Fig. 3). It was evident that also this enzymic activity was not stimulated by the analogue. The presence of GTP-binding proteins in nuclear fractions has been demonstrated by several reports [16-20]. Although some of these investigations have suggested that the proteins are localized to the nuclear envelope and might be involved in nuclear transport and/or fusion of nuclear membrane vesicles required for reassembly of the nucleus after mitosis [17,18], evidence seems to indicate that GTP-binding proteins are also present inside the nucleus and resist treatment with nonionic detergents, such Triton X-100 at a concentration as high as 1% [29]. These data are also supported by ultrastructural immunolocalization experiments [20] showing the presence of the GTP-binding protein $G_{s\alpha}$ inside the nucleus. Differences in nuclear GTP-binding proteins have been described after serum starvation of Swiss 3T3 cells [16] while exposure of quiescent Balb/

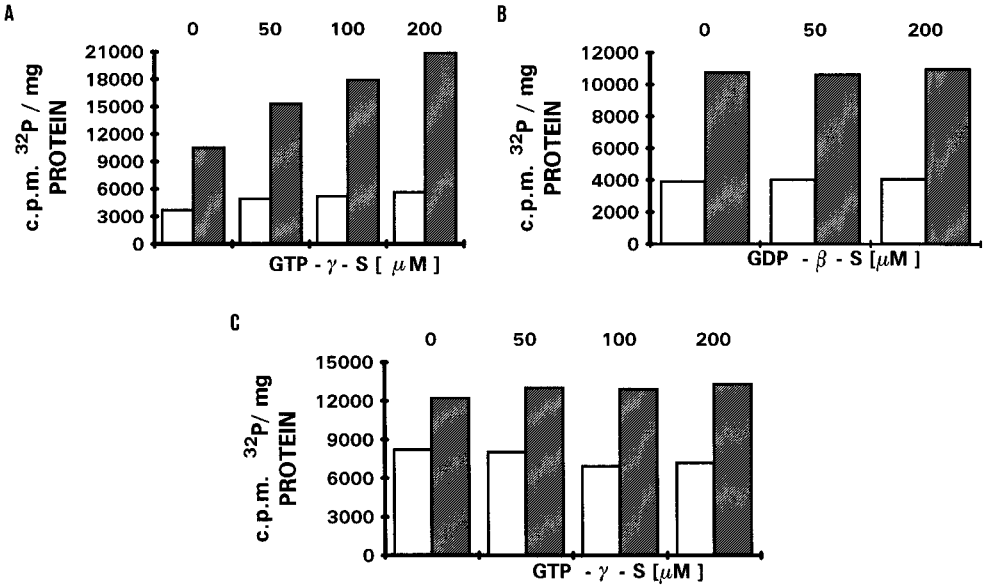

FIG. 1. Effect of different concentrations of GTP-γ-S and GDP-β-S on the in vitro phosphorylation of polyphosphoinositides in isolated nuclei prepared from control cells (panel A, B) and cells treated with DMSO (panel C). White bars: incorporation of the radiolabel into PIP_2; black bars: incorporation of the radiolabel into PIP. Results are the mean from three different experiments with a s.d. of 13%. Note that in nuclei from DMSO-treated cells both PIP and PIP_2 are synthesized to an extent greater than in control nuclei, as previously demonstrated [4].

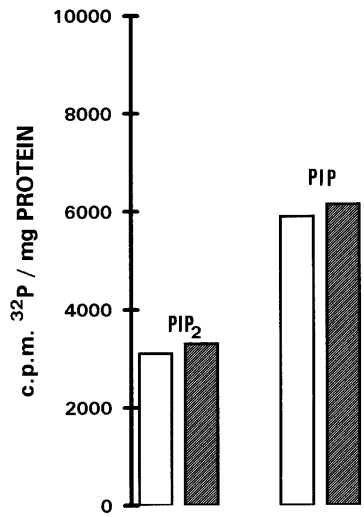

FIG. 2. Effect of 200 μM GTP-γ-S on phosphomonoesterase activity acting on polyphosphoinositides in isolated nuclei from control erythroleukemia cells. Isolated nuclei were incubated in the standard phosphorylation mix for 10 min at 30°C, then an aliquot was removed and incorporation of the radiolabel into both PIP and PIP_2 was determined. Incorporation into PIP_2 was 3,300 c.p.m./mg protein while into PIP was 10,750 c.p.m./mg protein. An excess of unlabelled ATP was then added and incubation proceeded for an additional 10 min. Then incorporation was determined again and the values are presented in the illustration. White bars: no GTP-γ-S; dark bars: plus GTP-γ-S. Results are the mean from three different experiments with a s.d. of 11%.

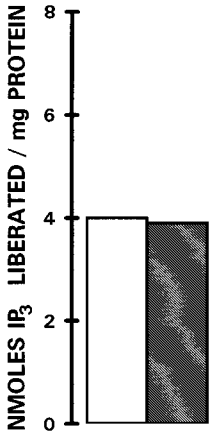

FIG. 3. PLC activity in isolated nuclei from mouse erythroleukemia cells in the absence (white bar) and in the presence (dark bar) of 200 μM GTP-γ-S. Results are the mean from three different experiments with a s.d. of 7%.

c3T3 to a combination of EGF and insulin resulted in a massive translocation to the nucleus of GTP-binding protein $G_{i\alpha}$ [19].

Although nuclear metabolism of polyphosphoinositides is quite extensively studied, only very limited information is at present available concerning factors that might be involved in controlling enzymes of the inositide cycle. In other cell districts it has been shown that phosphatidylinositol-4-kinase is under the control of a GTP-binding protein [22–25]. Indeed, a small GTP-binding protein, with a molecular mass between 20 and 25 kDa, has been partly purified from rat liver membranes and found to be capable of activating in vitro purified phosphatidylinositol-4-phosphate kinase [24]. Moreover, Stephens et al. have recently shown that in neutrophils and U937 cells a phosphatidylinositol-3-kinase activity is activated by G protein $\beta\gamma$ subunits [30].

Overall, our results suggest that in nuclei prepared from control (undifferentiated) mouse erythroleumia cells the activity of both phosphatidylinositol kinase and phosphatidylinositol-4-kinase is stimulated by the presence of GTP-γ-S and might be regulated by a GTP-binding protein. It is interesting to note that a concentration of 100 μM of GTP-γ-S resulted in an approximately 100% increase of phosphatidylinositol-4-phosphate kinase activity in rat brain membrane preparations [23], i.e. a value quite similar to what we have observed in isolated nuclei. Our data also ruled out that the increase in the phosphorylation of polyphosphoinositides could be due to activation of PIP_2 hydrolysis by PLC, or inhibition of hydrolysis by phosphomonoesterase activity [23]. A puzzling observation is that GTP-γ-S was unable to enhance inositol lipid phosphorylation in nuclei isolated from DMSO-treated (differentiated) mouse erythroleukemia cells. It is well documented that in this type of nuclei PLC-β1 activity is reduced in comparison with control nuclei [14] and this results in higher levels of nuclear PIP and PIP_2 as detected by in vivo labelling with [^{3}H]myo-inositol [31]. However, it might be possible that other differences in the polyphosphoinositide metabolism exist in nuclei from differentiated cells and one of these differences could be the absence of GTP-binding protein(s) controlling inositide kinases.

Future experiments should be directed at identifying the GTP-binding protein(s) involved in regulating nuclear inositide kinase activities.

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REFERENCES

1. Michell, R. H. (1992) *Current Biol.* **4,** 200–202.
2. Divecha, D., Banfic, H., and Irvine, R. F. (1993) *Cell* **74,** 405–407.

3. Cocco, L., Martelli, A. M., and Gilmour, R. S. (1994) *Cell. Signalling* **6,** 481–485.
4. Cocco, L., Gilmour, R. S., Ognibene, A., Letcher, A. J., Manzoli, F. A., and Irvine, R. F. (1987) *Biochem. J.* **248,** 765–770.
5. Capitani, S., Bertagnolo, V., Mazzoni, M., Santi, P., Previati, M., Antonucci, A., and Manzoli, F. A. (1989) *FEBS Lett.* **254,** 194–198.
6. Payrastre, B., Nievers, M., Boonstra, J., Breton, M., Verkleij, A. J., and van Bergen en Henegouwen, P.M. P. (1992) *J. Biol.Chem.* **287,** 5078–5084.
7. Banfic, H., Zizak, M., Divecha, N., and Irvine, R. F. (1993) *Biochem. J.* **290,** 633–636.
8. York, J. D., and Majerus, P. W. (1994) *J. Biol. Chem.* **269,** 7847–7850.
9. York, J. D., Saffitz, J. E., and Majerus, P. W. (1994) *J. Biol. Chem.* **269,** 19992–19999.
10. Fee, J. A., Monsey, J. D., Handler, R. J., Leonis, M. A., Mullaney, S. R., Rath Hope, H. M., and Silbert, D. F. (1994) *J. Biol. Chem.* **269,** 21699–21708.
11. Martelli, A. M., Gilmour, R. S., Bertagnolo, V., Neri, L. M., Manzoli, L., and Cocco, L. (1992) *Nature* **358,** 242–245.
12. Cataldi, A., Di Primio, R., Lisio, R., Rana, R. A., Robuffo, I., Bosco, D., and Miscia, S. (1993) *Cell. Signalling* **5,** 331–336.
13. Marmiroli, S., Ognibene, A., Bavelloni, A., Cinti, C., Cocco, L., and Maraldi, N. M. (1994) *J. Biol. Chem.* **269,** 1–4.
14. Martelli, A. M., Billi, A. M., Gilmour, R. S., Neri, L. M., Manzoli, L., Ognibene, A., and Cocco, L. (1994) *Cancer Res.* **54,** 2536–2540.
15. Buchner, K. (1995) *Eur. J. Biochem.* **228,** 211–221.
16. Takeda, S., Sugiyama, H., Natori, S., and Seikimizu, K. (1989) *FEBS Lett.* **244,** 469–472.
17. Rubins, J. B., Benditt, J. O., Dickey, B. F., and Riedel, N. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 7080–7084.
18. Seydel, U., and Gerace, L. (1991) *J. Biol. Chem.* **266,** 7602–7608.
19. Crouch, M. F. (1991) *FASEB J.* **5,** 200–206.
20. Saffitz, J. E., Nash, J. H., Green, K. G., Luke, R. A., Ransnas, L. A., and Insel, P. A. (1994) *FASEB J.* **8,** 252–258.
21. Becker, J., Melchior, F., Gerke, V., Bischoff, F. R., Ponstingl, H., and Wittinghofer, A. (1995) *J. Biol. Chem.* **270,** 11860–11865.
22. Urumow, T., and Wieland, O. H. (1988) *Biochim. Biophys. Acta* **972,** 232–238.
23. Smith, C. D., and Chang, K. J. (1989) *J. Biol. Chem.* **264,** 3206–3210.
24. Urumow, T., and Wieland, O. H. (1990) *FEBS Lett.* **263,** 15–17.
25. Urumow, T., and Wieland, O. H. (1990) *Biochim. Biophys. Acta* **1052,** 152–158.
26. Martelli, A. M., Falcieri, E., Gobbi, P., Manzoli, L., Gilmour, R. S., and Cocco, L. (1991) *Exp. Cell Res.* **196,** 216–225.
27. Manzoli, L., Billi, A. M., Gilmour, R. S., Martelli, A. M., Matteucci, A., Rubbini, S., Weber, G., and Cocco, L. (1995) *Cancer Res.* **55,** 2978–2980.
28. Gilmour, R. S., Prosser, C. G., Fleet, I. R., Cocco, L., Saunders, J. C., Brown, K. D., and Corps, A. N. (1988) *Br. J. Cancer* **58**(Suppl IX)**,** 23–30.
29. Takei, Y., Kurosu, H., Takahashi, K., and Katada, T. (1992) *J. Biol. Chem.* **267,** 5085–5089.
30. Stephens, L., Smrcka, A., Cooke, F. T., Jackson, T. R., Sternweis, P. C., and Hawkins, P. T. (1993) *Cell* **77,** 83–93.
31. Martelli, A. M., Cataldi, A., Manzoli, L., Billi, A. M., Rubbini, S., Gilmour, R. S., and Cocco, L. (1995) *Cell. Signalling* **7,** 53–56.