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Cytosolic Phospholipase C Activity: I. Evidence for Coupling With Cytosolic Guanine Nucleotide-Binding Protein, Giα

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Abstract In a previous report we showed that glucocorticoid inhibition of cytosolic PLC activity correlated with a reduction in cytosolic Giα levels, suggesting that there may be a functional relationship between cytosolic PLC and cytosolic Giα. In order to establish the nature of the coupling between cytosolic Giα and cytosolic PLC we examined the effects of G-protein activators, and inhibitors on cytosolic PLC activity from rat splenocytes and the rat lymphoma cell line Nb 2, with [^{3}H] PI and [^{3}H]PIP_2 as substrates. 1) Neither GTP nor its nonhydrolyzable analogue, GTPγS, at 100 μM had any effect on the calcium stimulated as well as the basal PLC activity. 2) However, affinity purified antibodies to Giα1 and Giα2 inhibited soluble PLC activity, by 85% and 55%, respectively, with PI as substrate; with PIP_2 as substrate, soluble PLC activity was inhibited 50–70% by antibodies to Gi1, whereas antibodies to Gi2 had little effect. 3) Administration of Giα1 antisense oligonucleotides to splenocytes for 48 h produced 25–40% decrease in cytosolic Giα1 levels compared to control. The soluble PLC activity with both PI and PIP_2 as substrates was also reduced by 25–50% compared to control conditions. This suggest that cytosolic Giα is associated with the activation of splenocyte soluble PLC. 4) Pertussis toxin administered in vivo significantly reduced cytosolic Giα immunoreactivity and soluble PLC activity when PI was used as substrate, providing additional evidence that cytosolic Giα is associated with the activation of soluble PLC. 5) Another agent that has been used extensively to define G-protein coupled processes is NaF/$AlCl_3$. NaF (5 mM; with or without $AlCl_3$) inhibited soluble PLC activity with PIP_2 as substrate, in contrast to the stimulatory effect that has been reported in the activation of membrane PLC. 6) Because NaF can act as a protein phosphatase inhibitor, we also tested the effects of trifluoperizine (50 μM, TFP), an inhibitor of protein phosphatase 2B; TFP (50 μM) significantly inhibited soluble PLC activity when PI was used as substrate. These results suggest a direct involvement of cytosolic Giα in the activation of soluble PLC from splenocytes. Other questions pertaining to the functional significance, the nature, and possible substrate preference of the splenocyte Giα coupled PLC is addressed in the second paper.

Key words: antisensense oligonucleotides, pertussis toxin, splenocytes, Nb2 cells, Giα

Phosphatidylinositol-specific phospholipase C (PLC) comprise a multigene family of proteins that hydrolyze inositol-specific phospholipids to generate diacylglycerol and inositol phosphates that may act as second messengers. There are at least three classes of PLC enzymes, which are believed to be activated via different mechanisms [Rhee and Choi, 1992] and there are at least two kinds of G-protein coupled PLC activities: A pertussis toxin insensitive and a pertussis toxin sensitive activity. The pertussis toxin insensitive PLC activity has been shown to be PLC β and the activation process is mediated by G_q/G_{11} class of G-proteins [Gutowski et al., 1991; Smrcka et al., 1991; Taylor et al., 1991]; the nature of the pertussis toxin sensitive PLC and coupled G-protein are not known [Deckmyn et al., 1990; Rhee et al., 1989]. In contrast to the activation of PLC β, the activation of soluble as well as membrane-associated PLC γ1 by tyrosine kinase-dependent growth factors may require tyrosine phosphorylation of PLC γ for full activation. Dephosphorylation, by a phosphoty-

Abbreviations used: GTPγS, guanosine-5′-o-(3-thiotriphosphate); GDPβS, guanosine-5′-o-(2-thiodiphosphate); TFP, trifluoperizine; G-protein, any GTP-binding protein that resembles a family of homologous proteins consisting of Gs, Gi, Go, and Gt; Giα, α subunit of the G-protein(s) that mediate the inhibition of adenylate cyclase; PI, phosphatidylinositol; PIP_2, phosphatidylinositol 4,5-biphosphate; PLC, phosphatidylinositol-specific phospholipase C. DAG, diacylglycerol.

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rosine-specific phosphatase, deactivates the enzyme [Nishibe et al., 1990; Wahl et al., 1992; reviewed by Majerus, 1992].

Although the site of action for phospholipase C is the membrane, in some cells most of the PLC activity is found in the soluble fraction [Allan and Michell, 1974a,b; Takenawa and Nagai, 1981; Hoffman and Majerus, 1982; Low and Weglicki, 1983; Bennet and Crooke, 1987]. Inspite of several reports indicating that G-protein subunits can activate soluble PLC in the absence of membranes, and the predominant location of PLC in the soluble fraction of several cell types [Blank et al., 1992; Boyer et al., 1992; Camps et al., 1992]; all the mechanisms envisioned for PLC activation assume a membrane localization for PLC and no serious consideration has been given to how soluble PLC may be activated. Our overall aim is to study the role that soluble PLC plays in cellular signal transduction, and how the soluble PLC system is regulated by hormones and other agents. In a previous study [Akompong et al., 1993] we showed that glucocorticoids administered in vivo and in vitro inhibit splenocyte soluble PLC activity. The inhibition of soluble PLC activity correlated with reductions in cytosolic Giα immunoreactivity suggesting that the two events (glucocorticoid reduction in PLC activity and the reduction in Giα levels) may be related.

We present in this paper evidence for cytosolic Giα involvement in soluble PLC activation and begin to explore the nature of the mechanism of the activation processes and whether such a mechanism occurs in intact splenocytes. A full examination of the nature of the splenocyte PLC that may be associated with soluble Giα is presented in the second paper.

MATERIALS AND METHODS

Materials

[^{3}H]PIP_2 and [^{3}H]PI were purchased from New England Nuclear (Boston, MA), GDPβS was from Boehringer Mannheim (Indianapolis, IN), and all the other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Antisense and scrambled oligonucleotides were purchased from Oligos (Newark, NJ) etc. Pertussis toxin was purchased from Calbiochem (La Jolla, CA). Affinity purified antibodies LD 103 and LE 102 that recognize Gi1 and Gi2, respectively, were kindly donated by Dr. Allen Spiegel (National Institutes of Health, Bethesda, MD); Nb 2 cells were a gift from Dr. Charles Clavenger (University of Pennsylvania, Philadelphia, PA).

In Vivo Administration of Pertussis Toxin

Pertussis toxin (15–25 μg in 100 μl saline) was injected ip to male rats (Sprague-Dawley, 210–270 g; Charles River) 48 h prior to death. Adrenalectomized (ADX) rats which received the toxin were ADX overnight prior to toxin administration the next day.

In Vitro ADP-Ribosylation

The ADP-ribosylation of membrane and cytosol were done as follows: membrane (20–40 μg) and cytosol (60–120 μg) were incubated for 1 hr at 30°C with pertussis toxin (10 μg/ml) preactivated with 50 mM dithiothreitol in 100 μl of ADP-ribosylation buffer containing 50 mM Tris, pH 8.0, 10 mM $MgCl_2$, 1 mM ATP, 0.5 mM GTP, 10 mM thymidine, 1 mM EDTA, 1 mM DMPC, 5 mM isoniazid, 250 μM NADP, 2 μCi [^{32}P]NAD, and 10 μM cold NAD. After the reaction the membrane was washed two times with 1 mL of 20 mM ice-cold Tris and pelleted in a microfuge.

Cell Culture

Spleens from male rats were removed and separated by mashing them through a wire mesh screen. The dispersed cells were rapidly passed over glasswool to remove fibrous and fatty particulate matter and then centrifuged at 1,600 rpm for 10 min and then resuspended in growth medium (RPMI 1640 complete media, supplemented with fetal bovine serum at a final concentration of 10%, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin and 0.2% mercaptoethanol); 5 ml of the resuspended cells, 3–5 million cells/ml, were placed in 25 cm^3 flasks. The cells were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The cells were harvested after 48 h by centrifugation at 1,600 rpm for 10 min and washed two times with phosphate-buffered saline and once with homogenization buffer at 1,600 rpm for 10 min. The cells were frozen at −70°C until use.

Nb 2 cells were maintained in Fischer's medium (supplemented with fetal bovine serum at a final concentration of 10%, horse serum at a final concentration of 10%, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 0.1 mM mercaptoethanol). The Nb 2 cells were grown in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Confluent cells used for the experiments were harvested as described for splenocytes above.

Gi1α Antisense Oligonucleotides

The sequences for the antisense oligonucleotides are near the initiation start (ATG) site of published cDNA sequences for Gi1α. The bases at the ends of the antisense oligonucleotides were modified to achieve a desirable GC/AT content that will result in a melting temperature around 37°C. The cDNA sequence for Gi1 is presented below together with the sequences for Gi2, Gi3, and Gs:

5′ GCC ACC ATG GGC TGC ACA CTG AGC GCT GAG 3′ Gi1
5′ GGC AGG ATG GGC TGC ACC GTG AGC GCC GAG 3′ Gi2
5′ GCC GTC ATG GGC TGC ACG TTG AGC GCC GAG 3′ Gi3
5′ GCC GCC ATG GGC TGC CTC GGC AAC AGT AAG 3′ Gs

The sequence for the two antisense and scrambled oligonucleotides synthesized from the Gi1 sequence is given below:

Antisense 1: 5′ ACA GCC CAT GGT GGT 3′
Antisense 2: 5′ CTC AGC GCT CAG TGT 3′
Scrambled 1: 5′ TGC ACT GAG GCG TAG 3′
Scrambled 2: 5′ AAT CTG TGG CTC GCC 3′

The antisense (30 μM) or scrambled (30 μM) oligonucleotides were added to the splenocytes and incubated for 48 h. The splenocytes were harvested and processed as outlined above.

Preparation of Cytosol and Membrane

The frozen cells were thawed and homogenized with a teflon hand pestle in a volume of 100–150 μl homogenization buffer (containing 50 mM Tris buffer, pH 7.2, 6 mM $MgCl_2$, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 3 mM benzamidine, 1 mM leupeptin, 1 μg/ml of pepsitenin, 1 μg/ml antipain, 1 μg/ml aprotinin, 10% (wt/vol) sucrose, and 1 μg/ml of soybean trypsin inhibitor). The homogenate was centrifuged in an airfuge at 130,000*g* (26 psig) for 20 min; the supernatant was collected and used as cytosol. The pellets were resuspended in homogenization buffer and used as membrane. Protein concentrations were determined according to the procedure of Bradford [1976] with bovine serum albumin as a protein standard.

Electrophoresis and Western blotting

Electrophoresis and western blotting were performed according to standard procedures as previously described [Akompong et al., 1993].

Phosphoinositide Hydrolysis

The PLC assay was performed as previously described [Akompong et al., 1993]. Briefly [^{3}H]PIP_2 or [^{3}H]PI (0.03 μCi/tube) together with cold PIP_2 or PI were dried under a stream of nitrogen; 1.5% sodium cholate was added to give a solution containing 28,000–50,000 cpm and 30–50 μM PI or PIP_2/25 μl; 25 μl of cytosol (25–50 μg) was added to a reaction buffer containing (final concentration in 100 μl) 50 mM NaHepes (pH 6.8), 1 mM EDTA, 3 mM EGTA, 1 mM dithiothreitol, 1 mM $MgCl_2$, 25 mM $LiCl_2$, and additions as indicated. This mixture was preincubated for 5 min at 37°C and the reaction was started by adding 25 μl of [^{3}H]PIP_2 or [^{3}H]PI in 1.5% sodium cholate to make a final reaction volume of 100 μl, which was further incubated for 10 min at 37°C. The reaction was stopped by adding 180 μl of methanol: chloroform: HCl (2:1:0.02, v/v). The phosphoinositides were extracted by adding 80 μl of chloroform followed by 160 μl of 2 M KCl. Each experiment contained a blank incubation with all components of the assay buffer except protein. The blank value (50–150 cpm) was substracted from all experimental points.

pH Dependency of PLC Activity (Fig. 1)

The pH dependency was determined with 50 μg of cytosolic proteins from spleen with calcium (1.5 μM) and 30 μM PIP_2 as substrate.

Protein and Substrate Concentrations

With PIP_2 as substrate, maximum activity using cytosol from spleen was reached at 50 μg

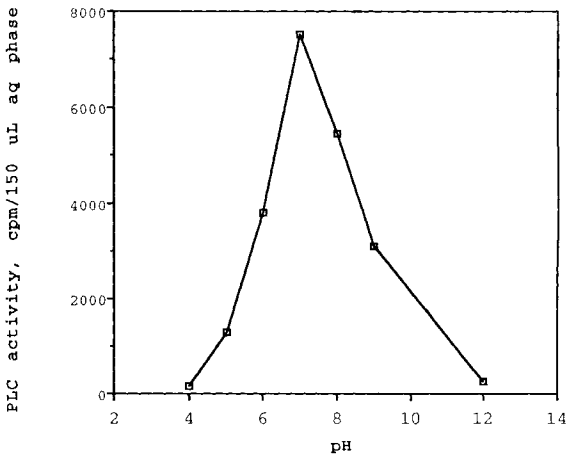

Fig. 1. pH dependency of PLC activity.

of protein, whereas with PI as substrate the maximum activity was reached at 100 μg of protein. The K_m for the soluble PI-PLC activity from spleen was 3.8 μM using PIP_2 as substrate and 1.4 μM using PI as substrate, (data not shown) this is similar to the K_m value (1.5 μM) obtained for purified spleen cytosolic PLC by Roy et al. [1991].

RESULTS

Effects of Guanine Nucleotides on Cytosolic PLC Activity in Splenocytes and Nb2 Rat Lymphoma Cells

Membrane-associated PLC activity is stimulated by GTP and its non-hydrolyzable analogs and inhibited by GDPβS [Deckmyn et al., 1990]. Cytosolic PLC activity has also been shown to be stimulated by GTP and GTPγS [Deckmyn et al., 1986; Camps et al., 1990]. However, as noted in the Introduction, while activation of PLCβ1 is coupled to G_q/G_{11}, activation of PLCγ1 from the membrane and cytosol may involve protein tyrosine phosphorylation and does not appear to be coupled to G-proteins. In view of these differences in the regulation of membrane PLCs, and the fact that both cytosolic PLC activity and cytosolic Giα levels are reduced by glucocorticoids [Akompong et al., 1993], we examined the possibility that cytosolic Giα may mediate the activation of cytosolic PLC from spleen cells. We measured soluble PLC activity in splenocytes and Nb2 cells (transformed rat T lymphocytes) with [^{3}H]PI and [^{3}H]PIP_2 in the presence of cold substrate.

To test the possibility that cytosolic Giα is coupled to the activation of splenocyte soluble PLC, we used GTP, GTPγS, and NaF [which have been shown to activate G-protein coupled processes; Gilman, 1987], to stimulate soluble PLC in splenocytes as well as Nb2 cells. We also used GDPβS in order to inhibit the G-protein coupled activation of soluble PLC. The soluble PLC activity from splenocytes measured with PI as substrate in the absence of calcium was very low and was not affected by GTP or GTPγS (100 μM). The calcium stimulated soluble PLC activity was also not affected by GTP or GTPγS (100 μM). However, the calcium stimulated soluble PLC activity was inhibited 40–60% by GDPβS at 1 mM (Table I).

It is possible that different immunocompetent cell types in splenocytes could be affected differentially by the guanine nucleotides to produce a composite result made of activating and inhibitory effects on the different cell types which will cancel each other out to give the net result of no effect of guanine nucleotides on soluble PLC activation. To rule out this possibility we stimulated soluble PLC in a pure population of cells, the Nb2 lymphoma cells with GTP or GTPγS (100 μM). As in splenocytes, neither GTP nor GTPγS had any effect on soluble PLC activity in Nb2 cells (Table I). Again, as in splenocytes, GDPβS produced a significant inhibition of

TABLE I. Effects of Guanine Nucleotides and Phosphatase Inhibitors on Cytosolic PLC Activity From Splenocytes and Nb2 Cells With and Without Added Calcium (10^{-6} M) and Using PI as Substrate†

	Substrate: PI (Sample cpm-background cpm)		
	Splenocytes		Nb2 cells
	NO calcium	$Ca^{2+} \times 10^{-6}$ M	$Ca^{2+} \times 10^{-6}$ M
Control	10 ± 3	3,139 ± 213	17,722 ± 205
GTP (100 μM)	17 ± 9	3,008 ± 392	18,295 ± 193
GTPγS (100 μM)	8 ± 2	2,476 ± 74	17,250 ± 428
GDPβS (1 μM)	3 ± 1	1,322 ± 54**	14,938 ± 726*
Control	20 ± 13	4,055 ± 759	20,573 ± 368
NaF (5 mM)	4 ± 1	3,230 ± 488	20,353 ± 515
TFP (50 μM)	ND	579 ± 90**	12,085 ± 316***

†PLC activity was measured with [^{3}H]PI as substrate; 25 μg of splenocyte and Nb2 cytosol proteins were used at pH 6.8, in the absence of calcium, and 1.5×10^{-6} M free calcium. The measurements with the guanine nucleotides [GTP(100 μM), GTPγS(100 μM, and GDPβS (1 mM)] were done in the same experiment with the same control and the measurements with the inhibitors [NaF(5 mM) and TFP(50 μM)] were done in a separate experiment with a separate control. The values represent Means ± SEM (n = 3) ND means not determined. PLC activity in the absence of calcium for Nb2 cells was not different from background and was not always measured. Statistics were one way analysis of variance with the Tukey test for multiple comparisons between means. *Significantly different from control $P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

soluble PLC activity in Nb2 cells when PI was used as substrate. When PIP_2 was used as substrate, the guanine nucleotides had no effect on PLC activity, both in splenocytes and Nb2 cells (Table II).

One explanation of the ineffectiveness of GTP and GTPγS to enhance the calcium stimulated soluble PLC activity may be that the concentration of calcium (10^{-6} M) used was sufficient to produce maximal activity which could not be increased further by other agents. In order to test whether GTPγS (100 μM) may shift the calcium dose response to the left, we used GTPγS (100 μM) with various concentrations of calcium to measure soluble PLC activity in spleen. As shown in Figure 2 (using PIP_2 as substrate) GTPγS had no significant effect on the calcium dose response. This suggests that the inability of GTPγS to stimulate calcium activated soluble PLC is not due to the use of saturating levels of calcium.

Effect of Protein Phosphatase Inhibitors on Cytosolic PLC Activity in Splenocytes and Nb2 Cells

Among the tools commonly used to determine whether G proteins are involved in the mediation of a second messenger signaling system is NaF/$AlCl_3$ which form AlF_4^- that can substitute for the γ phosphate on GDP-bound G-protein and act as a constant G-protein activator [Bigay et al., 1985]. However, NaF is also used extensively as a serine/threonine protein phosphatase inhibitor [Wosilait and Sutherland, 1956]. NaF (5 mM) with or without $AlCl_3$ (50 μM) tended to inhibit splenocyte soluble PLC activity with PI as substrate, but the effect was not statistically significant. As with the guanine nucleotides, NaF did not have any effect on soluble PLC activity in Nb2 cells when PI was used as substrate (Table I). With PIP_2 as substrate, however, NaF (5 mM) had a significant inhibitory effect on the calcium stimulated PLC activity in both splenocytes and Nb2 cells (Table II).

In view of the inability of GTP and its nonhydrolyzable analogue, GTPγS, to affect the calcium stimulated response (Tables I, II), we interpreted the NaF effect as a result of protein phosphatase inhibition rather than as a result of G-protein activation. Because of the calcium dependence of soluble PLC activity, we also examined the effects of trifluoperizine (TFP), which is a calmodulin antagonist, and a specific inhibitor of the calcium dependent protein phosphatase 2B activity [Cohen, 1989]. TFP (50 μM) inhibited the calcium stimulated soluble PLC activity in splenocytes by 80–90% and PLC activity from Nb2 cells by 40–45% with PI as substrate (Table I). With PIP_2 as substrate, however, TFP (50 μM) had no significant effect on soluble PLC activity (Table II).

Effects of Antibodies on Cytosolic PLC Activity in Splenocytes

In order to examine, whether cytosolic Giα may couple to soluble PLC by a mechanism

TABLE II. Effects of Guanine Nucleotides and Phosphatase Inhibitors on Cytosolic PLC Activity From Splenocytes and Nb2 Cells With and Without Added Calcium (10^{-6} M) and Using PIP_2 as Substrate†

	Substrate: PIP_2 (Sample cpm-background cpm)			
	Splenocytes		Nb2 cells	
	No Ca^{2+}	$Ca^{2+} \times 10^{-6}$ M	NO Ca^{2+}	$Ca^{2+} \times 10^{-6}$ M
Control	826 ± 62	3565 ± 220	2127 ± 172	3962 ± 81
GTP (100 μM)	967 ± 95	3771 ± 253	2101 ± 35	4149 ± 50
GTPγS (100 μM)	849 ± 77	3462 ± 223	1641 ± 47	4155 ± 29
GDPβS (1 μM)	646 ± 61	2845 ± 279	2598 ± 190	3925 ± 72
Control	873 ± 94	3963 ± 119	ND	3640 ± 53
NaF (5 mM)	879 ± 82	3040 ± 165*	ND	2118 ± 49***
TFP (50 μM)	ND	3751 ± 219	ND	3678 ± 102

†PLC activity was measured with [^{3}H]PIP_2 as substrate; 25 μg of splenocyte and Nb2 cytosol proteins were used at pH 6.8, in the absence of calcium, and 1.5×10^{-6} M free calcium. The measurements with the guanine nucleotides [GTP(100 μM), GTPγS(100 μM), and GDPβS (1 mM)] were done in the same experiment with the same control and the measurements with the inhibitors [NaF (5 mM) and TFP (50 μM)] were done in a separate experiment with a separate control. The values represent means ± SEM (n = 3); ND means not determined. Statistics were one way analysis of variance with the Tukey test for multiple comparisons between means. *Significantly different from control $P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

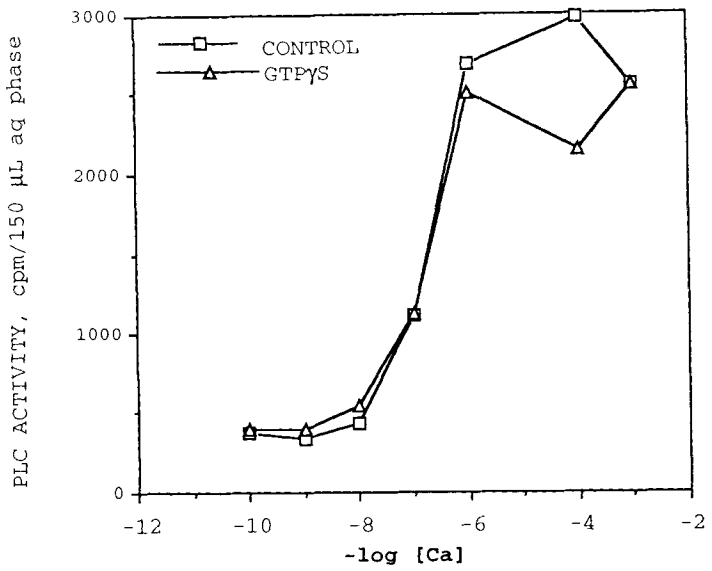

Fig. 2. Effects of GTPγS on the calcium dose response of spleen soluble PLC activity. Phospholipase C activity was measured with [^{3}H]PIP_2 as substrate; 50 µg of spleen cytosol proteins were used at pH 6.8. The free calcium concentration was calculated with EGTA 37, a program developed for the IBM computer by Seiriken. The PLC activity was measured with or without GTPγS (100 µM) (see Materials and Methods for other details).

different from the activation of membrane PLC by membrane Giα, we inactivated cytosolic Giα with affinity purified antibodies to Gi1 or Gi2. The affinity purified antibody LD (7.4 µg/10 µL), which recognizes Gi1α, and the affinity purified antibody LE (5.3 µg/10 µL), which recognizes Gi2α, significantly inhibited splenocyte soluble PLC activity with PI as substrate; 85–90% inhibition was obtained with Gi1 specific LD antibody and 55–70% inhibition, with Gi2 specific LE antibody (Fig. 3). With PIP_2 as substrate, the affinity purified antibody LD (7.4 µg/10 µL), inhibited splenocyte soluble PLC activity up to 70%, whereas affinity purified antibody LE (5.3 µg/10 µL) had very little effect on soluble PLC activity (Fig. 3).

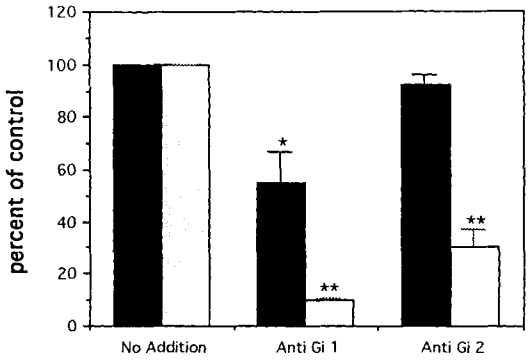

Fig. 3. Effect of anti G-protein antibodies on calcium stimulated PLC activity. PLC activity was measured with [^{3}H]PIP_2 (solid bars) and [^{3}H]PI (open bars) as substrate; 30–50 µg of spleen cytosol proteins were used at pH 6.8 and 1.5×10^{-6} M free calcium; 10 µl of anti Gi1 antibody, LD (0.74 µg/µl) and 10 µl of anti Gi2 antibody, LE (0.53 µg/µl) were preincubated for 5 min with buffer before adding substrate as described in Materials and Methods. The results were obtained from three different experiments. The bars represent the means ± SEM. Statistics used were one-way analysis of variance with the Tukey test for multiple comparisons between means. *Significantly different from no addition $P < 0.05$. **$P < 0.01$.

Effects of Gi1 Antisense Oligonucleotides on Cytosolic Gi1 Levels and Cytosolic PLC Activity

The inhibitory effects of Giα antibodies suggests that Giα is coupled to soluble PLC activation. One way of independently confirming this conclusion was to introduce Giα antisense oligonucleotides into splenocytes for 48 h, to specifically inhibit the transcription and translation of Gi and thus reduce the levels of Giα. The control conditions consisted of a scrambled version of the antisense oligonucleotides or no addition (see Materials and Methods for other details).

The incubation of splenocytes with Giα1 antisense oligonucleotides produced a 25–40% decrease in cytosolic Giα1 levels compared to scrambled oligonucleotides and no addition control (Fig. 4A). The membrane levels of Giα1 was

also reduced by a similar percentage (data not shown). The soluble PLC activity in the antisense treated group was reduced by 25–50% compared to the scrambled and no addition groups for either PI or PIP_2 as substrate (Fig. 4B). There was no significant difference between the scrambled oligonucleotide group and the no addition in Giα levels, or soluble PLC activity (Fig. 4).

Effects of Pertussis Toxin Administration on Con A-Induced PLC Activity

Pertussis toxin has been used in the past to describe two PLC activities in different cell types [reviewed by De Vivo and Gershengorn, 1990]. In order to examine further the association of cytosolic Giα with cytosolic PLC, we treated rats with pertussis toxin in an attempt to uncouple

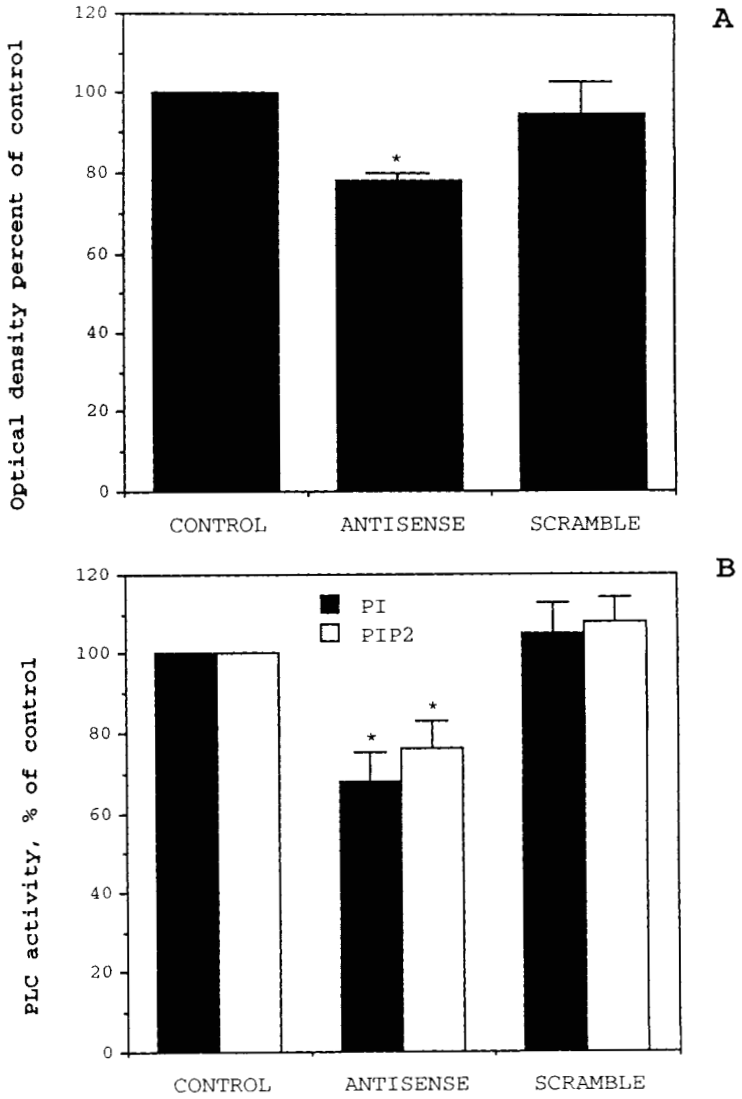

Fig. 4. Effects of Giα1 antisense oligonucleotides on cytosolic Gi1 levels and soluble PLC activity. Three groups of splenocytes cultured as described in Materials and Methods were used for these experiments. One group was incubated with two 15 mer antisense oligonucleotides, the second group was incubated with two 15 mer scrambled oligonucleotide sequences, and the third group was a No addition control group. **A:** 60 μg of cytosolic proteins were used in SDS-PAGE and western blots. The blots were incubated with Gi1 specific antibody LD and then ^{125}I-labeled goat anti-rabbit IgG. The optical densities were expressed as percent of control. **B:** PLC activity was measured with [^{3}H]PI and [^{3}H]PIP_2 as substrate; 25 μg of splenocyte cytosol proteins were used at pH 6.8, and 1.5×10^{-6} M free calcium. The results were obtained from three different experiments. The bars represent the means ± SEM. Statistics used were one-way analysis of variance with the Tukey test for multiple comparisons between means. *Significantly different from no addition $P < 0.05$.

cytosolic Giα from soluble PLC if they were indeed coupled.

Administration of pertussis toxin to adrenalectomized rats produced a 40–60% reduction in cytosolic Giα immunoreactivity (Fig. 5). It is important to note that pertussis toxin administration had no significant effect on membrane Giα immunoreactivity, though the membrane Giα tended to increase (Fig. 5). Moreover pertussis toxin had no effect on the membrane or cytosolic levels of the G-protein β-subunit (data not shown). We performed an in vitro ADP-ribosylation reaction with pertussis toxin to examine the extent of the in vivo ADP-ribosylation of membrane and cytosolic Giα. Administration of pertussis toxin to rats resulted in over 90% ADP-ribosylation of both membrane and cytosolic Giα. The pertussis toxin apparently had no direct effect on soluble PLC because none of the proteins ADP-ribosylated by pertussis toxin was in the molecular weight range of PLCs (namely, 60 kDa and above; data not shown).

We examined whether the pertussis toxin-mediated reduction in cytosolic Giα had any effect on soluble PLC activity. As can be seen in Figure 6 in vivo pertussis toxin treatment resulted in a 40–50% reduction of soluble PLC activity after splenocytes were removed and cultured for 24 h with or without Con A when PI was used as substrate (Fig. 6). With PIP_2 as substrate, however, the soluble PLC activity tended to decrease in the pertussis toxin treated group, but there was no significant difference between the pertussis treated and the control splenocytes (Fig. 6). After 24 h in culture, the cytosolic Giα immunoreactivity of splenocytes from pertussis toxin treated rats were about 50% lower than the cytosolic Giα immunoreactivity from splenocytes of control rats (data not shown).

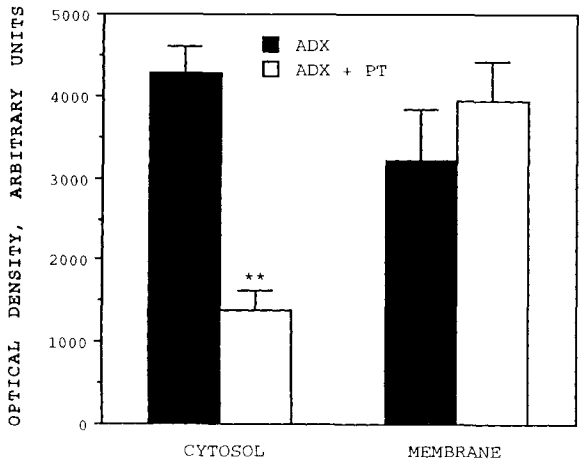

Fig. 5. Effects of Pertussis toxin on cytosolic and membrane Giα immunoreactivity in spleen of ADX rats. Equal amounts of cytosolic (60 μg) and membrane (20 μg) protein from Adrenalectomized (ADX) and ADX + Pertussis Toxin treated rats were used in SDS-PAGE and western blot analysis as described in Materials and Methods. Giα immunoreactivity was quantified by measuring optical densities from autoradiograms with a Drexel-Dumas image analysis program. The bars represent the means ± SEM (n = 3). Statistics were done by independent *t*-test. **Significantly different from control $P < 0.01$.

PI Versus PIP_2 as Substrate

We measured soluble PLC activity with PI and PIP_2 as substrates in all the conditions tested. The reason for using the two substrates is that whereas the hydrolysis of the more abundant PI leads to the production of 1 second messenger DAG, the hydrolysis of the less abundant PIP_2 produces DAG as well as another second messenger IP_3 (which mediates the release of calcium from internal stores) in addition to DAG. Thus the hydrolysis of the two sub-

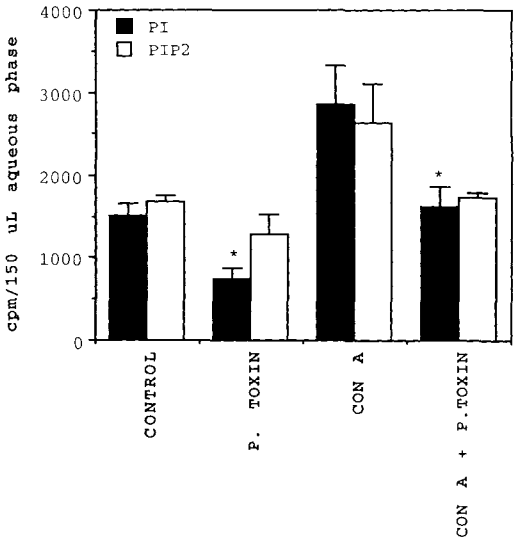

Fig. 6. Effects of in vivo administration of Pertussis Toxin on cytosolic PLC activity in splenocytes cultured for 24 h with and without Con A. Two groups of rats were used: one group received an injection of 25 μg pertussis toxin in 100 μL of saline; the other group received an injection of 100 μL of saline. Phospholipase C activity was measured with [^{3}H]PI and [^{3}H]PIP_2 as substrates; 30 μg of spleen cytosol proteins were used at pH 6.8 with 1.5 μM free calcium. The solid bars represent control (n = 3) and the open bars pertussis toxin treated rats (n = 3). The bars represent the means ± SEM. Statistics were done by independent *t*-test. *Significantly different from control $P < 0.05$.

strates may be differentially controlled by a given drug or hormone to influence physiological events. As can be seen from Tables I and II, splenocyte and Nb2 cytosolic PLC activity with PIP_2 as substrates was similar in level to the PLC activity observed with PI as substrate.

As shown in various experiments presented in this paper, the major substrate dependent differences were 1) lack of any activity above background when PI was used as substrate in the absence of calcium; 2) NaF had no significant inhibitory effect on the calcium stimulated activity when PI was used as substrate but inhibited activity when PIP_2 was used; 3) on the other hand TFP (50 μM) had no significant inhibitory effect when PIP_2 was the substrate while TFP (50 μM) significantly inhibited PLC activity when PI was used; 4) whereas both Giα1 and Giα2 antibodies significantly inhibited PLC activity with PI as substrate, only Giα1 antibody significantly inhibited PLC activity when PIP_2 was used as substrate (Fig. 3); and 5) in vivo treatment of rats with pertussis toxin resulted in a significant reduction in splenocytes soluble PLC activity with PI as substrate, but not with PIP_2 as substrate.

DISCUSSION

Phosphatidylinositol specific phospholipase C activation is stimulated by several hormones and drugs leading to the production of the second messengers inositol 1,4,5 trisphosphate and diacylglycerol in a wide variety of cells [Hokin, 1985; Berridge, 1987]. G-protein α-subunits have been implicated in the activation of soluble PLC, because of the stimulatory effects of GTP, GTPγS, and NaF [Deckymn et al., 1986; Camps et al., 1990; Baldassare and Fisher, 1986] on soluble PLC activation. The envisioned mechanism for this process is similar to the mechanism of activation of membrane PLC by G-proteins.

In this report we examined whether or not cytosolic Giα is associated with the activation of PLC in splenocytes. We approached this problem in a stepwise manner; First, we used the general activators of G-protein dependent processes, GTP, GTPγS, and NaF to establish the role of soluble G-proteins in the activation of soluble PLC, *assuming* that the mechanism of activation of the cytosolic PLC system was similar to that of membrane-associated G protein coupled PLC activation. The next step was to inactivate or reduce Giα in the cytosol to establish if the G-protein involved in the activation of soluble PLC was Giα.

Effects of Guanine Nucleotides on Cytosolic PLC Activity From Splenocytes

The data presented in this paper indicate that Giα is involved in the cytosolic PLC activation process in the spleen, but not by a guanine nucleotide dependent process. The inability of GTP and its non-hydrolyzable analogs as well as NaF (5 mM) to stimulate soluble PLC activity from splenocytes and Nb2 cells differs from the results of Deckmyn et al. [1986], who, using platelets, reported that GTPγS and Gpp(NH)p at 100 μM stimulated soluble PLC activity by 8–10 fold over basal; GTP and GDP at 100 μM and GDPβS at 1 mM also stimulated soluble PLC activity by 3–4-fold. In our study, in the presence of calcium (10^{-6} M), GTP and GTPγS at 100 μM had no effect on soluble PLC activity (Table I). This result is similar to that reported in platelet cytosol by Baldassare and Fisher (1986) who, using PI as substrate, found no further stimulation of calcium stimulated activity by 10 μM GTPγS, but it differs from the soluble PLC activity from HL-60 cells where the activity stimulated by calcium (0.1 μM) was enhanced up to 5-fold by GTPγS (30 μM) [Camps et al., 1990]. The differential effects of the G-protein activators on splenocyte and Nb2 cell soluble PLC activity and soluble PLC activity from other cells may reflect the presence of different PLC isoforms in the cytosol of the different cell types.

Although the G-protein activators had no effect on soluble PLC activity, GDPβS that inhibits G-protein coupled processes did inhibit PLC activity. There is no simple explanation for this paradox, but it is reasonable to suggest that the inhibitory effects of GDPβS may occur by sterically hindering the association between Giα and soluble PLC.

Possible Coupling of Cytosolic Giα to the Activation of Cytosolic PLC and the Mechanism of Activation

Although cytosolic Giα appears identical to membrane-associated Giα, on western blots [Akompong et al., 1993], it is possible that post-translational modification of soluble Giα, such as myristylation and phosphorylation may be

different from membrane-associated Giα and thus make it possible for the membrane and cytosolic pools of Giα to participate in different signal tranduction events independent of each other.

The inability of GTP and GTPγS as well as NaF to stimulate soluble PLC activity, with or without calcium, suggests a very limited role of guanine nucleotides in the activation of soluble PLC from splenocytes. On the other hand, inhibition of soluble PLC activity by affinity purified antibodies to Gi1 and Gi2 strongly suggest the involvement of cytosolic Giα in the activation of soluble PLC. Owing to nonspecific inhibitory effects of high protein concentrations on the PLC activity assay, we were not able to test the blockade of the antibody effect with excess of peptide antigen. Nevertheless the differential effect of the affinity purified antibodies (Gi1 and Gi2) suggests that the inhibitory effects are due to the specificity of the different IgGs. Nonspecific factors should result in similar inhibition by the two closely related antibody preparations.

The coupling between cytosolic Giα and soluble PLC is also supported by the correlated inhibition of cytosolic Giα1 levels and soluble PLC activity by antisense oligonucleotides to Giα1 administered to intact splenocytes. Additional evidence that is consistent with the notion that cytosolic Giα is associated with soluble PLC is provided by the correlated inhibition of cytosolic Giα immunoreactivity and soluble PLC activity by pertussis toxin. Pertussis toxin administered alone in vivo produced a 40–60% reduction in cytosolic Giα immunoreactivity. Soluble PLC activity from the pertussis toxin treated splenocytes cultured for 24 h was significantly reduced compared to control splenocytes, with or without Con A when PI was used as substrate. With PIP_2 as substrate, however, there was a trend towards reduction in the pertussis toxin treated splenocytes, but it was not statistically significant. The cytosolic Giα levels were also significantly reduced in the pertussis toxin treated splenocytes compared to the control, suggesting that pertussis toxin may reduce cytosolic PLC activity by reducing cytosolic Giα levels. The cytosolic PLC, sensitive to pertussis toxin, appear to prefer PI rather than PIP_2 as substrate.

Because pertussis toxin treatment also resulted in the ADP-ribosylation of cytosolic Giα, another interpretation of the results is that ADP-ribosylation of cytosolic Giα uncouples it from soluble PLC and thus reduces the efficiency of PLC activation. This view also suggest that cytosolic Giα is coupled to soluble PLC activation.

Owing to the inhibitory effects of NaF and TFP (serine/threonine phosphatase inhibitors) on soluble PLC activity, it is reasonable to suggest that serine/threonine phosphatases may be part of the mechanism of activating some form of soluble PLC in splenocytes/Nb2 cells. One possible site of the phosphatase action could be the soluble PLC itself. For the following reasons we favor an alternative mechanism where cytosolic Giα is the principal regulator; and serine/threonine dephosphorylation of this protein is the turn-on signal: First PLC is an unlikely target: ie, although all the four classes of phospholipase C (α,β,γ, and δ) have been shown to be phosphorylated (on serine/threonine) in vitro by protein kinase C, no changes in catalytic activity has been observed after phosphorylation [Rhee et al., 1989]; Second, Gi, when phosphorylated, shows an altered regulation of activity as shown by Katada et al. [1985]; phosphorylation of Gi by protein kinase C leads to the loss of Gi's ability to mediate the inhibition of adenylate cyclase by somatostatin. More recently Bushfield et al. [1990] have also shown that phosphorylation of Gi2 by protein kinase C disrupts the ability of low concentrations of the nonhydrolyzable GTP analogue p(NHppG) to inhibit forskolin-stimulated adenylate cyclase activity ("Gi" function) in hepatocytes.

Substrate Specific Soluble PI-PLCs

We used pure phosphatidylinositols (PIP_2 and PI) as substrates, and therefore the influence of other membrane lipids on the system is not factored into our analysis. Whereas activity with the two substrates responded to most of the agents tested in this study in a similar manner, there were notable differences between the two substrates. When PI was used as substrate there was no activity above background in the absence of added calcium. PIP_2, on the other hand, had a basal activity in the absence of calcium that was significantly above background. With PI as substrate the calcium stimulated activity was more sensitive to inhibition by GDPβS (60%) than when PIP_2 was the substrate (25%). The effect of TFP was also strikingly different between the two substrates (PI and PIP_2). That is the concen-

tration of TFP required to completely inhibit activity when PI was used as substrate (50 μM) was similar to that used to completely inhibit purified protein phosphatase 2B activity [Stewart et al., 1983; Cohen, 1989]. However, with PIP_2 as substrate a much higher concentration of TFP was required to completely abolish PLC activity. NaF (5 mM) inhibited soluble PLC activity when PIP_2 was the substrate but had little effect when PI was used as substrate. An analysis of how substrate specific PLCs may be important in the activation of splenocytes by mitogen is presented in the second paper.

In conclusion, the effect of Giα antibodies, antisense oligonucleotides to Giα1, and protein phosphatase inhibitors (NaF, and TFP) on the calcium stimulated activity suggest the involvement of Giα and serine/threonine protein phosphatases in the activation process. Unresolved questions including the relation between the Giα coupled soluble PLC activity and mitogen-induced PI-turnover in intact splenocytes, substrate preference, as well as, the characteristics of mitogen-induced PLC compared to other characterized PLCs will be considered in the second paper.

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