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Interferon-α selectively activates the β isoform of protein kinase C through phosphatidylcholine hydrolysis

(transmembrane signaling/diacylglycerol/antiviral/antiproliferative)

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ABSTRACT **The early events that occur after interferon binds to discrete cell surface receptors remain largely unknown. Human leukocyte interferon (interferon-α) rapidly increases the binding of [^{3}H]phorbol dibutyrate to intact HeLa cells (ED_{50} = 100 units/ml), a measure of protein kinase C activation, and induces the selective translocation of the β isoform of protein kinase C from the cytosol to the particulate fraction of HeLa cells. The subcellular distribution of the α and ε isoforms is unaffected by interferon-α treatment. Activation of protein kinase C by phorbol esters mimics the inhibitory action of interferon-α on HeLa cell proliferation and down-regulation of protein kinase C blocks the induction of antiviral activity by interferon-α in HeLa cells. Increased phosphatidylcholine hydrolysis and phosphorylcholine production is accompanied by diacylglycerol production in response to interferon. However, inositol phospholipid turnover and free intracellular calcium concentration are unaffected. These results suggest that the transient increase in diacylglycerol, resulting from phosphatidylcholine hydrolysis, may selectively activate the β isoform of protein kinase C. Moreover, the activation of protein kinase C is a necessary element in interferon action on cells.**

Interferons (IFNs) are cytokines that bind to discrete cell surface receptors, render cells resistant to a wide variety of viruses, and play a role in the host defense against tumorigenesis and viral infections (1). They inhibit the proliferation of transformed cells *in vitro* and in animal models and modulate cell motility and differentiation. Although the pleiotropic cellular effects of IFNs are well established, the precise mechanisms whereby the binding of IFN to its cell surface receptor is linked to the manifold changes in cell physiology are unclear. IFN-α action does not involve the well-recognized mechanisms of signal transduction (i.e., cyclic nucleotides or ion channels). Yet, several lines of evidence suggest that changes in protein phosphorylation may play an important role (1). IFN selectively enhances the phosphorylation of several proteins [i.e., ribosome-associated P_1 protein or α subunit of protein synthesis initiation factor eIF-2 (2–4)], while inhibiting the phosphorylation of others (5). Moreover, protein kinase inhibitors, such as staurosporine and H-7, selectively block the transcriptional activation of specific genes stimulated by IFN-α, and the IFN-α-mediated induction of antiviral activity in HeLa cells (6, 7). These results suggest that protein kinase C (PKC) activation is a necessary event in inducing IFN-α-stimulated gene expression and establishing antiviral activity.

We have investigated the early events that occur after human HeLa-S3 tumor cells are incubated with leukocyte IFN (IFN-α) to identify the biochemical pathways of transmembrane signaling. These cells are highly sensitive to the antiproliferative and antiviral actions of human leukocyte IFN (IFN-α) and exhibit a characteristic profile of IFN-stimulated gene responses (8–11). Here we report that IFN-α treatment of HeLa cells induces the translocation of the β isoform of PKC (PKC β) to the particulate fraction of the cell. Activation of PKC results from the IFN-α-induced generation of diacylglycerol (DAG) achieved through the breakdown of phosphatidylcholine (PC). In contrast, IFN-α does not stimulate the hydrolysis of inositol phospholipids. The activation of PKC by IFN-α appears to be a necessary element in the antiproliferative and antiviral actions of IFN-α in HeLa cells.

MATERIALS AND METHODS

Cell Cultures. Human HeLa-S3 cells were grown in suspension culture at cell concentrations between 1×10^5 and 1.5×10^6 cells per ml in Eagle's minimal essential medium modified for spinner culture and supplemented with 5% (vol/vol) calf serum. For analysis of the effect of IFN-α on cell proliferation, HeLa cells were suspended at 1×10^5 cells per ml and grown in the presence of IFN-α for 3 days, and then cell samples were counted in a Coulter counter.

Antiviral Assay. HeLa cells were suspended at 5×10^5 cells per ml and incubated overnight with IFN-α at the indicated concentrations. The cells were washed twice with medium and infected with vesicular stomatitis virus (VSV, Indiana strain) for 1.5 hr at a multiplicity of infection of 0.1 plaque-forming units per cell. Cell suspensions were washed with medium, and 24 hr later the virus yield in the medium was assayed by plaque formation on mouse L cells.

[^{3}H]Phorbol Dibutyrate (PBt_2) Binding to HeLa Cells. HeLa cells (1×10^6 cells per ml) were incubated for 30 min at 37°C in medium containing 20 nM [^{3}H]PBt_2 (19 Ci/mmol, 1 Ci = 37 GBq; DuPont). Cell suspensions were then exposed to IFN-α (1000 units/ml) and, at the indicated times, cell samples were filtered onto 0.45-μm (pore size) Durapore membranes. Control cultures received no IFN-α. The membranes were washed three times with phosphate-buffered saline. The results are expressed in terms of specific PBt_2 binding, which is the difference between binding in the absence and the presence of 10 μM unlabeled PBt_2.

Distribution of Isozymes of PKC in Cytosolic and Particulate Extracts of HeLa Cells. Cytosolic and particulate extracts of HeLa cells were prepared by lysing cells in 500 μl of a hypotonic buffer [25 mM Tris·HCl, pH 7.4/10 mM EGTA/2 mM EDTA/20 mM 2-mercaptoethanol/100 mM NaF/soybean trypsin inhibitor (0.5 mg/ml)/leupeptin (0.5 mg/ml)/10 mM benzamide]. The particulate and cytosolic extracts were prepared by centrifugation of cell lysates at 100,000 ×

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Abbreviations: IFN, interferon; PKC, protein kinase C; PC, phosphatidylcholine; PBt_2, phorbol dibutyrate; VSV, vesicular stomatitis virus; DAG, diacylglycerol; PA, phosphatidic acid; PMA, phorbol 12-myristate 13-acetate; EGF, epidermal growth factor.

g for 10 min at 4°C (12). The extracts were suspended in SDS sample buffer [25 mM Tris·HCl, pH 6.8/10% (wt/vol) SDS/10% (vol/vol) glycerol/5% (vol/vol) 2-mercaptoethanol]. Samples were boiled for 5 min and subjected to SDS/PAGE by the method of Laemmli (13). Proteins were transferred to nitrocellulose and immunoblots were prepared by using specific affinity-purified antisera against PKC isozymes and visualized with ^{125}I-labeled protein A (0.1 μCi/ml) (12). Blots were exposed to Kodak XAR film with intensifying screens at −70°C for 3 days.

Assay for Cellular DAG. At various times after the addition of IFN-α (1000 units/ml), HeLa cells were extracted with chloroform/methanol/6 M HCl, 200:100:1 (vol/vol). Samples in the organic phase were dried under N_2, dissolved in 20 μl of 7.5% (wt/vol) *n*-octyl glucoside/25 mM cardiolipin, and incubated with 1 mM [γ-^{32}P]ATP and *Escherichia coli* DAG kinase (14). The reaction was terminated by the addition of equal volumes of chloroform/methanol/6 M HCl and of distilled water and extracted lipid was quantitatively analyzed by silica gel G TLC with the chloroform/acetone/methanol/acetic acid/water, 10:4:2:2:1 (vol/vol), solvent system. The spot corresponding to phosphatidic acid (PA) was located by autoradiography and scraped off the plate, and radioactivity was measured in a β counter. The assay was linear with respect to DAG mass from 0.2 to 10 nmol.

Phospholipid Analysis. HeLa cells (2×10^5 cells per ml) were labeled for 48 hr with [^{3}H]choline (0.2 μCi/ml). The cells were then pelleted and resuspended at 1.2×10^6 cells per ml in medium containing 0.2% bovine serum albumin and incubated for 60 min. The cells were pelleted and resuspended in medium with bovine serum albumin and containing IFN-α (1000 units/ml). At various times after IFN-α addition, cell samples were rapidly pelleted in a microcentrifuge (10,000 × *g*, 10 sec), and the supernatants and cell pellets were extracted with chloroform/methanol/6 M HCl, 200:100:1 (vol/vol). The aqueous extracts were lyophilized and dissolved in 50% (vol/vol) ethanol, and water-soluble choline compounds were resolved by TLC using the solvent system methanol/0.5% NaCl/ammonia, 50:50:1 (vol/vol). Phosphorylcholine, glycerolphosphorylcholine, and choline were employed as standards. PC was determined in the organic phase of cell extracts by TLC analysis using chloroform/pyridine/formic acid, 50:30:1 (vol/vol), as solvent. Lipids were visualized by iodine staining, the radioactive spots were scraped off, and the radioactivity was quantitated by liquid scintillation spectrometry.

IFN. Recombinant DNA-derived IFN-α (1×10^9 units/mg of protein), designated IFN-αCON$_1$, a consensus analog of the known IFN-α subtypes (15), was used in these studies and was a generous gift of M. Narachi (Amgen). IFN activities are expressed in terms of international reference units/ml, as assayed by protection against the cytopathic effect of VSV on human fibroblasts, using the National Institutes of Health human IFN-α standard (catalog number G-023-901-527) for reference.

RESULTS

Effects of IFN-α on PKC in HeLa Cells. The activation of PKC has been correlated with its translocation from the cytosol to cellular membranes and a subsequent increase in the binding of [^{3}H]PBt$_2$ to intact cells (16, 17). To determine whether IFN-α causes PKC activation, the binding of [^{3}H]PBt$_2$ to intact HeLa cells was determined (16). The cells were prelabeled with [^{3}H]PBt$_2$, and the specific binding of PBt$_2$ was measured at various times after IFN-α addition (Fig. 1*A*). IFN-α stimulated the binding of [^{3}H]PBt$_2$ to HeLa cells within minutes. PBt$_2$ binding returned to control levels within 2 hr. The increase in [^{3}H]PBt$_2$ binding to HeLa cells represents a redistribution of PKC from a low-affinity form in the cytosol (not bound appreciably in intact cells at the PBt$_2$ concentration used) to a higher-affinity form in the cellular membranes (16, 17).

To show that the IFN-α-induced increase in [^{3}H]PBt$_2$ binding, a measure of PKC translocation, is relevant to the biologic actions of IFN-α, the dose–response relationship of the increase in PBt$_2$ binding was compared to the dose–response relationship for the inhibition of VSV replication and for the inhibition of cell proliferation in response to

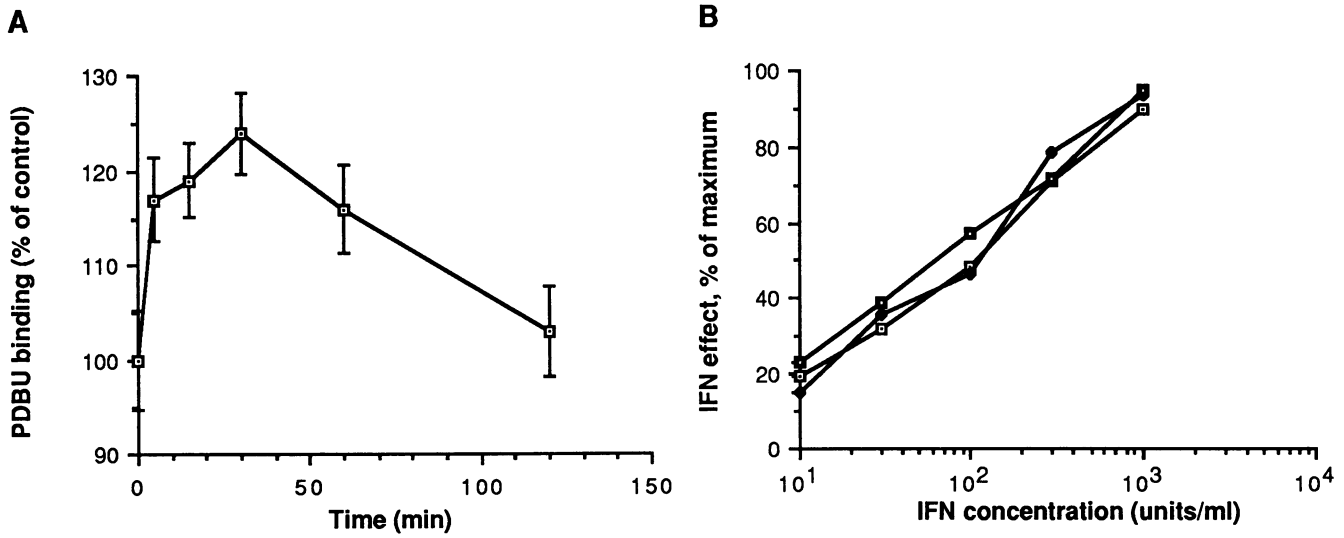

FIG. 1. IFN-α induces increased [^{3}H]PBt$_2$ binding to HeLa cells. HeLa cells were preincubated for 30 min with 20 nM [^{3}H]PBt$_2$. (*A*) Cells were exposed to IFN-α (1000 units/ml) for the indicated times and then were taken for [^{3}H]PBt$_2$ binding assays. Control cells bound 0.33 pmol of PBt$_2$ per 10^6 cells throughout the course of the experiment with an SEM <5%. (*B*) The dose–response curve of the effect of IFN-α on [^{3}H]PBt$_2$ binding was compared to that of cell proliferation and viral replication. [^{3}H]PBt$_2$ binding (□) was evaluated at 30 min after IFN-α addition. For analysis of the effect of IFN-α on cell proliferation (◆), the ratios of the cell number on day 3 to that on day 0 in IFN-α-treated HeLa cells were calculated as a percentage of the ratio in untreated control cells. For inhibition of viral replication (■), the data were calculated as the IFN-mediated log reduction in virus titer. The results on the effects of IFN-α on PBt$_2$ binding, cell proliferation, and viral replication at various IFN-α concentrations (10 units/ml to 10,000 units/ml) are presented as a percentage of the effect observed at saturating concentrations of IFN-α (10,000 units/ml). At this IFN-α concentration, a 34% increase in PBt$_2$ binding, a 63% inhibition of cell proliferation, and a 4.9 log reduction in virus replication were observed. PDBU, PBt$_2$.

IFN-α. The IFN-induced increase in PBt_2 binding closely correlated with the inhibition of HeLa cell proliferation by IFN-α and the induction of antiviral activity (Fig. 1*B*). For each activity, the effect of IFN-α was observed at concentrations as low as 10 units/ml, with an ED_{50} of ≈100 units/ml.

To determine whether IFN-α altered the distribution of specific PKC isozymes (12), extracts of HeLa cells were analyzed on immunoblots with antisera that selectively detect PKC isoforms α, β, γ, δ, or ε (11). Fig. 2 shows an immunoblot of cytosolic and particulate extracts of HeLa cells alone or treated with IFN-α for 10 min. PKC α, β, and ε were the only species among PKC isozymes detected in HeLa cells, with β being the predominant isoform (data not shown). The β and ε isoforms of PKC were found only in the cytosol of control cells, and PKC α is distributed equally between the cytosol and particulate fraction of control cells. IFN-α treatment resulted in a selective decrease in the cytosolic form of PKC β and an equivalent increase in the particulate form of the isozyme (Fig. 2). IFN-α had no effect on the distribution of PKC α or ε. Treatment with phorbol 12-myristate 13-acetate (PMA; 100 nM for 10 min) resulted in a similar shift in the distribution of PKC β. However, epidermal growth factor (EGF), which is mitogenic in HeLa cells and exhibits no antiviral activity, had no effect on the distribution of PKC β.

Effects of Phorbol Esters on IFN-α Action. Since phorbol esters activate PKC directly, we next investigated whether the activation of PKC may play a role in the biological actions of IFN-α in HeLa cells. HeLa cells were grown in the presence of PMA (0.1 nM to 1 μM). The proliferation of HeLa cells was inhibited dose dependently by PMA at concentrations as low as 0.1 nM (data not shown). As described (34), IFN-α also inhibited the proliferation of HeLa cells in a dose-dependent manner. Although PMA was not antiviral, overnight incubation of HeLa cells with 100 nM PMA, which results in down-regulation of PKC β (data not shown), largely blocked (99.9%) the induction of antiviral activity in HeLa cells against VSV (Table 1).

IFN-α Increases Cellular DAG Content. The physiological activation of PKC generally results from acute increases in cellular DAG content. To evaluate whether the translocation of PKC by IFN-α could be explained by the stimulation of DAG production, HeLa cells were incubated with IFN-α at 37°C and then immediately extracted for determination of DAG mass using the DAG kinase assay (Fig. 3). A 40% increase in DAG mass was observed 5 min after IFN-α addition. By 30 min, DAG returned to basal levels. This correlated well with the data regarding the time course of PKC β translocation (Figs. 1*B* and 2). DAG can be generated directly by phospholipase C or by the action of phospholipase D to yield PA, which is cleaved to DAG by PA phosphohydrolase. To determine whether IFN-α induced the production of PA, HeLa cells were prelabeled with [^{32}P]orthophosphate. At various times after exposure to IFN-α, cells were extracted and the production of [^{32}P]PA was evaluated by TLC. IFN-α caused a 65% increase in the incorporation of ^{32}P into PA within 2 min and declined thereafter (data not shown). Due to the apparent similarities in the time courses of DAG and PA production, these data do not reveal whether PA production precedes the increase in DAG and thus whether these increases represent the activities of phospholipase C, phospholipase D, or both.

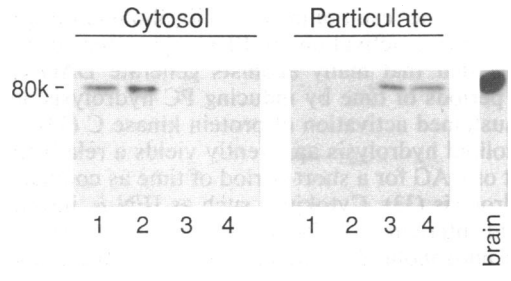

FIG. 2. IFN-α induces translocation of PKC β from the cytosol to the particulate fraction of HeLa cells. HeLa cells were treated with medium only or with EGF (10 nM), IFN-α (1000 units/ml), or PMA (100 nM) for 10 min (lanes 1–4, respectively) and cytosolic and particulate extracts were prepared. The extracts were subjected to SDS/PAGE, proteins were transferred to nitrocellulose, immunoblots were prepared with specific affinity-purified antisera against PKC β, and bands were visualized with ^{125}I-labeled protein A.

Table 1. Effect of PMA on the induction of antiviral activity by IFN-α in HeLa cells

Addition	VSV titer		Fold reduction in viral titer
	Control	Interferon	
None	7.23	3.79	2800
PMA	7.20	5.87	22

HeLa cells were incubated overnight with PMA (100 nM) prior to the addition of IFN-α (250 units/ml). After overnight incubation with IFN, the cells were infected for 1.5 hr with VSV, washed, and incubated with fresh medium, and 24 hr later the virus yield was assayed by plaque formation on mouse L cells. VSV titer was expressed as the $\log_{10}$ virus yield per ml of medium.

Effects of IFN-α on Phospholipid Turnover. To define the pathways that lead to the IFN-α-induced increase in cellular DAG and PA production, we explored the effects of the cytokine on phospholipid turnover. We found that IFN-α does not regulate inositol phospholipid turnover. ^{32}P labeling of inositol phospholipids was not immediately increased by the addition of cytokine, and the production of inositol phosphates was not detected (data not shown). Moreover, in contrast to results obtained by the addition of the calcium ionophore A23187 or platelet-derived growth factor to HeLa cells, IFN-α treatment did not increase free intracellular calcium concentration (19), as determined with both quin-2 and fura-2 as fluorescent indicators (data not shown). Similarly, neither IFN-α nor IFN-β increased calcium exchange or influx in HeLa cells (20). These experiments suggested that IFN-α does not stimulate inositol phospholipid turnover but rather that the increases in DAG and PA are derived from another lipid source.

One likely possibility was that DAG and perhaps PA production resulted from PC hydrolysis, as has been de-

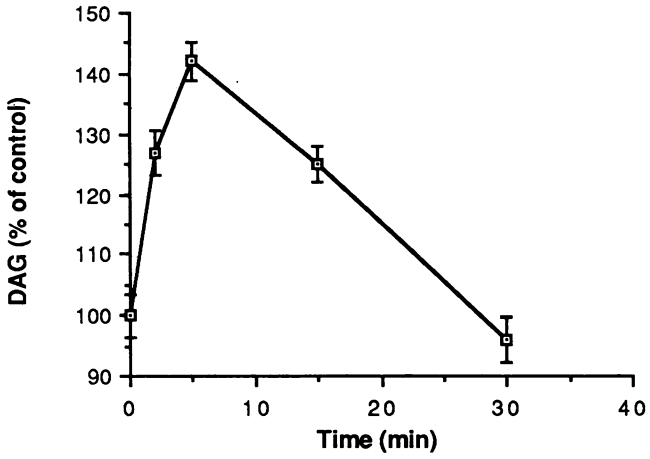

FIG. 3. IFN-α stimulates DAG production. At various times after the addition of IFN-α (1000 units/ml), the DAG mass in the organic phase of an extract of HeLa cells was measured by using *E. coli* DAG kinase (14). DAG mass was obtained from the standard curve and the results (duplicate determinations in two experiments) are expressed as DAG in treated cells as a percentage of DAG in control HeLa cells (which contain ≈1.2 nmol of DAG per 10^6 cells).

scribed for a number of agonists (21–24). To test this possibility, HeLa cells were labeled for 48 hr with [^{3}H]choline; IFN-α was added; at various times after IFN-α addition, the cells were extracted with chloroform/methanol/HCl; and the organic and aqueous phases were separated. Choline phospholipids that partitioned into the organic phase were analyzed by TLC. Treatment of cells with IFN-α caused the rapid hydrolysis of PC (Fig. 4*A*). Incorporation of radioactivity into lipid decreased by 25% within 2 min of IFN addition. The hydrolysis of PC was accompanied by the generation of choline metabolites into the aqueous phase. IFN-α stimulated the generation of intracellular phosphorylcholine within 2 min (Fig. 4*B*). The increase in phosphorylcholine was maximal 5 min after IFN-α addition and then declined thereafter. The release of water-soluble metabolites into the spent medium was also evaluated and the major metabolite was identified as phosphorylcholine by TLC (Fig. 4*C*). IFN-α treatment caused a 30–40% increase in the release of phosphorylcholine into the medium within 10 min. These data suggest that IFN-α stimulates the hydrolysis of PC by a phospholipase C, leading to the production of DAG (Fig. 3) and phosphorylcholine (Fig. 4 *B* and *C*).

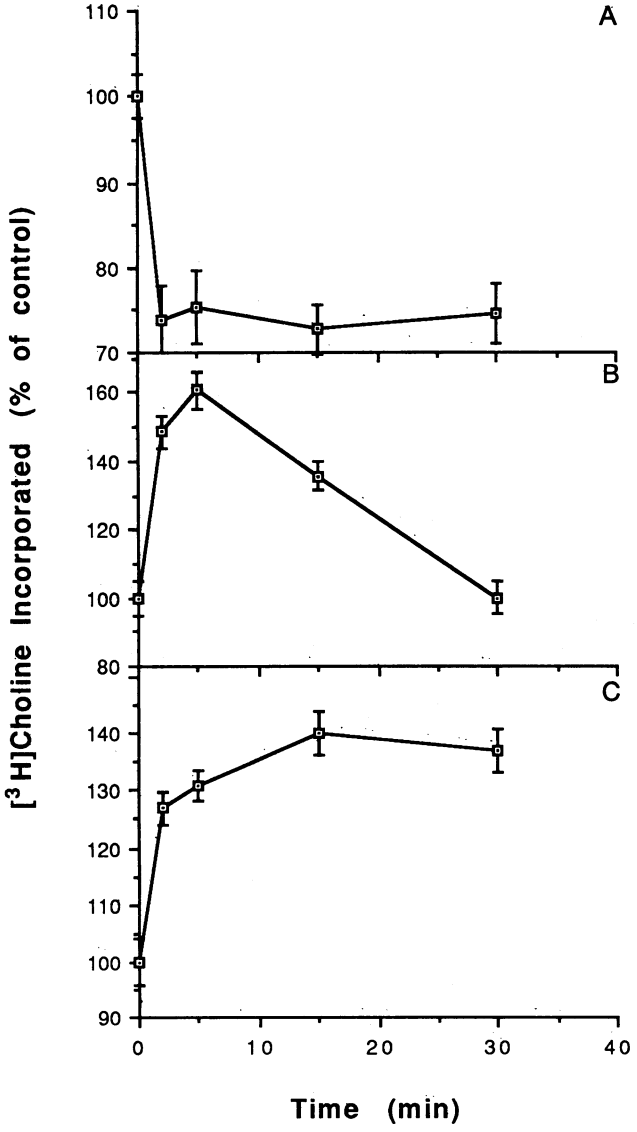

FIG. 4. IFN-α stimulates production and release of phosphorylcholine and hydrolysis of PC in HeLa cells. HeLa cells were labeled with [^{3}H]choline, washed, and then stimulated with IFN-α. At the indicated times, [^{3}H]choline-labeled lipids were analyzed by TLC. (*A*) PC hydrolysis in cell extracts (basal [^{3}H]choline incorporation into PC is 181,000 cpm per 10^6 cells). (*B*) Phosphorylcholine production in cell extracts (basal [^{3}H]choline incorporation into phosphorylcholine is 1300 cpm per 10^6 cells). (*C*) Phosphorylcholine release from HeLa cells (basal [^{3}H]phosphorylcholine release is 4200 cpm/ml of cell suspension).

DISCUSSION

Little progress has been made toward understanding the mechanisms whereby IFN-α–receptor interaction leads to the antiviral and antiproliferative effects of the cytokine. Previous reports have indicated the importance of protein phosphorylation (6, 7) and possibly a role for PKC (25) in IFN-α action. The biological significance of PKC activation by IFN-α is borne out by the findings that phorbol esters mimic the inhibitory action of IFN-α on the proliferation of HeLa cells, and down-regulate PKC activity and block the induction of antiviral activity in HeLa cells by IFN-α. However, the distinctive cellular effects of the agonist dictate that a selective signaling mechanism must exist for IFN-α. For instance, a number of mitogenic factors (i.e., platelet-derived growth factor and EGF) also activate PKC (albeit through inositol phospholipid turnover), although the biological effects of these factors differ from those of IFN-α. These paradoxical findings suggest that there must be some specificity in PKC activation by different agonists leading to distinctive patterns of gene expression. One possibility is that the selective stimulation of PKC isozymes (26, 27) by agonists could provide such specificity. We report herein that IFN-α causes the rapid translocation of the β isozyme of PKC but not of PKC α or ε. Interestingly, PKC β is unaffected by the mitogenic agent EGF.

Although the mechanism whereby IFN-α selectively activates PKC β remains unknown, the results reported here suggest that this specificity may reside in the particular lipid species that is hydrolyzed in response to the cytokine. IFN-α causes the rapid hydrolysis of PC in HeLa cells, with a corresponding increase in cellular DAG levels. DAG production is not accompanied by an increased intracellular calcium concentration, since inositol phospholipids are not hydrolyzed and thus, inositol trisphosphate is not produced in response to this cytokine. Interestingly, *in vitro* studies indicate that the activity of PKC β is highly sensitive to the DAG concentration but is not sensitive to changes in calcium concentrations at physiological conditions (28, 29). The unique signaling pathway utilized by IFN-α for PKC activation in HeLa cells may contribute to the spectrum of biological effects of the cytokine.

Although a number of hormones and growth factors have been shown to stimulate PC hydrolysis through the activation of phospholipase C or D (18, 21, 22, 30, 31), in most cases PC hydrolysis is the result of inositol phospholipid turnover and the subsequent activation of PKC (32). Recent evidence indicates that that many agonists generate DAG for prolonged periods of time by inducing PC hydrolysis and thus cause sustained activation of protein kinase C (33). Inositol phospholipid hydrolysis apparently yields a relatively small amount of DAG for a short period of time as compared with PC hydrolysis (33). Cytokines such as IFN-α, interleukin 1 (23), and interleukin 3 (24) induce PC hydrolysis, but not inositol phospholipid turnover, thus providing a source of DAG without increasing the intracellular free calcium concentration. Identification of the cellular and subcellular distribution, IFN sensitivity and substrate specificity of different PKC isozymes may reveal much about the signal transduction pathways involved in the action of cytokines. This is important as the application of IFNs in the treatment of viral infections and human malignancies is an area of intense investigation.

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