Interferon- γ -stimulated and GTP-binding-proteins-mediated phospholipase A_2 activation in human neuroblasts

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Interferon- γ (IFN- γ) is a potent growth-inhibitory cytokine also endowed with differentiating activity on neural cells. Binding of IFN- γ to its high-affinity receptor induces a rapid and transient activation of phospholipase A_2 (PLA $_2$). The mechanism coupling the IFN- γ receptor (IFN- γ -R) to PLA $_2$ activation is not clearly defined, and no information is available on this mechanism in neuroblast cells. We have tested the hypothesis that GTP-binding proteins (G-proteins) may couple the IFN- γ -R to PLA $_2$ in the human neuroblastoma (NB) cell line LAN-5. Incubation of NB cells with IFN- γ resulted in a rapid increase in [3 H]arachidonic acid (AA) release, and this effect was blocked by pretreatment with anti-IFN- γ antibodies. IFN- γ -stimulated AA release was still observed in permeabilized cells that were blocked by pretreatment with anti-IFN- γ -R antibodies. Exposure of permeabilized LAN-5 cells to guanosine 5'-[γ -thio]triphosphate (GTP[S]), a non-hydrolysable GTP analogue, induced a dose-dependent release of [3 H]AA. A non-specific nucleotide effect was excluded, since similar stimulatory effects on AA mobilization were not observed by GTP, ATP, CTP, ADP and GDP. IFN- γ -stimulated AA release was completely blocked by the

guanine nucleotide analogue that inhibits G-protein function, guanosine 5'-[β -thio]diphosphate (GDP[S]). A role for G-proteins in IFN- γ -R coupling to PLA $_2$ was further supported by the inhibition of IFN- γ -induced [3 H]AA release by treatment of permeabilized cells with pertussis toxin and with the antiserum against the common α -subunits of G-proteins. To determine a possible contribution to AA mobilization by the phospholipase C and diacylglycerol lipase pathway or by protein kinase C activation, the effects of neomycin, a phospholipase C inhibitor, and PMA (phorbol 12-myristate 13-acetate), a direct activator of protein kinase C, were investigated. Neither neomycin nor PMA affected either basal or IFN- γ -stimulated AA release. Ca^{2+} concentration, which has been shown to regulate the activity of some PLA $_2$ s, does not appear to play an important role in the regulation of the IFN- γ -stimulated PLA $_2$ activity, since incubating permeabilized cells in different concentrations of Ca^{2+} induced AA release without affecting the IFN- γ response. Altogether, these findings suggest the existence of IFN- γ -R, which couples a Ca^{2+} -independent PLA $_2$ activation via pertussis-toxin-sensitive G-proteins.

INTRODUCTION

Many lines of evidence suggest that the type II (γ) interferon (IFN- γ), a lymphokine produced by activated T lymphocytes and NK cells, exerts a variety of biological effects, including immunomodulatory, anti-viral and anti-proliferative activities (Trinchieri and Perussia, 1985; Vilcek et al., 1985). In addition, IFN- γ may play a role in the interaction between the nervous and the immune system (Ljungdahl et al., 1989), and is endowed with differentiation-promoting capability on neural cells (Parodi et al., 1989; Higuchi et al., 1990; Wuarin et al., 1991).

IFN- γ action is mediated via a high-affinity cell-surface receptor (IFN- γ -R) that has recently been shown to be an ubiquitous protein occurring in different molecular forms depending on the cell types (Novick et al., 1989; Van Loon et al., 1991). Stimulation of IFN- γ -R may involve a role for protein kinase C (PKC) (Fan et al., 1988) and Ca^{2+} /calmodulin-dependent kinase pathways (Koide et al., 1988) as well as for a GTP-binding protein (G-protein) (Gariglio et al., 1988). However, the intracellular mechanisms mediating IFN- γ -induced responses have yet to be clarified. Although an IFN- γ -dependent tyrosine phosphorylation of a latent cytoplasmic transcription factor has been reported in fibroblasts (Schindler et al., 1992),

more recently we reported the induction of arachidonic acid (AA) mobilization by IFN- γ treatment of neuronal cells (Ponzoni et al., 1992).

Experimental evidence indicates that two enzymatic pathways may be involved in AA release: (1) direct mobilization of AA by phospholipase A_2 (PLA $_2$)-catalysed hydrolysis of mainly phosphatidylcholine and phosphatidylethanolamine (Van Corven et al., 1989; Broekman, 1986); and (2) activation of a phosphoinositide-specific phospholipase C (PLC) leading to the formation of diacylglycerol, which may be further metabolized by diacylglycerol lipases to yield AA (Rittenhouse, 1983; Mason-Garcia et al., 1992). Although we clearly demonstrated stimulation of receptor-coupled PLA $_2$ by IFN- γ induction of neuroblastoma (NB) cells (Ponzoni et al., 1992), the relative importance of the two pathways has yet to be resolved, since simultaneous PLA $_2$ and PLC functional coupling to the same receptor, possibly through distinct G-proteins, has been observed in many systems (Slivka and Insel, 1988; Le Gouvello et al., 1990). Moreover, the mechanisms of signal transduction leading to AA mobilization are not yet well understood. There is considerable evidence of G-proteins regulating PLA $_2$ activity in many models (Jelsema, 1987; Silk et al., 1989; Murayama et al., 1990; Gupta et al., 1990; Axelrod, 1990). Activation of PLA $_2$ secondary to activation of PKC (Parker et al., 1987) or to increased intracellular Ca^{2+}

Abbreviations used: IFN- γ , interferon- γ ; IFN- γ -R, IFN- γ receptor; AA, arachidonic acid; G-protein(s), GTP-binding protein(s); GTP[S], guanosine 5'-[γ -thio]triphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate; PTX, pertussis toxin; PMA, phorbol 12-myristate 13-acetate; PLA $_2$, phospholipase A_2 ; PLC, phospholipase C; PKC, protein kinase C; NB, neuroblastoma; TL, tetanolysin; MoAb, monoclonal antibody.

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(Rittenhouse, 1984; Jordan and Russo-Marie, 1992) have been reported.

In this paper we show that IFN- γ stimulation of NB cells results in activation of a Ca^{2+} -independent PLA_2 whose stimulation is independent of PLC and PKC activation, but is mediated by a pertussis-toxin (PTX)-sensitive G-protein.

EXPERIMENTAL

Materials

Human recombinant IFN- γ was obtained from Genzyme Corp. (Boston, MA, U.S.A.). IFN- γ (1000 i.u./ μl) was kept in batches in PBS (Flow Laboratories, Milan, Italy) plus 0.1% BSA at -80°C . Phorbol 12-myristate 13-acetate (PMA), neomycin sulphate, digitonin and PTX were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Guanosine 5'-[γ -thio]triphosphate (GTP[S]), guanosine 5'-[β -thio]diphosphate (GDP[S]) and all nucleotides were purchased from Boehringer G.m.b.H. (Mannheim, West Germany). Tetanolysin (TL), a gift from Dr. E. Bonvini (FDA, Bethesda, MD, U.S.A.), was obtained as a partially purified preparation from a tetanus-toxin-negative mutant of *Clostridium tetani*. The mouse anti-(human-IFN- γ) monoclonal antibody (MoAb) was from Genzyme. Anti-IFN- γ -R MoAb (γ -R-38) was generously given by Dr. G. Garotta (Hoffman-La Roche, Basel, Switzerland). The rabbit anti-G-proteins antiserum (GA/1), specific for common α subunits of GTP-binding proteins, was obtained from NEN (Du Pont de Nemours, Dreieich, W. Germany). [5,6,8,9,11,12,14,15- ^3H]AA (60–100 Ci/mmol) was from Amersham International (Amersham, Bucks, U.K.).

Cell cultures

LAN-5 NB cell line was given by Dr. R. Seeger (Seeger et al., 1982). Cells were maintained in the exponential phase of growth in 75 cm^2 plastic culture flasks (Costar, Cambridge, MA, U.S.A.) in RPMI 1640 medium (Seromed; Biochrom KG, Berlin, Germany), supplemented with 15% heat-inactivated foetal-calf serum (Seromed), sodium penicillin G (50 i.u./ml) and streptomycin sulphate (50 μl /ml) (complete medium) at 37°C in a humidified incubator under air/ CO_2 (19:1). Cells were split after treatment with 1 mM EDTA in Hanks' salts solution (Seromed), washed, counted, and re-plated in fresh complete medium.

Cell labelling, permeabilization procedures, and release of [^3H]AA

LAN-5 cells (5×10^5 /well in 24-well plates) were labelled for 16–24 h at 37°C with [^3H]AA (20 μCi /ml) in RPMI 1640 complete medium. After labelling, monolayer cells were washed three times with complete medium at 37°C . For time-course experiments, RPMI 1640 with IFN- γ (1000 i.u./ml) was added at zero time after washing. During the different time points examined, samples (200 μl) of the supernatants were measured for [^3H]AA released in the culture medium by using a β scintillation counter (Tri-carb; Packard Instruments, Milan, Italy). For dose-dependence experiments, cells were treated for 2 min 30 s with different doses of IFN- γ . In order to confirm uptake of label into appropriate pools, [^3H]AA-labelled phospholipids were analysed by t.l.c. (Bar-Sagi and Feramisco, 1986). Typically, about 35% of added [^3H]AA was incorporated into lipids. Under these conditions, the lipid profile of incorporated [^3H]AA was phosphatidylcholine ($47 \pm 6\%$), phosphatidylethanolamine ($20 \pm 4\%$), phosphatidylinositol ($10 \pm 1.5\%$), phosphatidylserine ($8 \pm 2\%$), phosphatidic acid ($4.5 \pm 1\%$), lysophosphatidylcholine

($6 \pm 1\%$), lysophosphatidylethanolamine LPE ($3 \pm 0.75\%$), PtdInsP + PtdInsP₂ ($1.3 \pm 0.5\%$). For inhibition experiments, after washing, labelled LAN-5 cells were pretreated for 10 min at 37°C with PMA (200 nM), neomycin (0.5 mM) or anti-IFN- γ MoAb (20 μg /ml), and then treated with or without 1000 i.u./ml IFN- γ . After 2 min 30 s at 37°C , samples were counted for [^3H]AA release. PTX treatment (20 ng/ml) of LAN-5 cells was for the last 18 h during the 24 h [^3H]AA labelling of cell lipids.

Permeabilization of LAN-5 cells was performed in a buffer [potassium glutamate/Hepes (KG) buffer] composed of 20 mM Hepes, pH 7.15 at 37°C , 138 mM potassium glutamate, 7 mM magnesium acetate, 1 mM EGTA, 0.1 mg/ml BSA, 10 mM D-glucose, 100 μM myo-inositol, 5 mM ATP and 0.285 mM CaCl_2 to give a free Ca^{2+} concentration of 100 nM at 37°C . The exact free Ca^{2+} concentration in KG buffer, as well as the formulations of the Ca^{2+} /Mg $^{2+}$ /EGTA buffers employed in the Ca^{2+} curve were calculated as described by Bonvini *et al.* (1991) by using a modification of the computer program CHELATE (kindly provided by Dr. E. Bonvini) and the stability constants given by Sillen and Martell (1964) and Martell and Smith (1974). In some experiments ATP was omitted from the KG buffer. Permeabilization was performed as described by Conti *et al.* (1993). Briefly, cell suspensions were mixed for 10 min on ice with TL (0.45 $\mu\text{g}/10^7$ cells/ml), diluted ≥ 5 -fold with KG buffer, and incubated for 5 min at 37°C to allow permeabilization to occur. In some preliminary experiments TL was reduced with 5 μM dithiothreitol for 10 min at room temperature. This procedure did not improve cell permeabilization, so it has been avoided during the final experiments. TL binds in the cold and produces pores of diameter 25–50 nm in cholesterol-containing membranes upon exposure at 37°C (Smyth and Duncan, 1978). In several experiments, labelled LAN-5 cells were permeabilized with 15 μM digitonin for 7 min at 37°C . In all experiments, cell permeabilization, measured as the percentage of cells stained by the non-permeating probes Trypan Blue or ethidium bromide, was better than 95%. The permeabilizing treatments were terminated by washing the cells (twice) in KG buffer. In Table 3, the labelled and permeabilized LAN-5 cells were incubated for 15 min at 37°C in 0.5 mM neomycin, 17 μg /ml anti-IFN- γ -R MoAb (γ -R-38, IC₅₀ 1.7 μg /ml) (Garotta *et al.*, 1990), 17 μg /ml mouse IgG1 (Sigma), 20 μg /ml anti-G-proteins (anti-G-com) antiserum, or 20 μl /ml normal rabbit serum (Sigma), and then treated or not with IFN- γ for 2 min 30 s at 37°C .

RESULTS

IFN- γ -R-stimulated AA release

To investigate a possible release of AA in response to IFN- γ -R stimulation in neuronal cells, prelabelled cultures of LAN-5 human NB cells were triggered with different doses of IFN- γ and assessed for [^3H]AA release. As shown in Figure 1, there was a concentration-dependent enhancement of AA release, with half-maximal increase observed with 150 i.u./ml IFN- γ . Addition of the immunological antagonist anti-IFN- γ to the incubation medium completely blocked the IFN- γ -induced AA mobilization (Table 1) confirming our previous data (Ponzoni *et al.*, 1992) indicating hydrolysis of phosphatidylcholine by PLA_2 in IFN- γ -treated LAN-5 cells, and suggesting this metabolite as part of the signal-transduction pathway activated by IFN- γ .

IFN- γ -induced AA release is not secondary to polyphosphoinositide hydrolysis

Since these results could not exclude the possibility that AA could also be derived from the sequential activation of PLC and

Figure 1 Dose-dependent effect of IFN- γ on [3 H]AA release by neuroblasts

For measurement of AA release, cells were preincubated with 20 μ Ci/ml [3 H]AA for 24 h. Cells were washed and then various concentrations of IFN- γ were added for 2 min 30 s, and assays were completed as described in the Experimental section. The data (means $\pm$ S.D.) are expressed as percentages of the control level obtained from four independent experiments assayed in duplicate.

Table 1 Effects of anti-IFN- γ antibody, pertussis toxin, neomycin and PMA on the basal and IFN- γ -stimulated AA release

Cells were labelled with 20 μ Ci/ml [3 H]AA for 24 h, washed, preincubated with the anti-IFN- γ MoAb (20 μ g/ml), or neomycin (NEO, 0.5 mM), or PMA (200 nM), and then treated or not with 1000 i.u./ml IFN- γ for 2 min 30 s. Pretreatment with PTX (20 ng/ml) was for 18 h. Data shown are means $\pm$ S.D. of three separate experiments each done in duplicate.

Addition	[3 H]AA release (%)				
	None	PTX	NEO	PMA	Anti-IFN- γ
None	100	95 $\pm$ 3	104 $\pm$ 5.5	99 $\pm$ 6	101 $\pm$ 3.9
IFN- γ	174 $\pm$ 3.1	103 $\pm$ 2.9	169 $\pm$ 3.2	173 $\pm$ 4.6	98 $\pm$ 6.2

diacylglycerol lipase (Bell et al., 1979), neomycin, an inhibitor of PLC, was used. The aminoglycoside antibiotic neomycin avidly binds polyphosphoinositides and makes them unavailable to PLC (Schwartz et al., 1984). As shown in Table 1, treatment of LAN-5 cells with 0.5 mM neomycin did not affect either basal or IFN- γ -stimulated AA release. The same dose of neomycin inhibited retinoic acid-induced polyphosphoinositide hydrolysis in NB cells (Ponzoni and Lanciotti, 1990) (results not shown). Although neomycin has been shown to work on intact cells (Slivka and Insel, 1988), it is preferentially used on permeabilized cells (Nielson et al., 1991) to rule out the possibility that it does not get inside the cells. Treatment of permeabilized cells with neomycin did not affect the AA release induced by IFN- γ (Table 3).

Since it has been shown that ATP can also interact with a cell-surface receptor which is coupled to phosphoinositide-specific PLC via a PTX-sensitive G-protein (Cockcroft and Stutchfield, 1989), the possibility of an indirect activation of PLA₂ has been ruled out by treating permeabilized cells in KG buffer not containing ATP. Under these experimental conditions IFN- γ still induced an almost 2-fold increase in AA release.

Figure 2 Dose-dependent effect of [Ca^{2+}] on the basal and IFN- γ -stimulated AA release

After labelling, cells were washed and permeabilized with TL as described in the Experimental section. The exact free Ca^{2+} concentrations were calculated by using the computer program CHELATE. Incubations were carried out for 2 min 30 s in the presence of different doses of Ca^{2+} alone ($\circ$) or Ca^{2+} plus 1000 i.u./ml IFN- γ ($\bullet$). The data are means $\pm$ S.D. of three independent experiments each done in duplicate.

Role of PKC and Ca^{2+} in IFN- γ -stimulated AA release

In other cellular models, PLA₂ activation has been assumed either to result from elevation of cytoplasmic Ca^{2+} concentration induced by receptor-agonist interactions (Rittenhouse, 1984; Siess and Lapetina, 1983) or to be secondary to PKC activation (Parker et al., 1987; Burch, 1987). We tested the effect of PKC stimulation on IFN- γ -induced or basal AA release. PMA is thought to exert its effects on cells via its ability to mimic diacylglycerol in the activation of the phospholipid-dependent PKC (Nishizuka, 1986). PMA did not stimulate AA release and, furthermore, pretreatment of LAN-5 cells with 200 nM PMA for 10 min before addition of IFN- γ did not modify AA release (Table 1), although the same schedule of treatment with PMA induced a 3-fold increase in total PKC activity (results not shown). In order to investigate the role of free Ca^{2+} , we tested the effects of different concentrations of Ca^{2+} on AA mobilization. As shown in Figure 2, addition of increasing amounts of Ca^{2+} to the incubation medium induced a dose-dependent enhancement of AA release, reaching a maximum at 1 mM free Ca^{2+} . In contrast, IFN- γ -stimulated AA release was completely independent of the presence of extracellular Ca^{2+} , since the shapes of the basal and IFN- γ -induced curves of AA release in the presence of increasing concentration of free Ca^{2+} were identical (Figure 2).

Moreover, using the Ca^{2+} -sensitive dye Fura-2 we never observed Ca^{2+} fluxes in non-permeabilized LAN-5 cells after IFN- γ stimulation (M. Ponzoni, unpublished work). On the contrary, treatment of LAN-5 cells with 1 μ M ionomycin enhanced AA release by about 250% of control level.

Interaction of G-proteins with IFN- γ -R and PLA₂

To determine the possible G-protein regulation of the NB membrane PLA₂, the effects on AA mobilization of the guanine nucleotide analogue GTP[S] were investigated in permeabilized LAN-5 cells. Initial characterization of the model was performed by studying the PLA₂ activation by IFN- γ treatment of intact and permeabilized cells. As shown in Figure 3, IFN- γ -stimulated [3 H]AA release was observed in both intact and TL-permeabilized cells with the same entity and almost in the same time-dependent

Figure 3 Comparison of the time-dependent release of AA by intact or permeabilized cells

LAN-5 cells (1×10^6 /ml) were labelled as in Figure 1. Permeabilized cells were obtained by exposure to TL in KG buffer as described in the Experimental section and treated with 1000 i.u./ml IFN- γ . The incubation was terminated at the time points indicated. The data (means $\pm$ S.D.) shown are representative of four different experiments each done in triplicate.

Figure 4 Dose-response effect of GTP[S] on $[^3\text{H}]\text{AA}$ release from permeabilized NB cells

LAN-5 cells were labelled and permeabilized with TL as described in the Experimental section. Cells were then treated with buffer alone (●, control, unstimulated cells) or IFN- γ (○; 1000 i.u./ml) in the presence of the indicated concentration of GTP[S]. The incubation was terminated after 2 min 30 s, and AA release was determined as described in the Experimental section. Each point represents the mean $\pm$ S.D. of four independent experiments each done in duplicate.

Table 2 Effects of nucleotides on AA mobilization

LAN-5 cells were labelled and permeabilized with TL as described in the Experimental section. All buffers and quantification of the released AA are described in the Experimental section. Values are means $\pm$ S.D. from triplicate observations: * significant difference compared with the control ($P < 0.05$ evaluated by Student's *t* test).

Additions	$[^3\text{H}]\text{AA}$ released (d.p.m./ 10^6 cells)
Control	1710 $\pm$ 171
EDTA (100 μM)	1790 $\pm$ 214
GTP[S] (100 μM)	2690 $\pm$ 173*
GTP (100 μM)	1833 $\pm$ 149
ATP (100 μM)	2126 $\pm$ 112
CTP (100 μM)	1599 $\pm$ 312
ADP (100 μM)	1685 $\pm$ 235
GDP (100 μM)	1850 $\pm$ 113

Figure 5 Dose-response effect of GDP[S] on the release of AA in response to IFN- γ or GTP[S] in NB cells

LAN-5 cells were labelled and permeabilized with TL as described in the Experimental section. Cells were preincubated with different doses of GDP[S] for 10 min at 37 °C, and then buffer alone (□; control) or IFN- γ (○; 1000 i.u./ml) or GTP[S] (●; 250 μM) was added and the incubation continued for an additional 2 min 30 s. The data shown are means $\pm$ S.D. of three independent experiments assayed in duplicate.

manner. In the experiments where digitonin-permeabilized cells were used, the enhancement of the AA release induced by IFN- γ was only marginally decreased (Table 3). Addition of GTP[S] to permeabilized cells induced a significant enhancement of $[^3\text{H}]\text{AA}$ release, compared with cells exposed to buffer alone (Figure 4). A maximal effect of GTP[S] was observed at 250 μM , with half-maximal response between 20 and 25 μM . A similar dose-response to GTP[S] was noted when permeabilized LAN-5 cells were simultaneously activated by perturbation of IFN- γ -R, by treatment with 1000 i.u./ml IFN- γ (Figure 4, ○ symbols). The possibility of a non-specific nucleotide effect on the PLA_2 activity was also investigated. The effects of ATP, GTP, CTP, ADP and GDP were compared with those of GTP[S]. Table 2 clearly indicates that AA mobilization was significantly stimulated only by treatment of permeabilized LAN-5 cells with 100 μM GTP[S].

Events mediated by G-proteins are known to be inhibitable by non-hydrolysable GDP analogues (Gilman, 1987). Preincubation of permeabilized NB cells for 10 min with GDP[S] inhibited AA release in response to IFN- γ -R perturbation in a dose-dependent fashion (Figure 5). Complete inhibition was observed at 5 mM GDP[S], with 50% inhibition between 1.5 and 2 mM. GDP[S] was less effective in blocking AA release stimulated by GTP[S]. This was not surprising, owing to competition between the two non-hydrolysable nucleotides.

IFN- γ -stimulated AA release is prevented by PTX and anti-G-proteins-antiserum treatment

Since the above results suggested the involvement of G-protein(s) in AA release by IFN- γ , the effect of PTX was investigated. PTX, an agent that is known to inactivate several G-proteins by inducing their ADP-ribosylation, is used as a probe to study mechanisms involved in receptor-mediated signal transduction in a variety of cell types (Murayama et al., 1990; Bizzarri et al., 1990; Gupta et al., 1990). As in other cell types, in LAN-5 cells PTX catalyses the ADP-ribosylation of a 40 kDa protein (M. Lanciotti and M. Ponzoni, unpublished work). The stimulatory effect of IFN- γ on AA release was completely abolished by an

Table 3 Effects of neomycin, anti-G-proteins and anti-IFN- γ -R antibodies on IFN- γ -stimulated AA release

The [³H]AA-labelled and permeabilized (digitonin) LAN-5 cells were first incubated with KG buffer, neomycin (NEO, 0.5 mM) control serum, anti-G-com or anti-IFN- γ -R antiserum for 15 min at 37 °C and further incubated with the indicated additions for AA release. [³H]AA released is plotted as a percentage of the control values obtained by pretreating and then treating cells with KG buffer. The data are mean $\pm$ S.D. from three separate experiments

Addition	[³ H]AA release (%)				
	None	NEO	Control serum	Anti-G com	Anti-IFN- γ -R
None	100 $\pm$ 5.8	102 $\pm$ 3.5	103 $\pm$ 2	94 $\pm$ 8.8	98 $\pm$ 7.5
IFN- γ (1000 i.u./ml)	163 $\pm$ 3.2	158 $\pm$ 2.2	161 $\pm$ 3.9	99 $\pm$ 5.9	95 $\pm$ 7.7

18 h pretreatment of the cells with PTX at 20 ng/ml, whereas PTX did not affect basal [³H]AA release (Table 1).

In Table 3, the effects of an anti-G-proteins antiserum were examined. Pretreatment of permeabilized cells with an antibody specific for common α -subunits of G proteins blocked IFN- γ -stimulated AA release. To confirm the specificity and the integrity of the receptor-coupled PLA₂ activation under these permeabilization conditions, LAN-5 cells were pretreated with an anti-IFN- γ -R, which reacts with an extracellular domain of IFN- γ -R, inhibiting IFN- γ activities (Garotta et al., 1990). Pretreatment of permeabilized LAN-5 cells with a 10-fold higher concentration of this MoAb with respect to its IC₅₀, totally abolished IFN- γ -induced AA mobilization (Table 3). In contrast, pretreatment of permeabilized cells with irrelevant antibodies was without effect.

DISCUSSION

The results presented herein show, for the first time to our knowledge, that human neuroblasts have a pathway for IFN- γ -receptor-mediated activation of phospholipase A₂ that is Ca²⁺-independent, is not secondary to stimulation of PLC and PKC, but is mediated by PTX-sensitive GTP-binding proteins.

The PLA₂s are a family of enzymes that exist in a variety of tissues; their activity can be positively modulated by different mechanisms, including PLC activation, increase in intracellular Ca²⁺ levels, PKC-mediated inactivation of the endogenous PLA₂ inhibitor lipocortin, or the actions of G-proteins (for a review see Chang et al., 1987). The possibility that PLA₂ is directly activated by the interaction between the IFN- γ -R and a G-protein(s) is the most intriguing in the NB model.

We assessed the relationship between phosphoinositide hydrolysis and AA release, using the PLC inhibitor neomycin (Fuse and Tai, 1987). The results, showing that pretreatment of LAN-5 cells with 0.5 mM neomycin did not affect basal and IFN- γ -stimulated AA release, lead to an important conclusion: InsP₃ and diacylglycerol formation and, in turn, Ca²⁺ mobilization and PKC activation, respectively are not prerequisites for AA release. Thus we conclude that IFN- γ -R-mediated AA release is neither secondary to nor dependent on polyphosphoinositides hydrolysis. This conclusion is confirmed and extended by the findings obtained with the PKC activator PMA (Nishizuka, 1986). Pretreatment of LAN-5 cells with 200 nM PMA for 10 min did not modify IFN- γ -induced AA release (Table 1), further indicating that the PLA₂ species involved is not regulated by PKC.

Moreover, the lack of absolute requirement for MgATP for IFN- γ -stimulated PLA₂ activation would indicate that PLC- as well as PKC-mediated phosphorylations do not play obligatory roles.

Furthermore, the data regarding the Ca²⁺-dependence, reported in Figure 2, clearly indicate that this IFN- γ -R-coupled PLA₂ behaves similarly to other mammalian PLA₂s recently purified (Wijkander and Sundler, 1989; Hazen et al. 1990; Jordan and Russo-Marie, 1992), which also hydrolyse the *sn*-2-acyl position of phospholipids in the absence of Ca²⁺.

Our data do not completely eliminate the possibility that diacylglycerol, produced by PLC-mediated hydrolysis of phospholipids other than polyphosphoinositides (Slivka and Insel, 1988), participates in IFN- γ -mediated AA release. This diacylglycerol could provide a substrate for diacylglycerol lipase, giving rise to further AA release, and/or activate PKC and enhance PLA₂ activity (Parker et al., 1987). To this regard, we have obtained evidence suggesting that IFN- γ is not able to promote any hydrolysis of phosphatidylcholine by PLC in LAN-5 cells (M. Ponzoni, unpublished work).

Although the conformation of the IFN- γ -R (Aguet et al., 1988) differs from that of the classical G-protein-coupled receptor (one versus seven predicted transmembrane domains), other non-classical growth-factor receptors, those for erythropoietin and platelet-derived growth factor, have been reported to be coupled to an early release of AA via a receptor-G-protein interaction (Mason-Garcia et al., 1992; Gronich et al., 1988). In view of such data and the recently emerging evidence of G-proteins regulating a number of PLA₂s (Fuse and Tai, 1987; Jelsema, 1987; Silk et al., 1989; Kajiyama et al., 1989; Axelrod, 1990; Murayama et al., 1990; Gupta et al., 1990; Mason-Garcia et al., 1992), we investigated the existence of a similar regulating mechanism for the IFN- γ -R-coupled PLA₂ in human neuroblast cells. It has been concluded that G_i-like proteins regulate receptor-coupled phospholipases because these pathways are inhibited by treatment of cells with pertussis toxin, which ADP-ribosylates and inhibits G_i and G_o proteins (Burch et al., 1986; Raben et al., 1987; Gilman, 1987), and because GTP and GDP analogues stimulate and inhibit, respectively, phospholipase activities in permeabilized cells (Fain et al., 1988; Murayama et al., 1990; Bonvini et al., 1991).

The following findings suggest that activation of PLA₂ is coupled to IFN- γ -R by G-protein(s) in human neuroblasts. (1) The non-hydrolysable guanine nucleotide analogue GTP[S] induced, in a dose-dependent manner, AA release in permeabilized LAN-5 cells. Activation by GTP[S] was specific. None of the other nucleotides used seemed to have any PLA₂ stimulatory effect. (2) IFN- γ activation of the PLA₂ was completely inhibited by GDP[S]. The concentrations of GDP[S] used to inhibit signalling were substantially in agreement with those previously reported (Gold et al., 1987; Bonvini et al., 1991). The inhibition by GDP[S] that we observed was not due to a non-specific toxic effect, but appeared to be related to the ability of GDP[S] to compete for a guanine-nucleotide-binding site. Indeed, GTP[S]-induced AA release was decreased by only 50%, as for T lymphocytes (Bonvini et al., 1991). (3) IFN- γ -stimulated AA release was thoroughly blocked by pretreatment of LAN-5 cells with PTX as well as with anti-G-proteins antiserum. These results clearly indicate that a PTX-sensitive G-protein(s) is involved in IFN- γ -receptor-mediated activation of PLA₂.

Very recently, it has been suggested that interferon-dependent tyrosine phosphorylation of a latent cytoplasmic transcription factor is involved in signal transduction of this inhibitory cytokine (Schindler et al., 1992). This transcription factor is a multimeric protein complex termed interferon-stimulated gene-factor 3

(ISGF3), composed of a regulatory component (ISGF3 α), and the DNA-binding component (ISGF3 γ) which recognizes the IFN-stimulated responsive element (ISRE). Although the involvement of a novel type of latent cytoplasmic tyrosine kinase in the early events of IFN- α signalling has been clearly documented (Fu, 1992; Velazquez et al., 1992; David and Larner, 1992; Schindler et al., 1992), the relatively late phosphorylation by IFN- γ (about 15 min) of only one of the ISGF3 proteins (Schindler et al., 1992), and the fact that ISGF3 α seems to be membrane-associated (David and Larner, 1992), open the possibility that another enzymic reaction (e.g. the action of a lipase, as suggested by David and Larner, 1992) may precede ISGF3 α phosphorylation. This in turn allows the association of ISGF3 α with ISGF3 γ and the eventual binding to the specific interferon-stimulated genes. In view of the above hypothesis and of our results, indicating the activation of a receptor-coupled PLA₂ as a very early event in the IFN- γ signalling pathway, membrane PLA₂ seems a very attractive candidate, although its exact role in the intracellular environment remains unknown.

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