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REGULATION OF INOSITOL 1,4,5-TRISPHOSPHATE METABOLISM BY GUANINE NUCLEOTIDES IN MEMBRANES OF CULTURED NEWBORN RAT CARDIOMYOCYTES

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Abstract—Membranes of cultured newborn rat cardiomyocytes contain enzymatic activities that regulate the formation and the breakdown of inositol 1,4,5-trisphosphate (1,4,5-IP_3). GTPγS increased the rate of exogenous [^{3}H]phosphatidyl 4,5-bisphosphate ([^{3}H]PIP_2) hydrolysis (EC_{50}: 40 μM). This effect was dependent on the presence of deoxycholate and maximal at 2 mM deoxycholate. GTPγS increased the efficacy of phospholipase C (PLC) (by 2.3-fold), but did not alter the apparent affinity of the enzyme for PIP_2. Other nucleotides, GDPβS and ATPγS, and pyrophosphate also stimulated PIP_2 hydrolysis, while AlF_4^- was ineffective. The effect of GTPγS was not inhibited by GDPβS. The agonists norepinephrine and thrombin, which by themselves had no effect, did not potentiate the response to GTPγS. In contrast, 1,4,5-IP_3 hydrolysis was decreased by GTPγS (EC_{50}: 100 μM) as well as by other nucleotides and by pyrophosphate, but not by AlF_4^-. GDPβS did not antagonize the GTPγS-induced inhibition of IP_3 hydrolysis. These results suggest that GTP can stimulate the hydrolysis of exogenous PIP_2 by an action on membrane-bound PLC at a site beyond the G protein activating PLC and inhibit the hydrolysis of 1,4,5-IP_3 by a mechanism common to all nucleotides. Thus, GTP can regulate 1,4,5-IP_3 metabolism by stimulating its formation and inhibiting its breakdown.

Phosphoinositides are membrane-bound phospholipids that are rapidly hydrolyzed by phospholipase C (PLC‖) to inositol phosphates and diacylglycerol in response to a variety of hormones and transmitters [1]. Inositol 1,4,5-trisphosphate (1,4,5-IP_3), the major physiologically active inositol phosphate, mediates the release of calcium from nonmitochondrial stores. The phosphoinositides and the inositol phosphates are metabolically closely related. They coexist in a dynamic equilibrium which is maintained by a complex system of specific kinases and phosphatases [2]. Kinetic studies in many intact tissues and isolated cells have provided indirect evidence that stimulation of phosphatidyl 4,5-bisphosphate (PIP_2) hydrolysis by PLC is the preferred pathway for agonist-induced formation of 1,4,5-IP_3 [1]. The agonist-induced activation of PLC, like adenylate cyclase activation, has been shown in a number of tissues to be dependent upon guanine nucleotides, implying that a guanine nucleotide binding protein (G protein) is involved in the transduction of the hormonal signal from the receptor binding site to PLC [3, 4]. This has been confirmed recently by Smrcka *et al.* [5] who demonstrated that a G protein α subunit purified from bovine brain selectively stimulates PIP_2 hydrolysis by a partially purified PLC.

The recent studies of Steinberg *et al.* [6] show that, in intact cardiomyocytes, norepinephrine-induced stimulation of phosphoinositide hydrolysis results in the rapid formation of 1,4,5-IP_3. In addition, in myocyte membranes, the α_1-adrenergic stimulation of inositol phosphate formation from endogenous phosphoinositides is dependent upon the presence of a GTP analogue, an indication that the receptor is coupled to PLC by a G protein [6]. Those studies, however, did not directly demonstrate a role for a guanine nucleotide in the hydrolysis of PIP_2 to 1,4,5-IP_3. Furthermore, Coleman and Bilezikian [7] have shown recently that, in renal cortical membranes, guanine nucleotides also increase the level of 1,4,5-IP_3 by inhibiting its breakdown.

In the present study, we have used exogenous radiolabeled [^{3}H]PIP_2 and [^{3}H]1,4,5-IP_3 to explore whether guanine nucleotides have a direct regulatory role on PIP_2 and 1,4,5-IP_3 hydrolysis. Cardiomyocyte membranes from newborn rats served as the source of PLC and phosphatase. The results of our study show that guanine nucleotides can modulate 1,4,5-IP_3 levels by a dual action, stimulation of PIP_2 hydrolysis and inhibition of 1,4,5-IP_3 breakdown.

METHODS

Chemicals and drugs. (−)Norepinephrine, GTPγS, GDPβS, Gpp(NH)p, ATP, ATPγS, 2,3-diphosphoglycerate, hypoxanthine, and fibronectin were obtained from the Sigma Chemical Co. (St. Louis,

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‖ Abbreviations: PLC, phospholipase C; 1,4,5-IP_3(IP_3), inositol 1,4,5-trisphosphate; PIP_2, phosphatidyl 4,5-bisphosphate; and DOC, deoxycholate.

MO); phosphatidylinositol 4,5-bisphosphate and 1,4,5-inositol trisphosphate from Boehringer Mannheim (Indianapolis, IN); pertussis toxin from the List Biological Co. (Campbell, CA); and [^{3}H]-phosphatidylinositol 4,5-bisphosphate and [^{3}H]-inositol 1,4,5-trisphosphate from Dupont-NEN (Boston, MA). α-Thrombin was a gift from Dr. David M. Stern of the Department of Medicine at this institution. All other chemicals were reagent grade and were obtained from standard chemical suppliers.

Cardiomyocyte cultures and membrane preparation. Hearts from 2-day-old Wistar rats from Camm Research (Wayne, NJ) were removed under sterile conditions and placed in sterile salt solution. The atria were trimmed away and the ventricular cells were isolated according to a trypsin dispersion protocol described previously [8]. Briefly, isolated cells were pooled, centrifuged at 200 *g* for 5 min, and resuspended in Dulbecco's Minimum Essential Medium (MEM) supplemented with 10% horse serum, 5 μM hypoxanthine and 12 mM $NaHCO_3$. The cells were preplated for 60 min at 37° to decrease fibroblast contamination. The muscle cells were then resuspended in MEM supplemented with 10% horse serum and grown in 100 mm plates, which had been coated previously with fibronectin (25 ng/mm^2) for 45 min. For some experiments, myocytes were pretreated with pertussis toxin (100 ng/mL) for the last 24 hr in culture. This treatment has been shown previously to be associated with complete ADP-ribosylation of the pertussis toxin sensitive G protein in cardiomyocytes [9].

After 3–4 days of culture, the cells were detached and suspended in buffer containing 250 mM sucrose, 0.1 mM phenylmethylsulfonyl fluoride, 20 mM Tris–HCl, pH 7.5, homogenized and centrifuged at 43,000 *g* at 4° for 30 min. The pellet was taken up in homogenization buffer, and recentrifuged at the same speed. The final pellet was resuspended in homogenization buffer at a protein concentration of ~2 mg/mL, and stored in aliquots at −70°. Protein concentration was determined by the modified method of Lowry *et al.* [10].

Since PLC and phosphatase activities are present in both the membrane and the cytosol [11, 12], the activity of lactate dehydrogenase, a cytosolic marker, was measured in four membrane suspensions, with the Yellow Spring Instrument WSY-model 27. Negligible amounts of lactate dehydrogenase were found, indicating that the activities measured in our experiments were from membrane-associated PLC and phosphatases.

PIP_2 hydrolysis assay. The reaction mixture consisted, unless otherwise indicated, of [^{3}H]PIP_2, 8 μM (0.01 mCi); $MgCl_2$, 0.5 mM; KCl, 25 mM; LiCl, 25 mM; 2,3-diphosphoglycerate, 1 mM; sodium deoxycholate, 2 mM; sodium cholate, 2 mM; the agents under study; the membrane suspension containing 2 μg of protein; sodium phosphate buffer, 100 mM, pH 7.0, in a total volume of 50 μL. After the addition of the membrane suspension, the reaction mixture was kept at 4° for 10 min. After this preincubation, the reaction proceeded at 30° for 5 min unless otherwise stated, and was terminated by the addition of 1.5 mL of ice-cold acidified chloroform–methanol (1:2, v/v), followed by 0.5 mL chloroform and 1 mL distilled water to separate the aqueous and organic phase. Before use, PIP_2 was dried under a stream of N_2, resuspended by sonication (2 × 30 sec) in 2 mM sodium deoxycholate and 2 mM sodium cholate in 100 mM phosphate buffer, pH 7.0. Lithium and 2,3-diphosphoglycerate were present in the reaction mixture to prevent the breakdown of IP_3 by phosphatases [11, 13]. IP_3, the product of PIP_2 hydrolysis, was used as an index of PIP_2 hydrolysis. An assay was performed in the absence of membrane (blank) to estimate the non-enzymatic hydrolysis of the substrate (PIP_2) during the time of incubation. These blank values, <0.5% of total [^{3}H]PIP_2, were subtracted from the values obtained in the presence of membrane.

IP_3 hydrolysis assay. The reaction mixture was the same as for PIP_2 hydrolysis, except that 20 nM [^{3}H]1,4,5-IP_3 (about 10,000 cpm) was included as substrate instead of [^{3}H]PIP_2; lithium and 2,3-diphosphoglycerate were omitted; 20 μg protein was routinely used per assay. The reaction proceeded at 30° for 20 min unless otherwise stated. Loss of IP_3 was used as index of IP_3 hydrolysis. An assay was also performed in the absence of membrane. In these conditions there was no significant loss of [^{3}H]-IP_3, indicating the lack of non-enzymatic hydrolysis during the time of incubation. This value was used as 100% to calculate percent hydrolyzed.

Identification of inositol phosphates. Inositol phosphates in the aqueous phase were separated and identified by anion exchange chromatography, as described by Berridge *et al.* [14]. The radioactivity of the eluate fractions, corresponding to [^{3}H]IP_1, -IP_2, -IP_3 and -IP_4, was determined by liquid scintillation spectrometry, using Aquasol-II as scintillant.

Data analysis. Triplicate determinations were performed with each membrane preparation in a single experiment. The membranes for repeated experiments were obtained from different cultures. Statistical differences were determined using ANOVA for repeated measures and, when the F value indicated significance, Student's paired *t*-tests with Bonferroni correction for multiple comparisons; P values <0.05 were considered significant. K_D and V_{max} values for each experiment were determined by linear regression [15].

RESULTS

Effect of GTP analogues on PIP_2 hydrolysis. Membrane preparations of cultured cardiomyocytes from newborn rats contained an enzymatic activity that hydrolyzed [^{3}H]PIP_2 (Fig. 1). There was no significant accumulation of IP_2, IP_1, or IP_4 over the 5-min time course of the assay. Thus, IP_3 formation reflected the hydrolysis of PIP_2 by PLC. The rate of IP_3 formation was linear with time up to 8 min (data not shown). The detergent deoxycholate (DOC) increased the rate of hydrolysis of PIP_2 with a maximum effect at 2 mM. At higher concentrations of DOC the rate of IP_3 formation declined and DOC became inhibitory (Fig. 1). Comparable biphasic effects of DOC on PIP_2 hydrolysis have been observed in other heart preparations [16, 17].

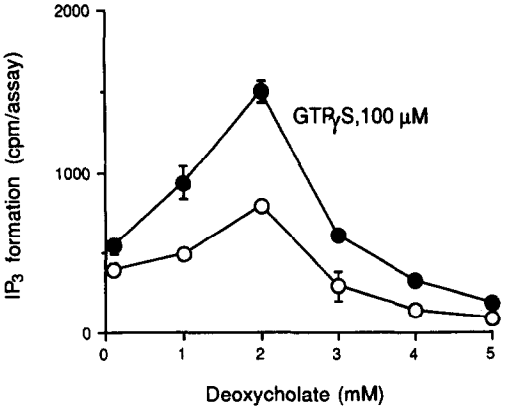

Fig. 1. Effect of sodium deoxycholate on basal- and GTPγS-stimulated PIP_2 hydrolysis. Cardiomyocyte membranes were incubated in the presence of increasing concentrations of sodium deoxycholate without (○) and with (●) GTPγS (100 μM) as described in Methods, using exogenous [^{3}H]-PIP_2 as substrate. The results are expressed in cpm [^{3}H]IP_3 formed per assay. Values are means ± SD of triplicate determinations from one of four experiments with membrane preparations from different cultures.

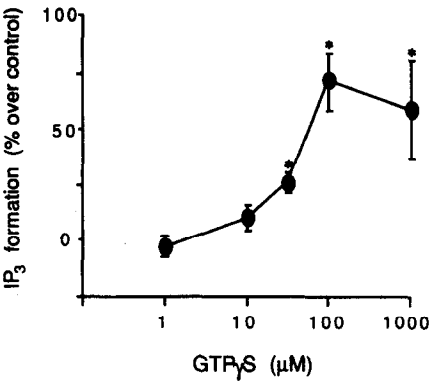

Fig. 2. Concentration-dependent effect of GTPγS on PIP_2 hydrolysis. Cardiomyocyte membranes were incubated in the absence and in the presence of increasing concentrations of GTPγS under standard assay conditions as described in Methods, using exogenous [^{3}H]PIP_2 as substrate. Values, expressed in percent over control, i.e. IP_3 formed in the absence of GTPγS, are means ± SEM from eight experiments. Control value: 940 ± 110 cpm/assay. Key: (*) significantly different from control ($P < 0.05$).

GTPγS, a phosphatase-resistant GTP analogue, did not increase significantly IP_3 formation in the presence of 0.2 mM DOC (+13 ± 14.3%, N = 4), but produced a gradual increase in IP_3 formation over the concentration range of DOC associated with stimulation, reaching a maximum at 2 mM (+57.9 ± 17.7%, N = 4,: $P < 0.05$) (Fig. 1). At inhibitory concentrations of DOC, the GTPγS response also declined. In all the subsequent experiments, DOC was used at 2 mM.

GTPγS produced a concentration-dependent increase in IP_3 formation; half-maximum activation occurred at 40 μM and the maximum at 100 μM (Fig. 2). Figure 3 shows the effect of 100 μM GTPγS on PLC activity at increasing PIP_2 concentrations. Analysis of PIP_2 hydrolysis, according to Lineweaver–Burk, indicated that 100 μM GTPγS significantly increased the apparent V_{max} of PLC (2.3 ± 0.25 fold, N = 4, $P < 0.05$), but did not alter the apparent affinity of the enzyme for PIP_2 (K_D 11.1 ± 2.4 vs 16.0 ± 4.0 μM in the absence and the presence of GTPγS, respectively).

Several other compounds were tested to evaluate the selectivity of the action of GTPγS for activation of the G protein coupled to PLC (Fig. 4). The analogue of GDP, GDPβS, which competes with GTP and prevents G protein activation, by itself stimulated IP_3 formation and did not antagonize the increase induced by GTPγS, even when the membrane was preincubated with GDPβS for 30 min before addition of GTPγS. ATPγS was about as effective as GTPγS. AlF_4^-, which activates G proteins, did not increase IP_3 formation. Sodium pyrophosphate (1000 μM) had a weak stimulatory effect (+37%).

Since GTP is an absolute requirement for agonist activation of G protein-mediated receptor–effector pathways, we next studied the effect of agonists on

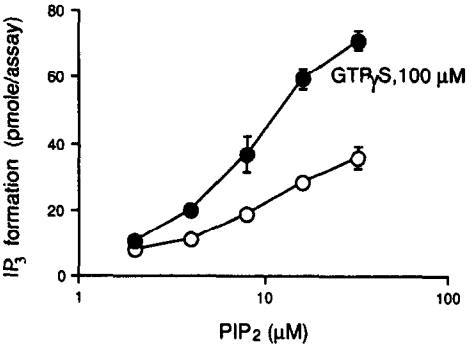

Fig. 3. PIP_2 hydrolysis as a function of substrate concentration and the effect of GTPγS. Cardiomyocyte membranes were incubated with increasing concentrations of exogenous PIP_2, labeled with a fixed amount of [^{3}H]-PIP_2, in the absence (○) or in the presence (●) of GTPγS. Values are means ± SD of triplicate determinations from one of four experiments and are expressed in pmol IP_3 formed per assay, calculated from the specific activity of exogenous PIP_2.

PIP_2 hydrolysis. Norepinephrine and thrombin, at concentrations which stimulate inositol phosphate accumulation in intact myocardial tissue [9, 18], did not increase IP_3 formation. GTPγS was not able to promote a response to either norepinephrine or thrombin (Fig. 4). The agonists were also ineffective in the presence of ATP, which was necessary in some preparations to demonstrate G protein activation and agonist potentiation of the guanine nucleotide response [19, 20]. Similarly, elimination of the 10-min preincubation period of membranes with detergent at 4°, which has been reported to decrease GTPγS and carbachol stimulation of PIP_2

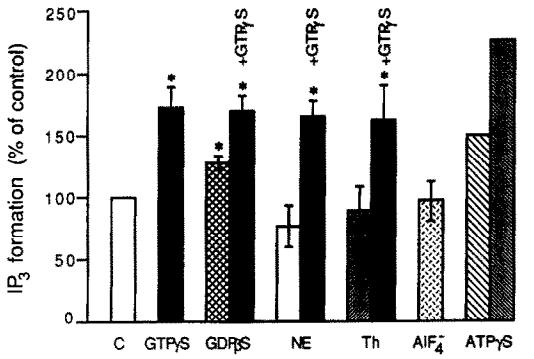

Fig. 4. Influence of nucleotides, agonists and fluoroaluminate on PIP_2 hydrolysis. Cardiomyocyte membranes were incubated in the absence (C) or in the presence of the indicated test agents under standard assay conditions as described in Methods, using exogenous [^{3}H]PIP_2 as substrate. The concentration of GTPγS, alone and in combination, and of GDPβS was 100 μM; that of ATPγS was 100 μM (lighter shade) and 1000 μM (darker shade). AlF_4^- consisted of a mixture of 10 mM sodium fluoride and 20 μM aluminum chloride. Abbreviations: NE, norepinephrine, 50 μM; Th, α-thrombin, one unit. Values, expressed as percent of corresponding control (C), are means ± SEM from three to six experiments, except for ATPγS where only two experiments were performed. The SD of the triplicates in each experiment with ATPγS was less than 12% at 100 μM and 3% at 1000 μM. The control values, expressed in IP_3 formed per assay, were: 1100 ± 190 cpm/assay for the GTPγS and GDPβS groups; 1260 ± 140 for the norepinephrine and thrombin groups; 1090 ± 180 for AlF_4^-; and 930 for ATPγS. Key: (*) significantly different from corresponding control (C) ($P < 0.05$).

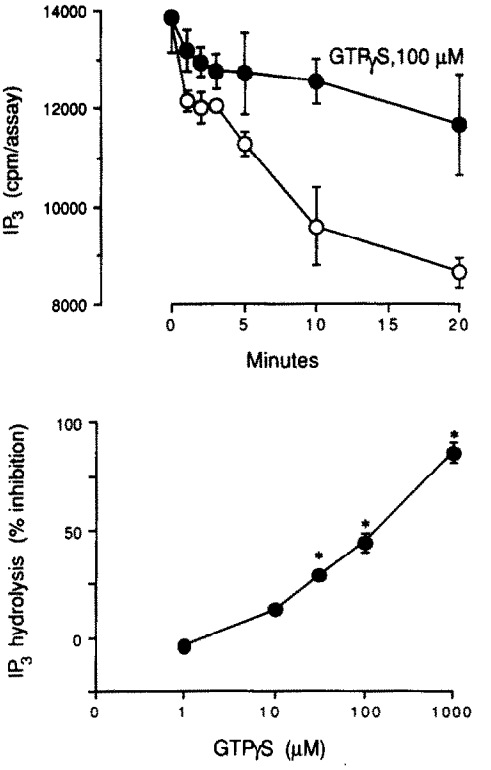

Fig. 5. Top panel: Time course of IP_3 hydrolysis. Cardiomyocyte membranes were incubated in the absence (○) and in the presence of GTPγS (●) for the times indicated under standard assay conditions as described in Methods, using exogenous [^{3}H]1,4,5-IP_3 as substrate. The results are expressed in cpm [^{3}H]IP_3 remaining at the end of the incubation period. Values are means ± SD of triplicate determinations from one of two experiments. Bottom panel: Concentration-dependent effect of GTPγS on IP_3 hydrolysis. Cardiomyocytes were incubated in the absence (control) and in the presence of increasing concentrations of GTPγS under standard assay conditions as described in Methods, using exogenous [^{3}H]1,4,5-IP_3 as substrate. The results are expressed in percent inhibition of IP_3 hydrolysis under control conditions, calculated from the equation $(C-D)/C \times 100$, where C = cpm [^{3}H]IP_3 hydrolyzed under control conditions; and D = cpm [^{3}H]-IP_3 hydrolyzed in the presence of GTPγS. Control: 5190 ± 730 cpm [^{3}H]1,4,5-IP_3 hydrolyzed in 20 min, corresponding to 47% of [^{3}H]1,4,5-IP_3 incubated in the absence of membranes. Values are means ± SEM from five experiments. Key: (*) significantly different from control ($P < 0.05$).

hydrolysis [21], was also without effect (data not shown).

Several studies have suggested that PLC, like adenylate cyclase, is positively and negatively coupled to the agonist receptor by G proteins. Litosch [22] observed in rat cerebral cortical membranes that GTP analogues have a dual effect on exogenous PIP_2 hydrolysis, a stimulatory effect and a pertussis toxin-sensitive inhibitory effect. Since α_1-adrenergic agonists produce a positive and a pertussis toxin-sensitive negative chronotropic response in cardiomyocytes [23], cells were pretreated with pertussis toxin to test whether GTPγS might also regulate cardiomyocyte PLC by a pertussis toxin-sensitive inhibitory mechanism. Pretreatment of cardiomyocytes with 100 ng/mL of pertussis toxin, a concentration sufficient to ADP-ribosylate virtually all accessible sites [9], decreased, though not significantly, both basal and GTPγS-stimulated PIP_2 hydrolysis. However, the response to GTPγS (1–100 μM), as a function of control activity, was similar in treated and untreated membranes (+54.3 and 51.7% at 100 μM GTPγS, respectively). These results are in agreement with those obtained using membranes with endogenous phosphoinositide substrates [6] and indicate that the effect of GTPγS on PIP_2 hydrolysis does not involve a pertussis toxin-sensitive inhibitory component.

Effect of GTP analogues on IP_3 hydrolysis. In view of the results of a recent study suggesting that guanine nucleotides may also increase IP_3 levels by inhibiting the rate of IP_3 hydrolysis [7], the next set of experiments was aimed at defining a role for GTP in IP_3 hydrolysis. The rate of 1,4,5-IP_3 hydrolysis in myocyte membranes was very slow. At the protein concentration (2 μg) used in the PIP_2 assay, there was no detectable activity. This observation is consistent with the lack of accumulation of IP_2 and IP_1 in the PIP_2 assay. At a higher protein concentration (20 μg), 18% IP_3 was hydrolyzed in 5 min and 34% after 20 min ($P < 0.05$) (Fig. 5, top

Table 1. Effect of nucleotide analogues, aluminum fluorate and sodium pyrophosphate on 1,4,5-IP_3 hydrolysis

Test agent	% Inhibition
GTPγS, 100 μM	49.6 ± 3.1*
GTPγS, 1000 μM	93.7
Gpp(NH)p, 100 μM	23.4 ± 1.8*
Gpp(NH)p, 1000 μM	72.0 ± 4.2*
GDPβS, 100 μM	31.7 ± 3.2*
GDPβS, 1000 μM	59.5
GDPβS, 100 μM + GTPγS, 100 μM	50.1 ± 3.2*
GDPβS, 1000 μM + GTPγS, 100 μM	51.4
ATPγS, 100 μM	42.9
ATPγS, 1000 μM	92.6 ± 13.2*
AlF_4^-†	0.3 ± 13.2
Sodium pyrophosphate, 100 μM	15.7 ± 4.1*

Cardiomyocyte membranes were incubated in the absence (control) or in the presence of a test agent under the standard assay conditions as described in Methods, using exogenous [^{3}H]1,4,5-IP_3 as substrate. The results are expressed in percent inhibition of IP_3 hydrolysis under control conditions, calculated from the equation: $(C - D)/C \times 100$, where C = cpm [^{3}H]IP_3 hydrolyzed under control conditions; and D = cpm [^{3}H]IP_3 hydrolyzed in the presence of a test agent. The average control value was 2340 ± 460 (SEM) cpm [^{3}H]1,4,5-IP_3 hydrolyzed in 20 min, corresponding to 40% of [^{3}H]1,4,5-IP_3 incubated in the absence of membranes. Values are from 2 to 4 experiments; when 3–4 experiments were performed, values are means ± SEM; when only 2 experiments were done, no SEM is shown (the SD of the triplicates in each experiment ranged from 0.2 to 5%).

* Significantly different from corresponding control ($P < 0.05$).

† AlF_4^- consisted of a mixture of 10 mM sodium fluoride and 20 μM aluminum chloride.

panel). In the following experiments, the assay contained 20 μg of protein and proceeded for 20 min. There was no significant accumulation of IP_4, IP_1 or inositol during the incubation. After correction for recovery, the combined amounts of [^{3}H]IP_3 and [^{3}H]-IP_2 at the end of incubation were similar to the total amount of [^{3}H]IP_3 (data not shown). This indicated that the decrease in IP_3 reflects mainly phosphatase activity.

GTPγS reduced the rate of 1,4,5-IP_3 hydrolysis (Fig. 5, top panel). The effect was concentration dependent between 10 and 1000 μM, with half-maximum inhibition at 100 μM (Fig. 5, bottom panel). Other nucleotides, AlF_4^- and sodium pyrophosphate were tested for their effect on IP_3 hydrolysis to evaluate the selectivity of the GTP effect (Table 1). Gpp(NH)p, another GTP analogue, as well as GDPβS, inhibited IP_3 hydrolysis, though less effectively than GTPγS. When the membranes were preincubated with GDPβS before the addition of GTPγS, the rate of IP_3 hydrolysis was similar to that of GTPγS alone. The adenosine derivative, ATPγS, was about equieffective as GTPγS as an inhibitor of IP_3 hydrolysis. AlF_4^- had no effect, while sodium pyrophosphate, at the same concentration as GTPγS, produced a small, though significant, inhibitory effect on IP_3 hydrolysis. Norepinephrine, 50 and 500 μM, alone and in the presence of 100 μM GTPγS, had no effect on IP_3 hydrolysis (data not shown).

DISCUSSION

The results of this study demonstrate the presence in cardiomyocyte membranes from newborn rats of GTP-sensitive enzymatic activities that regulate the formation and the hydrolysis of 1,4,5-IP_3. The stimulatory effect of the GTP analogue GTPγS on the hydrolysis of exogenous PIP_2 confirms the results of earlier studies with intact cells and with prelabeled membranes using endogenous PIP_2 [6]. However, the mechanism of GTP stimulation may be different when an exogenous substrate is used. GTP stimulation of IP_3 formation from endogenous substrate has been attributed to activation of the G protein regulating phosphoinositide hydrolysis. In our preparation GTPγS appears to have a broader role. The GTPγS response was not specific, as GDP and ATP analogues were also stimulatory. The concentrations of GTPγS stimulating PLC far exceeded those for activation of G protein-dependent processes. Furthermore, GDP analogues, which compete with GTP and prevent the activation of the G protein, did not antagonize the stimulation of GTPγS. AlF_4^-, a potent activator of G proteins, was ineffective. Moreover, norepinephrine and thrombin in the presence of GTPγS, which is an absolute requirement for agonist stimulation of G protein-mediated receptor pathways, had no effect on PIP_2 hydrolysis.

The reason for the difference in the mechanism of GTP activation of endogenous and exogenous PIP_2 hydrolysis is not clear. The need to treat the membranes with a detergent to observe a GTP effect suggests that the accessibility and/or the presentation of the substrate PIP_2 to its binding sites is critical to demonstrate R-G protein-activation of PLC. Even though the detergent deoxycholate facilitates the stimulation of PLC by GTPγS, it did not promote agonist stimulation, as was the case in brain membranes [21]. It is possible that disruption of the membrane structure by the detergent, while facilitating access of the substrate to PLC, dissociates PLC from the R-G protein complex. In a detailed study comparing the effects of several detergents on the activation of PLC by exogenous PIP_2, Carter *et al.* [21] showed, in rabbit cortical membranes, that stimulation by GTPγS and carbachol required deoxycholate and was maximal at 1 mM. From their results, and those obtained in other similar studies, they concluded that the concentration of detergents that maintain a functionally active R-G protein–PLC complex and that which uncouples the complex varies among tissues and is critical to demonstrate R-G protein-activation of PLC. Future studies will explore whether with other detergents agonist activation of exogenous PIP_2 hydrolysis can be observed in cardiomyocytes.

The lack of nucleotide selectivity observed in our preparation suggests additional mechanisms to G protein activation for GTP stimulation of PIP_2 hydrolysis. Non-specific stimulation of exogenous PIP_2 hydrolysis by nucleotides has also been observed in other preparations and appears to be related to

activation of PLC or an associated protein downstream of the G protein [24, 25]. Ryu *et al.* [26] have shown that ATP and GTP activate a purified brain PLC. The stimulatory effect of sodium pyrophosphate suggests that the nucleotide effect may be due to a phosphorylation reaction. In rabbit thymocyte membranes [24], hydrolysis of endogenous PIP_2 was selectively stimulated by GTPγS, while that of exogenous PIP_2 was stimulated by GTP- as well as GDP- and ATP-analogues. In contrast, in permeabilized 3T3 cells, which have a functionally activatable G protein, ATPγS stimulated the hydrolysis of endogenous PIP_2 [25]. These observations suggest that the GTP sensitivity of the processes involved in PLC activation may depend, in part, upon the structural properties of the membrane components of the pathway, as well as their orientation with the plane of the membrane.

The results of our study demonstrate that GTP analogues can also regulate 1,4,5-IP_3 hydrolysis. The rate of IP_3 hydrolysis, however, was much slower than its formation by PLC. This apparently low phosphatase activity probably reflects a small amount of membrane-bound enzyme, since the amount of protein present in the PIP_2 assay had to be increased in order to observe IP_3 hydrolyzing activity. It has been reported that in canine renal cortical membranes or in coronary artery membranes, 80 and 20% [^{3}H]-IP_3, respectively, was hydrolyzed in 1 min [7, 12]. Even though it is difficult to compare these preparations because of differences in the assay conditions and the unknown endogenous IP_3 concentration, it appears that the membrane bound phosphatase activity which hydrolyzes 1,4,5-IP_3 varies greatly from tissue to tissue.

GTPγS had an inhibitory effect on IP_3 hydrolysis. The effect is not selective for GTPγS, since other nucleotides were also inhibitory. The specificity of the effect appears to be in the phosphate group of the nucleotide, suggesting a phosphorylating mechanism. IP_3 hydrolysis does not appear to be regulated by norepinephrine, since the agonist had no significant effect in the absence or in the presence of GTPγS. This could indicate that the phosphatase hydrolyzing 1,4,5-IP_3 is not linked to the α_1-adrenergic receptor. However, this possibility cannot be ruled out completely since in the same membrane preparations the α_1-adrenergic receptor was uncoupled from PLC. Our results are comparable to those obtained in renal cortical membranes [7]. In this preparation, nucleotides inhibited 1,4,5-IP_3 formation and showed specificity best associated with the phosphate group of the nucleotide, GTPγS and ATPγS being most potent. In addition, parathyroid hormone, which stimulates phosphoinositide hydrolysis, has no effect on 1,4,5-IP_3 hydrolysis in the absence or in the presence of GTPγS [7]. Furthermore, in cardiomyocyte membranes the characteristics of the effect of GTPγS on the phosphatase and PLC are similar, suggesting that the nucleotide regulates the activity of both enzymes by a similar mechanism.

The results of our study indicate that, besides its action on the G protein mediating PLC activation observed in preparations using endogenous PIP_2, GTP can modulate phosphoinositide metabolism by actions at multiple sites along this pathway. Our data suggest that GTP can regulate 1,4,5-IP_3 at the level of membrane-bound PLC and 5′-phosphatase, stimulating its formation from PIP_2 and inhibiting its breakdown. Other possible sites of control have been described recently. GTPγS has been shown to stimulate the formation of the phosphoinositides in rat liver and brain plasma membranes [27, 28]. The relative influence of these various regulatory sites on the ultimate IP_3 level will depend upon many factors such as the amount of enzyme activities *per se*, the availability of substrates, as well as their accessibility to the components of the sytem involved.

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REFERENCES

1. Berridge MJ, Inositol trisphosphate and diacylglycerol as second messengers. *Biochem J* **220**: 345–360, 1984.
2. Majerus PW, Connolly TM, Bansal VS, Inhorn RC, Ross TS and Lips DL, Inositol phosphates: Synthesis and degradation. *J Biol Chem* **263**: 3051–3054, 1988.
3. Fain JN, Regulation of phosphoinositide-specific phospholipase C. *Biochim Biophys Acta* **1053**: 81–88, 1990.
4. Litosch I and Fain JN, 5-Methyltryptamine stimulates phospholipase C-mediated breakdown of exogenous phosphoinositides by blowfly salivary gland membranes. *J Biol Chem* **260**: 16052–16055, 1985.
5. Smrcka AV, Hepler JR, Brown KO and Sternweis PC, Regulation of polyphosphoinositide-specific phospholipase C activity by purified G_q. *Science* **251**: 804–807, 1991.
6. Steinberg SF, Kaplan LM, Inouye T, Zhang JF and Robinson RB, *Alpha*-1 adrenergic stimulation of 1,4,5-inositol trisphosphate formation in ventricular myocytes. *J Pharmacol Exp Ther* **250**: 1141–1148, 1989.
7. Coleman DT and Bilezikian JP, Parathyroid hormone stimulates formation of inositol phosphates in a membrane preparation of canine renal cortical tubular cells. *J Bone Miner Res* **5**: 299–306, 1991.
8. Drugge ED, Rosen MR and Robinson RB, Neuronal regulation of the development of α-adrenergic chronotropic response in the rat heart. *Circ Res* **57**: 415–423, 1985.
9. Steinberg SF, Chow YK, Robinson RB and Bilezikian JP, A pertussis toxin substrate regulates α_1-adrenergic dependent phosphatidylinositol hydrolysis in cultured rat myocytes. *Endocrinology* **120**: 1889–1895, 1987.
10. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
11. Storey DJ, Shears SB, Kirk CJ and Michell RH, Stepwise enzymatic dephosphorylation of inositol 1,4,5-trisphosphate to inositol in liver. *Nature* **312**: 374–376, 1984.
12. Sasaguri T, Hirata M and Kuriyama H, Dependence on Ca^{2+} of the activities of phosphatidylinositol 4,5-bisphosphate phosphodiesterase and inositol 1,4,5-trisphosphate phosphatase in smooth muscles of the porcine coronary artery. *Biochem J* **231**: 497–503, 1985.
13. Downes CP, Mussat MC and Michell RH, The inositol trisphosphate monoesterase of the human erythrocyte membrane. *Biochem J* **203**: 169–177, 1982.
14. Berridge MJ, Dawson RMC, Downes CP, Helsop JP and Irvine RF, Changes in the levels of inositol

phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. *Biochem J* **212**: 473–482, 1983.
15. Snedecor GW and Cochran WG, *Statistical Methods*. Iowa State University Press, Ames, IA, 1967.
16. Schwertz DW and Halverson J, Characterization of phospholipase C-mediated polyphosphoinositide hydrolysis in rat heart ventricles. *Arch Biochem Biophys* **269**: 137–147, 1989.
17. Edes I and Kranias EG, Characterization of cytoplasmic and membrane-associated phosphatidylinositol 4,5-bisphosphate phospholipase C activities in guinea pig ventricles. *Basic Res Cardiol* **85**: 78–87, 1990.
18. Steinberg SF, Robinson RB, Lieberman HB, Stern DM and Rosen MR, Thrombin modulates phosphoinositide metabolism, cytosolic calcium, and impulse initiation in the heart. *Circ Res* **68**: 1216–1229, 1991.
19. Litosch I, Wallis C and Fain JN, 5-Hydroxytryptamine stimulates inositol phosphate production in a cell-free system from blowfly salivary glands. *J Biol Chem* **260**: 5464–5471, 1985.
20. Chiu AS, Li PP and Warsh JJ, G-protein involvement in central-nervous-system muscarinic-receptor-coupled polyphosphoinositide hydrolysis. *Biochem J* **256**: 995–999, 1988.
21. Carter HR, Wallace MA and Fain JN, Activation of phospholipase C in rabbit brain membranes by carbachol in the presence of GTPγS; Effects of biological detergents. *Biochim Biophys Acta* **1054**: 129–135, 1990.
22. Litosch I, Guanine nucleotides mediate stimulatory and inhibitory effects on cerebral-cortical membrane phospholipase C activity. *Biochem J* **261**: 245–251, 1989.
23. Steinberg SF, Drugge ED, Bilezikian JP and Robinson RB, Acquisition by innervated cardiac myocytes of a pertussis toxin-specific regulatory protein linked to the α_1-receptor. *Science* **230**: 186–188, 1985.
24. Sommermeyer H, Behl B, Oberdisse E and Resch K, Effects of nucleotides on the activity of phospholipase C in rabbit thymus lymphocytes. Differences in assays using endogenous [^{3}H]inositol-prelabeled membranes or exogenous [^{3}H]phosphatidylinositol 4,5-bisphosphate as substrate. *J Biol Chem* **264**: 906–909, 1989.
25. Higashi K and Ogawara H, ATP-dependent regulation of phospholipase C in permeabilized 3T3 cells. *FEBS Lett* **267**: 51–54, 1990.
26. Ryu SH, Cho KS, Lee K-Y, Suh P-G and Rhee SG, Purification and characterization of two immunologically distinct phosphoinositide-specific phospholipase C from bovine brain. *J Biol Chem* **262**: 12511–12518, 1987.
27. Benistant C, Thomas AP and Rubin R, Effect of guanine nucleotides on polyphosphoinositide synthesis in rat liver plasma membranes. *Biochem J* **271**: 591–597, 1990.
28. Smith CD and Chang K-J, Regulation of brain phosphatidylinositol-4-phosphate kinase by GTP analogues. A potential role for guanine nucleotide regulatory proteins. *J Biol Chem* **264**: 3206–3210, 1989.