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Guanine nucleotides stimulate hydrolysis of phosphatidylinositol and polyphosphoinositides in permeabilized Swiss 3T3 cells

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Hydrolysis-resistant analogues of GTP specifically stimulate the formation of [^{3}H]inositol mono-, bis- and trisphosphates by saponin-permeabilized Swiss 3T3 cells prelabelled with [^{3}H]inositol. Each inositol phosphate is formed largely by hydrolysis of its parent lipid and not by dephosphorylation of inositol 1,4,5-trisphosphate [(1,4,5)IP_3]. Although hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) is most sensitive to guanine nucleotides, hydrolysis of phosphatidylinositol (PI) and phosphatidylinositol 4-phosphate (PIP) is quantitatively more important. These results suggest that a guanine nucleotide-dependent regulatory protein(s) (G-protein) is involved in regulating the hydrolysis of PI and PIP, as well as PIP_2, and so may allow formation of diacylglycerol (DG) without simultaneous production of (1,4,5)IP_3 and mobilization of intracellular Ca^{2+}.

Phosphatidylinositol; Polyphosphoinositide; G-protein; Inositol phosphate; (Swiss 3T3 cell)

1. INTRODUCTION

Guanine nucleotide-dependent regulatory proteins (G-proteins) mediate the effects of many cell-surface receptors on their intracellular effector enzymes [1]. Activation of one such effector, phosphoinositidase C, leads to hydrolysis of a membrane phospholipid, PIP_2, to give two intracellular messengers, (1,4,5)IP_3 and DG. The former regulates the mobiliztion of intracellular Ca^{2+} pools [2] and perhaps also Ca^{2+} transport at the plasma membrane [3], whilst the latter stimulates the activity of certain protein kinase C [4].

PI and PIP are the substrates from which PIP_2 is synthesised, but it is unclear to what extent they may, in addition, be substrates for a G-protein-regulated phosphoinositidase C. Without complex kinetic analyses, which are subject to many assumptions, it is difficult in intact cells to determine whether IP_1 and (1,4)IP_2 are formed by hydrolysis of PI and PIP or by successive dephosphorylations of (1,4,5,)IP_3. Nevertheless, it is important to determine whether hydrolysis of PI and PIP occurs because their hydrolysis could allow activation of one limb of this signalling pathway, the DG-protein kinase C limb, without the simultaneous increase in cytosolic Ca^{2+} that is evoked by (1,4,5)IP_3 but not by IP_2 or IP_1 [2]. Here, we have examined the effects of guanine nucleotides on permeabilized Swiss 3T3 cells to determine whether G-protein(s) regulate(s) the activity of the phosphoinositidase(s) C that catalyse(s) hydrolysis of phosphoinisitides.

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Abbreviations: DG, diacylglycerol; GDP, guanosine 5′-diphosphate; GDPβS, guanosine 5′-[β-thio]diphosphate; GTP, guanosine 5′-triphosphate; GTPγS, guanosine 5′-[γ-thio]triphosphate; GppNHp, guanosine 5′-[β,γ-imido]triphosphate; IP_1, IP_2 and IP_3, inositol mono-, bis- and trisphosphates; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate

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2. MATERIALS AND METHODS

Swiss 3T3 cell cultures were maintained as described [5]. Cells were seeded into 24-well culture trays (Nunc) in Dulbecco's modified Eagles medium supplemented with 10% calf serum. *myo*-[^{3}H]Inositol (10 μCi/m) was added directly to the growth medium 5 or 6 days after seeding. After a further 48 h, when the cells were confluent and quiescent, they were washed in phosphate-buffered saline [6] supplemented with glucose (10 mM) and permeabilized by first washing them in a high-K^+ medium (mM: KCl, 140; NaCl, 20; $MgCl_2$, 2; ATP, 2; EGTA, 1; Pipes, 20; free Ca^{2+} = 130 nM; pH 6.8) followed by addition of saponin (60 μg/ml) for 4 min. The cells were then rinsed in 1 ml high-K^+ medium and incubated in 300 μl of this medium containing the indicated concentrations of guanine nucleotides. All incubations contained Li^+ at a final concentration of 4 mM to provide a control for Li^+ added with the maximal concentrations of the Li^+ salts of guanine nucleotides. Reactions were stopped by addition of 300 μl cold $HClO_4$ (6% v/v) and then 50 μl of a hydrolysate of phytic acid (about 3 mg phosphate/ml) [7]. Inositol phosphates were allowed to extract for 30 min, the extract was removed and the wells rinsed with 300 μl $HClO_4$ (3% v/v). The combined extract and wash was neutralized by vigorous mixing with 1 ml of a 1:1 mixture of tri-*n*-octylamine and 1,1,2-trichlorotrifluoroethane [8]. The neutralized extract was loaded onto anion-exchange columns [9] and [^{3}H]inositol was eluted with water (8 ml), [^{3}H]glycerophosphoinositol with 60 mM $NaCO_2H$ (8 ml), [^{3}H]IP_1 with 200 mM NH_4CO_2H in 0.1 M HCO_2H (8 ml), [^{3}H]IP_2 with 500 mM NH_4CO_2H in 0.1 M HCO_2H (8 ml) and [^{3}H]IP_3 with 800 mM NH_4CO_2H in 0.1 M HCO_2H (8 ml). Samples were counted in 10 ml Liquiscint scintillation cocktail (National Diagnostics).

For extraction of [^{3}H]inositol-labelled lipids, cells were scraped from the dishes into $HClO_4$ (3% v/v; final volume 0.9 ml) and 3.3 ml $CHCl_3$/MeOH/6 M HCl (50:100:1, v/v) was added followed after 30 min by 1.1 ml $CHCl_3$ and 1.1 ml of 2 M KCl. After mixing, the organic layer was saved and pooled with the organic layer obtained after re-extraction with a further 1.5 ml $CHCl_3$. The organic extract was washed 4 times with a mixture of MeOH and HCl (1 M) (1:1, v/v) to remove water-soluble [^{3}H]inositol-labelled compound and then dried under vacuum before deacylation and separation [10,11].

Neomycin sulphate and guanine nucleotides (Li or Na salts) were supplied by Boehringer, *myo*-[^{3}H]inositol (88Ci/mmol) was from Amersham (England), and AG1X8 (200–400 mesh, formate form) anion-exchange resin was from BioRad. All other reagents were from Sigma or the suppliers listed in earlier publications.

3. RESULTS AND DISCUSSION

The non-hydrolysable analogues of GTP, GTPγS and GppNHp, stimulated formation of [^{3}H]inositol mono-, bis- and trisphosphates in saponin-permeabilized Swiss 3T3 cells previously labelled with [^{3}H]inositol (fig.1). The effects are specific, since neither GTP nor GDP is effective, and while GDPβS slightly stimulates formation of

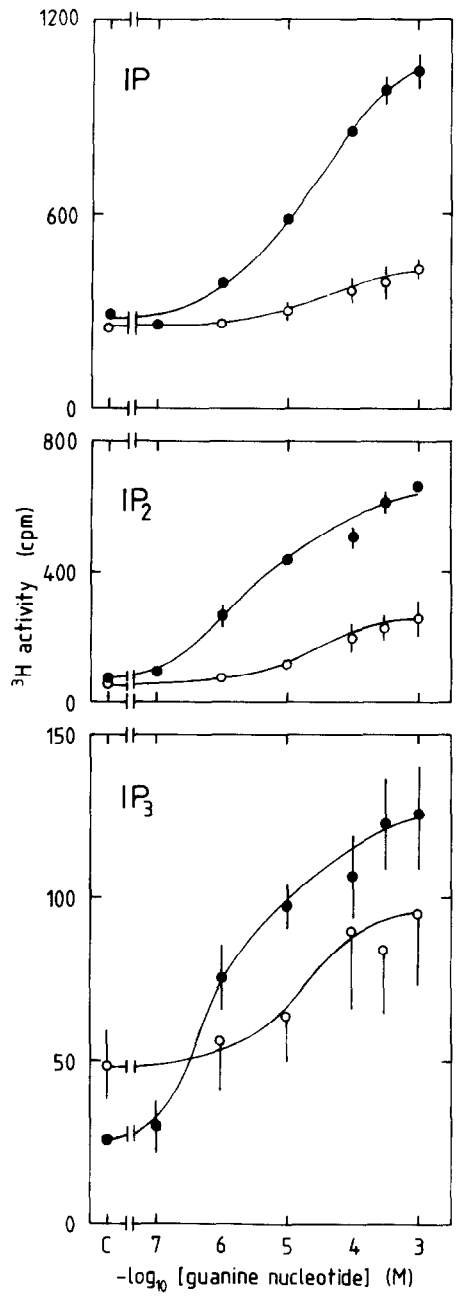

Fig.1 Concentration-response relationships for the stimulation of [^{3}H]inositol phosphate formation by GTPγS and GppNHp in permeabilized Swiss 3T3 cells. Permeabilized cells were incubated for 10 min with GTPγS (●) or GppNHp (○) at the indicated concentrations. [^{3}H]Inositol phosphates were then extracted and measured as described in section 2. Results are means ± SE of duplicate determinations from 4 independent experiments.

Table 1

Effects of guanine nucleotides, alone or combined, on [^{3}H]inositol phosphate formation by permeabilized cells

	[^{3}H]Inositol phosphates (cpm)			
	IP_1	IP_2	IP_3	$IP_1+IP_2+IP_3$
Control	293 ± 48	64 ± 15	30 ± 9	387 ± 70
GTPγS	1530 ± 166	1011 ± 231	164 ± 33	2704 ± 393
GDPβS	309 ± 35	114 ± 16	39 ± 5	462 ± 56
GDP	237 ± 34	55 ± 11	28 ± 8	321 ± 52
GTP	241 ± 43	71 ± 15	34 ± 8	347 ± 65
GTPγS +				
None	684 ± 63	639 ± 84	118 ± 9	1440 ± 150
GDPβS	352 ± 45	139 ± 26	38 ± 11	529 ± 82
GDP	307 ± 45	88 ± 16	33 ± 10	428 ± 65
GTP	289 ± 21	105 ± 17	38 ± 8	432 ± 46

Permeabilized cells were incubated for 10 min with single guanine nucleotides (GTPγS, 300 μM; GDPβS, GDP or GTP, 1000 μM) or with a combination of GTPγS (10 μM) and GDPβS, GDP or GTP (1000 μM). Results (cpm/culture well) are means ± SE of 4 experiments

[^{3}H]inositol phosphates (table 1), we cannot eliminate the possibility that such stimulation results from contamination. High concentrations of GDP, GTP or GDPβS (1000 μM) each substantially inhibit the ability of GTPγS (10 μM) to stimulate the formation of [^{3}H]inositol phosphates (table 1). These results imply that each guanine nucleotide binds to a G-protein that stimulates phosphoinositidase C, but under the conditions of these experiments only the non-hydrolysable anlogues of GTP lead to significant activation. The effects of GTPγS on [^{3}H]inositol phosphate formation are observed only after permeabilization of the cells (not shown), thus confirming the expected intracellular site of action of guanine nucleotides.

While [^{3}H]IP_3 is likely to come only from hydrolysis of PIP_2, (1,4)IP_2 and IP_1 may have been formed either directly by hydrolysis of PIP and PI or by successive dephosphorylations of (1,4,5)IP_3. The results shown in table 2 indicate that the major

Table 2

Effects of neomycin and 2,3-bisphosphoglycerate on GTPγS-stimulated formation of [^{3}H]inositol phosphates

	GTPγS	[^{3}H]Inositol phosphates (cpm)			
		IP_1	IP_2	IP_3	$IP_1+IP_2+IP_3$
Control					
	−	286 ± 20	75 ± 12	23 ± 5	384 ± 27
	+	1135 ± 163	677 ± 17	119 ± 14	1931 ± 184
Neomycin					
	−	292 ± 56	97 ± 23	41 ± 13	430 ± 91
	+	968 ± 63	342 ± 53	47 ± 10	1357 ± 114
2,3-Bisphosphoglycerate					
	−	322 ± 39	130 ± 26	68 ± 23	520 ± 87
	+	1022 ± 109	665 ± 28	193 ± 2	1880 ± 138

Permeabilized cells were incubated for 2 min with neomycin (1 mM) or 2,3-bisphosphoglycerate (5 mM) before addition of a maximal concentration (1000 μM) of GTPγS. Incubations were terminated after a further 10 min. Results, expressed as cpm/culture well, are means ± SE of duplicate determinations from 3 independent experiments

source of each of the [^{3}H]inositol phosphates is direct hydrolysis of the parent lipids. Addition of 2,3-bisphosphoglycerate (5 mM), which inhibits Ins(1,4,5)P_3 5-phosphatase [1,2] substantially increased the unstimulated level of [^{3}H]IP_3, slightly increased (from 96 to 125 cpm) [^{3}H]IP_3 formation in response to a maximal concentration of GTPγS, but most importantly had minimal effects on the GTPγS-stimulated formation of either [^{3}H]IP_2 or [^{3}H]IP_1 (table 2). Neomycin, an aminoglycoside antibiotic reported to bind selectively to polyphosphoinositides and particularly to PIP_2 [13,14], abolished GTPγS-stimulated formation of [^{3}H]IP_3, substantially reduced formation of [^{3}H]-IP_2 but had no significant effect on formation of [^{3}H]IP_1 (table 2). In a further series of experiments (not shown), addition of high concentrations (100 μM) of unlabelled (1,4,5)IP_3 or (1,4)IP_2 had no significant effect on either the basal or GTPγS-stimulated formation of [^{3}H]IP_1, [^{3}H]IP_2 or [^{3}H]IP_3. Taken together, these results indicate that the major source of each of the inositol phosphates formed in response to stimulation by GTPγS is hydrolysis of the parent lipid.

Since different concentrations of GTPγS or GppNHp were required for half-maximal stimulation of inositol phosphates formation ([^{3}H]IP_3 < [^{3}H]IP_2 < [^{3}H]IP_3; fig.1), we were concerned that hydrolysis of PI and PIP might simply reflect a failure of permeabilized cells to maintain adequate pools of PIP_2 during stimulation. However, this appears unlikely because the different sensitivities to guanine nucleotides were similar in cells stimulated with either GTPγS or GppNHp, even though the magnitude of the response to GTPγS was substantially larger (fig.1). Furthermore, after stimulation with a maximal concentration of GTPγS there was a lag of about 1 min after which formation of each of the [^{3}H]inositol phosphates was essentially linear for 10 min. Finally, when the lipids of intact or permeabilized cells, before and after stimulation by GTPγS, were analysed, the results revealed that permeabilization slightly increased the ratio of polyphosphoinositides to PI, and that subsequent stimulation with GTPγS reversed the effect (table 3). This suggests that while maximal stimulation for 10 min may lead to small losses of PIP_2 and PIP, the effects are probably too small to explain the observed differences in sensitivity to guanine nucleotides.

Table 3

Effects of GTPγS on [^{3}H]phosphoinositides of permeabilized cells

	[^{3}H]Phosphoinositide (% total)		
	PI	PIP	PIP_2
Intact cells	93.2 ± 0.8	2.5 ± 0.5	4.3 ± 0.2
Permeabilized cells			
Control	91.7 ± 0.3	2.9 ± 0.1	5.4 ± 0.4
+ GTPγS	93.3 ± 0.4	2.4 ± 0.5	4.3 ± 0.02

Intact or permeabilized cells were incubated for 10 min with or without GTPγS (300 μM). The incubations were terminated, lipids were extracted and deacylated and [^{3}H]glycerophosphoinositides separated as described in section 2. Results are means ± SE of duplicate determinations from 3 independent experiments. Comparison of the total [^{3}H]phosphoinositide activities of intact and permeabilized cells is not possible because during permeabilization some cells detach and are lost. In permeabilized cells the total [^{3}H]phosphoinositide activity was 12 045 ± 241 cpm for unstimulated cells and 11 484 ± 1335 cpm after stimulation with GTPγS

Several studies of broken cells have reported receptor- or G-protein-stimulated hydrolysis of both PIP and PIP_2, but they have not, in contrast to our findings, generally reported hydrolysis of PI [15–17]. However, in certain intact cells, an initial hydrolysis of polyphosphoinositides seems to be followed by a more sustained hydrolysis of PI [18–20]. Our results raise the possibility that G-protein(s) is (are) involved in regulating the hydrolysis of PI and PIP as well as PIP_2 in Swiss 3T3 cells and may thereby permit formation of DG without an accompanying increase in intracellular Ca^{2+} concentration.

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REFERENCES

[1] Gilman, A.G. (1987) Annu. Rev. Biochem. 56, 615–649.
[2] Berridge, M.J. and Irvine, R.F. (1984) Nature 312, 315-321.
[3] Putney, J.W. jr (1986) Cell Calcium 7, 1–12.
[4] Nishizuka, Y. (1984) Nature 308, 693–697.
[5] Brown K.D. and Blakeley, D.M. (1983) Biochem. J. 212, 465-472.
[6] Heslop, J.P. Blakeley, D.M., Brown, K.D. Irvine, R.F. and Berridge, M.J. (1986) Cell 47, 703–709.
[7] Wreggett, K.A. and Irvine, R.F. (1987) Biochem. J. 245, 655–660.

[8] Sharp, E.D. and McCarl, R.L. (1982) Anal. Biochem. 124, 421–424.
[9] Berridge, M.J., Dawson, R.M.C., Downes, C.P. Heslop, J.P. and Irvine, R.F. (1983) Biochem. J. 222, 195–201.
[10] Taylor, C.W., Blakeley, D.M., Corps, A.N., Berridge, M.J. and Brown, K.D. (1988) Biochem. J. 249, 917–920.
[11] Clarke, N.G. and Dawson, R.M.C. (1981) Biochem. J. 195, 301–306.
[12] Downes, C.P., Mussat, M.C. and Michell, R.H. (1982) Biochem. J. 203, 169–177.
[13] Schacht, J., Weiner, N.D. and Lodhi, S. (1978) in: Cyclitols and Phosphoinositides (Wells, W.W. and Eisenberg, F., eds) pp. 153–165, Academic Press, London.
[14] Downes, C.P. and Michell, R.H. (1981) Biochem. J. 198, 133–140.
[15] Hrbolich, J.K., Culty, M. and Haslam, R.J. (1987) Biochem. J. 243, 457–465.
[16] Straub R.E. and Gershengorn, M.C. (1986) J. Biol. Chem. 261, 2712–2717.
[17] Baukal, A.J., Balla, T., Hunyady, L., Hausdorff, W., Guillemette, G. and Catt, K.J. (1988) J. Biol. Chem. 263, 6087–6092.
[18] Majerus, P.W., Connolly, T.M., Deckmyn, H., Ross, T.S., Bross, T., Ishii, H., Bansal, V.S. and Wilson, D.B. (1986) Science 324, 1519–1526.
[19] Griendling, K.K., Rittenhouse, S.E., Brock, T.A., Ekstein, L.S., Gimbrone, M.A. and Alexander R.W. (1986) J. Biol. Chem. 261, 5901–5906.
[20] Imai, A. and Gershengorn, M.C. (1986) Proc. Natl. Acad. Sci. USA 83, 8540–8544.