

## Rapid and transient rise in diacylglycerol concentration in Daudi cells exposed to interferon

(phorbol ester/inositol phosphate/calcium/protein kinase C)

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**ABSTRACT** Human  $\beta$ -interferon stimulates a 4-fold increase in the concentration of diacylglycerol and a 2-fold increase in the concentration of inositol monophosphate in Daudi (human B-lymphoblastoid) cells within 30 sec of exposure of the cells to interferon. The increase in diacylglycerol and in inositol monophosphate is transient and the concentrations of these compounds decrease to basal levels within 10 min. Preincubation of human  $\beta$ -interferon with anti-interferon antibodies inhibits this effect as well as the binding of interferon to Daudi cells. Diacylglycerol concentrations were unaffected in mouse A<sub>9</sub> cells (fibroblasts) incubated with human  $\beta$ -interferon and in Daudi cells incubated with human  $\gamma$ -interferon. Mouse A<sub>9</sub> cells are insensitive to human interferon and Daudi cells are insensitive to human  $\gamma$ -interferon. The magnitude of the increase in diacylglycerol concentration stimulated by interferon can be correlated to the interferon-induced inhibition of Daudi cell division in a dose-responsive manner. Phorbol 12-myristate 13-acetate also inhibits Daudi cell division in a dose-responsive manner. It is likely that the sharp and transient increase in diacylglycerol concentration represents one of the early biochemical changes in Daudi cells exposed to interferon.

Although much is known of the biological effects as well as the biochemical reactions associated with interferon *in vitro*, little is known of the role of interferon *in vivo* (1-4). Equally little is known of the mechanism by which interferon produces its effect at the level of its receptor. Presumably, interferon interacts with its receptor, initiating signals from the cell surface into the cell and its nucleus. The transmission of these cellular signals into the nucleus initiates the transcription of specific genes (5-10). The products of these genes convert the cell into an altered physiological state. Alternatively, the interaction between interferon and its receptor itself may be sufficient to produce this state. Recent advances in interferon receptor studies show that there are two types of human interferon receptors. One, which binds human  $\beta$ -interferon, is a product of a chromosome 21 gene (11, 12). Another, which binds human  $\gamma$ -interferon, is a product of a chromosome 6 gene (13).

In this paper, we investigate the early events that occur after Daudi cells are incubated with human  $\beta$ -interferon. We report that a rapid and transient increase in diacylglycerol concentration occurs in Daudi cells within seconds of their exposure to interferon.

### MATERIALS AND METHODS

**Interferons, Anti-Interferon Antibodies, and Cells.** Human  $\beta$ -interferon was prepared in 2-mg batches from primary diploid human fibroblasts by the method of Tan and cowork-

ers (14, 15) and purified to homogeneity by Blue Sepharose column chromatography followed by chromatography on a reversed-phase HPLC column (16). The purity of the interferon was verified by NaDodSO<sub>4</sub>/PAGE. The purified interferon was used as an immunogen to generate antibodies in New Zealand White rabbits as described (17). Recombinant human  $\gamma$ -interferon was a gift from W. Berthold (Dr. Karl Thomae GmbH, Biberach, F.R.G.). Human interferon standards used in this study were GO2-902-527 ( $\beta$ -interferon) and Gg-23-901-503 ( $\gamma$ -interferon) from the National Institutes of Health (Bethesda, MD). Human anti- $\beta$ -interferon was standardized by the radioimmunoassay procedure of Inoue and Tan (18). The cells used in this study were the human B-lymphoblastoid cell line Daudi and the mouse L-cell-derived cell line A<sub>9</sub>. Inhibition of division of Daudi cells by interferon as well as by phorbol 12-myristate 13-acetate (PMA) was assayed as described (19).

**Preparation of Radioiodinated Interferon and its Crosslinking to Daudi Cells.** Human  $\beta$ -interferon was radioiodinated as described (20). The labeled interferon was analyzed by NaDodSO<sub>4</sub>/PAGE followed by autoradiography using a Cronex Lightning Plus intensifying screen and preflashed Kodak XAR film. Labeled interferon of a radiospecific activity of about 2000 cpm/international unit (IU) was used in the binding and crosslinking of interferon to cells. About  $5 \times 10^7$  to  $10 \times 10^7$  Daudi cells were washed twice in Dulbecco's phosphate-buffered saline with Ca<sup>2+</sup> and Mg<sup>2+</sup> (PBS), and the washed cells were used for binding as well as for crosslinking of labeled human  $\beta$ -interferon to Daudi cells as described (20).

**Assay of Diacylglycerol, Inositol Phosphates, Phosphatidylinositol, Phosphatidylinositol 4-Phosphate, Phosphatidylinositol 4,5-Bisphosphate, and Cytosolic Ca<sup>2+</sup> in Daudi Cells.** The assay of diacylglycerol concentrations in Daudi cells was according to the procedure of Habenicht *et al.* (21). Daudi cells were grown in RPMI 1640 medium containing 10% (vol/vol) fetal bovine serum (regular medium) and [2-<sup>3</sup>H]glycerol (Amersham; 6  $\mu$ Ci/ml; 1 Ci = 37 GBq) for about 42 hr. The cells were washed to remove unincorporated labeled glycerol and resuspended in regular medium at  $1.25 \times 10^7$  cells per ml. Aliquots (0.4 ml,  $5 \times 10^6$  cells) in triplicate were dispensed into Eppendorf tubes and subjected to treatment with various concentrations of interferon for the times indicated in the figures. Generally, the range of radioactivity incorporated into the cells was 500,000-800,000 cpm for each set of  $5 \times 10^6$  cells. The cells were extracted for lipid by the method of Bligh and Dyer (22). The extracted lipid with added unlabeled diacylglycerol was quantitatively analyzed by silica gel G thin-layer plastic plate (Eastman Kodak) chromatography. The chromatography was performed in

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Abbreviations: PMA, phorbol 12-myristate 13-acetate; IU, international unit(s).

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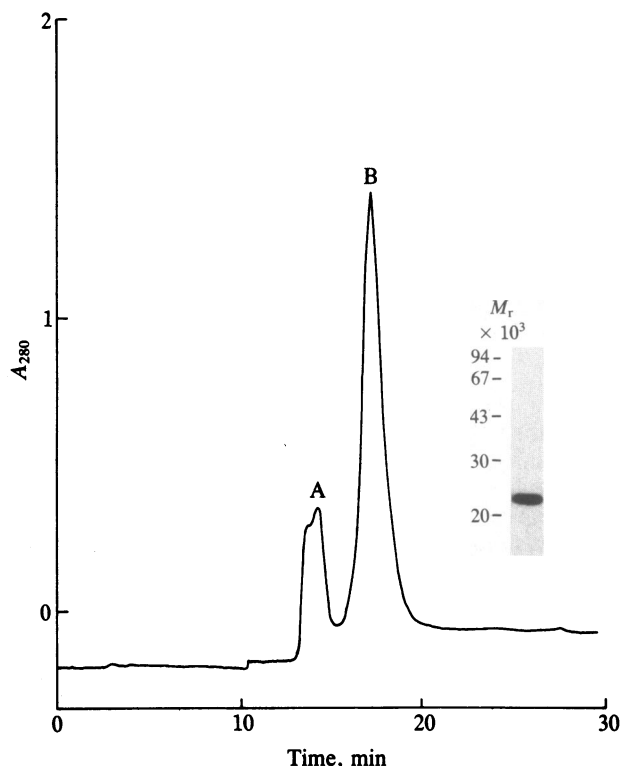


FIG. 1. Purity of HPLC-purified human  $\beta$ -interferon used in these studies. Blue Sepharose-purified interferon was applied to a Brownlee Aquapore RP-300 column and eluted with a propanol/formic acid mixture. Peak A represents human serum albumin and peak B represents biologically active human  $\beta$ -interferon with a specific activity of  $3.0 \times 10^8$  IU/mg of protein. (Inset) An aliquot of the material in peak B was radioiodinated and analyzed by NaDodSO<sub>4</sub>/PAGE followed by autoradiography. Positions of molecular weight markers run in parallel are at left.

Table 1. Inhibition of binding of labeled human  $\beta$ -interferon to Daudi cells by anti-interferon antibodies

Exp.	Treatment	Radioactivity bound, cpm per $10^7$ cells
a	$^{125}\text{I}$ -IFN- $\beta$	$6805 \pm 63$
b	$^{125}\text{I}$ -IFN- $\beta$ + $100\times$ IFN- $\beta$	$3390 \pm 252$
c	$^{125}\text{I}$ -IFN- $\beta$ + $10\times$ anti-IFN	$3441 \pm 428$
d	$^{125}\text{I}$ -IFN- $\beta$ + $3\times$ anti-IFN	$3520 \pm 189$

Triplicate sets of Daudi cells ( $10^7$ ) were incubated with 1000 IU of  $^{125}\text{I}$ -labeled  $\beta$ -interferon ( $^{125}\text{I}$ -IFN- $\beta$ ). The labeled interferon was crosslinked to the cells with 0.1 mM Lomant's reagent as described (20). The amount of labeled interferon bound to the cells was estimated by measuring the amount of radioactivity crosslinked to the cells. Similar experiments were performed in the presence of a 100-fold excess of unlabeled human  $\beta$ -interferon (Exp. b). Exp. a was repeated with 1000 IU of  $^{125}\text{I}$ -IFN- $\beta$  pretreated with a 3-fold (Exp. c) or 10-fold (Exp. d) excess of rabbit anti-interferon serum. Values reported are the means  $\pm$  standard deviations of triplicate samples.

hexane/ether/methanol/acetic acid (180:40:6:4) and the diacylglycerol was stained with iodine vapor and identified on the silica gel plates. The identified diacylglycerol spots were cut out and the radioactivity of these spots was measured with the aid of an LKB Rackbeta counter. The mean and standard deviation of diacylglycerol concentrations was calculated for each set of triplicates. The assay of inositol phosphate concentrations in Daudi cells was according to the method of Berridge *et al.* (23), using *myo*-[ $^3\text{H}$ ]inositol (Amersham). Phosphatidylinositol, phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate were assayed by the method of Cockcroft and Gomperts (24). Comparative levels of cytosolic free  $\text{Ca}^{2+}$  in Daudi cells treated and not treated with interferon were determined by the method of Blackmore and Exton (25), using quin-2 tetrakis(acetoxymethyl) ester (quin-2 AM) purchased from Sigma.

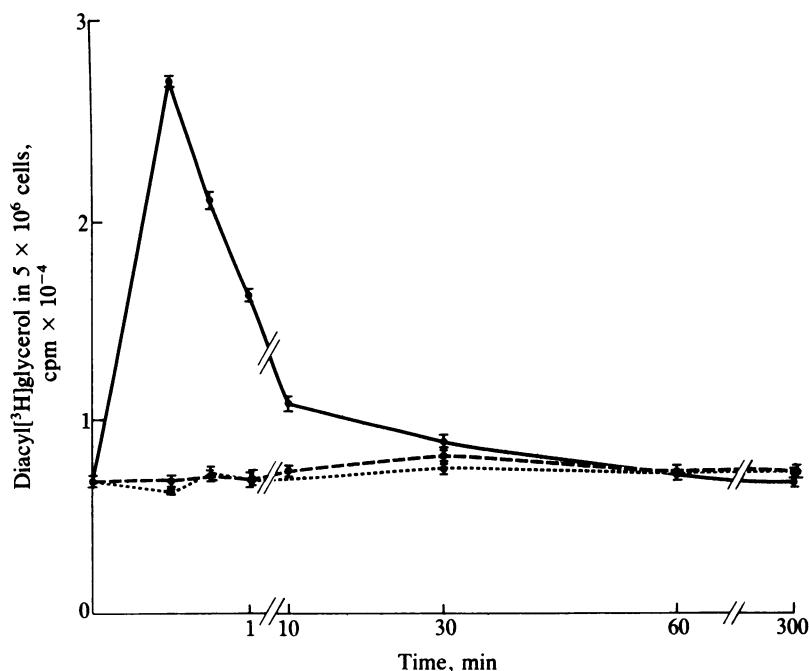


FIG. 2. Determination of the kinetics of the increase in diacylglycerol concentration in Daudi cells as a result of exposure to human  $\beta$ -interferon (20,000 IU/ml). Diacylglycerol was assayed as described in *Materials and Methods*. The concentration of diacylglycerol in cells treated with interferon is indicated by a solid line, the concentration of diacylglycerol in cells treated with an interferon/anti-interferon mixture is indicated by a dashed line, and the concentration of diacylglycerol in cells not treated with interferon is indicated by a dotted line. Values shown are the means  $\pm$  standard deviations for three replicate aliquots of cells.

Table 2. Comparison of the effect of human  $\beta$ - and  $\gamma$ -interferon on the concentration of diacylglycerol in Daudi and mouse A<sub>9</sub> cells

Treatment	Diacyl[ <sup>3</sup> H]glycerol in $5 \times 10^6$ cells, cpm
Daudi cells (0.5 min)	
Control	7,571 $\pm$ 272
$\beta$ -Interferon	29,969 $\pm$ 313
$\gamma$ -Interferon	7,487 $\pm$ 296
Daudi cells (1 min)	
Control	7,521 $\pm$ 2
$\beta$ -Interferon	18,553 $\pm$ 193
$\gamma$ -Interferon	7,737 $\pm$ 46
Mouse A <sub>9</sub> cells (0.5 min)	
Control	4,543 $\pm$ 14
$\beta$ -Interferon (200 IU/ml)	4,773 $\pm$ 76
$\beta$ -Interferon (10,000 IU/ml)	4,606 $\pm$ 485

Daudi cells ( $5 \times 10^6$  in 0.4 ml) were incubated with human  $\beta$ - or  $\gamma$ -interferon (20,000 IU/ml) at 37°C for 0.5 or 1 min as indicated in parentheses. The cells were washed at 4°C to remove the interferon, and the concentration of diacylglycerol in the cells was determined as described in *Materials and Methods*. Similarly, mouse A<sub>9</sub> cells ( $5 \times 10^6$  in 0.4 ml) were incubated with human  $\beta$ -interferon (200 or 10,000 IU/ml) for 0.5 min. Values reported are the means  $\pm$  standard deviations for three aliquots of cells.

## RESULTS

**Purity of Human  $\beta$ -Interferon.** The elution of human  $\beta$ -interferon from a reversed-phase HPLC column is described in Fig. 1, and the purity of the interferon in fraction B is indicated by NaDodSO<sub>4</sub>/PAGE analysis (see Fig. 1 *Inset*).

**Polyclonal Anti-Interferon Antibody Inhibits the Binding of Interferon to its Receptor.** Daudi cells were incubated with saturating levels of <sup>125</sup>I-labeled human  $\beta$ -interferon in the

absence or presence of a 3- and 10-fold excess of anti-interferon antibody, as well as in the absence or presence of a 100-fold excess of unlabeled human  $\beta$ -interferon. Unlabeled human  $\beta$ -interferon competed with <sup>125</sup>I-labeled  $\beta$ -interferon for binding to Daudi cells. Rabbit anti-interferon antibody reduced the binding of <sup>125</sup>I-labeled interferon to Daudi cells (Table 1).

**Increased Diacylglycerol Concentration in Daudi Cells Treated with Interferon.** When Daudi cells were incubated with human  $\beta$ -interferon (20,000 IU/ml) at 37°C, the concentration of diacylglycerol was found to increase within seconds. By 30 sec, the concentration of diacylglycerol was about 4-fold higher than that in cells not treated with interferon. Thereafter, diacylglycerol concentration declined rapidly (within minutes) and was restored to about basal level 10 min after interferon treatment (Fig. 2). This observation is consistently reproducible in Daudi cells incubated with human  $\beta$ -interferon. A rise in diacylglycerol was not observed if the human  $\beta$ -interferon was rendered biologically inactive by preincubation with rabbit anti- $\beta$ -interferon serum (Fig. 2). Human  $\gamma$ -interferon did not stimulate a rise in diacylglycerol concentration in Daudi cells, nor did human  $\beta$ -interferon stimulate an increase in diacylglycerol concentration in mouse A<sub>9</sub> cells (Table 2).

The increase in diacylglycerol concentration was proportional to the concentration of human  $\beta$ -interferon applied to the cells. Concentrations of interferon up to 0.2 IU/ml did not stimulate a significant increase in diacylglycerol in Daudi cells, whereas interferon concentrations exceeding 2.0 IU/ml did. The concentration of human  $\beta$ -interferon required to fully stimulate the production of diacylglycerol was about 200 IU/ml. Further increases in the concentration of interferon up to 20,000 IU/ml did not correspondingly increase the concentration of diacylglycerol (Fig. 3).

**Dose-Dependent Inhibition of Daudi Cell Division by Interferon.** The division of Daudi cells in culture is inhibited by human  $\beta$ -interferon but not by human  $\gamma$ -interferon (data not shown). Concentrations of  $\beta$ -interferon exceeding 2.0 IU/ml

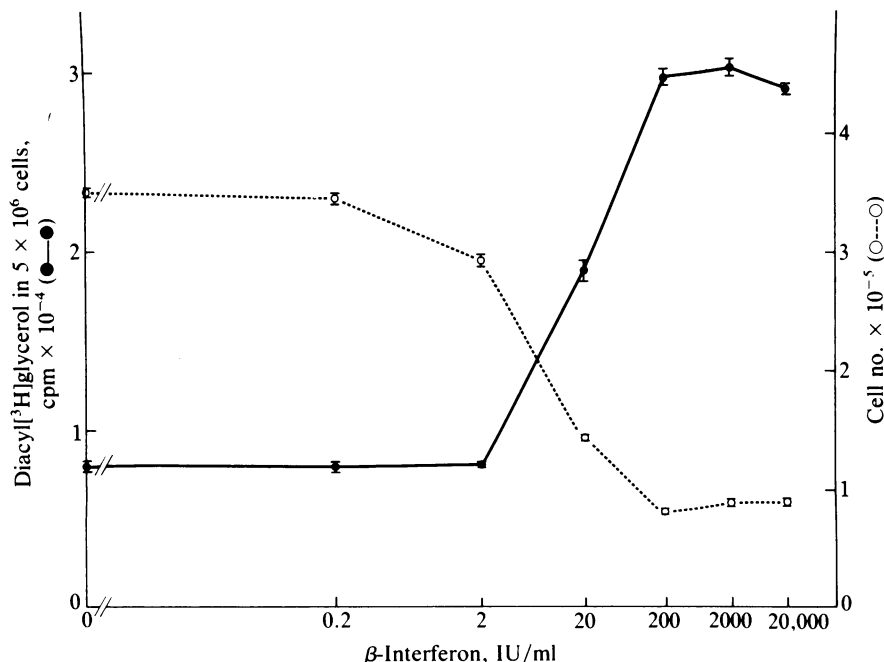


Fig. 3. Correlation between the increase in diacylglycerol concentration in Daudi cells and the inhibition of Daudi cell division at various concentrations of human  $\beta$ -interferon. Cellular diacylglycerol levels are indicated by a solid line. Inhibition of Daudi cell division can be directly estimated by comparing the number of cells counted on day 4 (dotted line) with the number of cells counted on day 0 ( $3.0 \times 10^5$ ). Values reported are the means  $\pm$  standard deviations of triplicate cultures. The diacylglycerol levels in Daudi cells were determined after a 0.5-min incubation with the various concentrations of interferon. Preincubation of human  $\beta$ -interferon with 3-fold excess of anti-interferon abolished the inhibitory effect of interferon (20,000 IU/ml) on Daudi cell division.

Table 3. Inhibition of Daudi cell division by PMA

PMA, nM	Cells per ml
0	153,111 ± 6673
0.3	119,952 ± 4109
3	90,216 ± 2100
30	74,917 ± 2290
300	94,395 ± 4246

Daudi cells ( $2.5 \times 10^4$  per ml) were grown in 25-cm<sup>2</sup> tissue culture flasks in the presence of PMA as indicated. On the fourth day the cells were counted with the aid of a Coulter counter (model ZM). Values reported are the means of viable cell density ± standard deviation for triplicate cultures. At these PMA concentrations, cell viability assessed by trypan blue exclusion was >98%, indicating that PMA does not destroy Daudi cells.

inhibit the division of Daudi cells. Maximal inhibition of cell division was observed at 200 IU/ml. Interferon concentrations higher than 200 IU/ml and up to 20,000 IU/ml did not further inhibit Daudi cell division (Fig. 3).

#### Dose-Dependent Inhibition of Daudi Cell Division by PMA.

Daudi cells were grown in the presence of 0.3, 3, 30, and 300 nM PMA. The division of these cells is inhibited dose dependently by PMA up to 30 nM (Table 3) but not by biologically inactive phorbol or phorbol 13-acetate.

**Measurement of Inositol Phosphates and Cytosolic Free Ca<sup>2+</sup>.** The concentrations of inositol phosphates and cytosolic free Ca<sup>2+</sup> were measured in Daudi cells after the cells were incubated with 20,000 IU interferon per ml. A measurable increase of inositol monophosphate was observed after 30 sec of incubation of Daudi cells with 20,000 IU of human  $\beta$ -interferon per ml (Table 4). However, by 10 min the inositol monophosphate concentration had returned to near the control value (Table 4).

No significant increase of cytosolic free Ca<sup>2+</sup> was detected after up to 1 hr of treatment of Daudi cells with human  $\beta$ -interferon.

## DISCUSSION

Although interferon is known to induce a number of physiological changes in cells, nothing is known of the mechanisms by which interferon transmits its effect from the cell surface into the cell to bring about these changes. The data presented in this paper demonstrate that a few seconds of exposure of Daudi cells to human  $\beta$ -interferon is sufficient to produce a 4-fold increase in diacylglycerol in these cells (Figs. 2 and 3 and Table 2). It is likely that this is an interferon-associated effect (Fig. 1). One of the first reactions between interferon and cells is the binding of interferon to its receptor, resulting in formation of an interferon-receptor complex (20). Preincubation of human  $\beta$ -interferon with its antibody prevents interferon from binding to its receptor sites and thus neutralizes the inhibition of Daudi cell division by interferon (Table 1 and Fig. 3). Similarly, preincubation of interferon with its antibody also abolishes the rapid and transient rise in diacylglycerol concentration in Daudi cells (Fig. 2). In contrast, human  $\gamma$ -interferon has no effect on cell division (data not shown) and does not stimulate an increase in diacylglycerol concentration in Daudi cells (Table 2). There is evidence suggesting that human  $\beta$ - and  $\gamma$ -interferons interact with different receptor sites (11–13, 26), and apparently Daudi cells are insensitive to the biological effects of  $\gamma$ -interferon. The results suggest that the binding of human  $\beta$ -interferon to its putative receptor sites is necessary to stimulate an increase in diacylglycerol in Daudi cells.

The degree of inhibition of cell division was compared to the magnitude of increase in diacylglycerol concentrations in Daudi cells at increasing concentrations of interferon. The degree of inhibition of Daudi cell division corresponds closely

Table 4. Inositol phosphate concentrations in Daudi cells incubated with human  $\beta$ -interferon

	cpm		
	Control	0.5 min	10 min
Inositol monophosphate	813 ± 50	1913 ± 168	880 ± 30
Inositol bisphosphate	83 ± 15	99 ± 7	69 ± 5
Inositol trisphosphate	79 ± 3	90 ± 9	108 ± 6

Daudi cells ( $2 \times 10^7$  cells) prelabeled with *myo*-[<sup>3</sup>H]inositol were exposed to human  $\beta$ -interferon (20,000 IU/ml) for 0.5 or 10 min. Inositol phosphates were assayed by the method of Berridge *et al.* (23) as described in *Materials and Methods*. Values given are radioactivity of [<sup>3</sup>H]inositol phosphate fractions (mean ± standard deviation for three aliquots of cells).

to the size of the increase in cellular diacylglycerol (Fig. 3). Therefore, the interferon-stimulated increase in diacylglycerol concentration can be related to a biological effect of interferon in a dose-responsive manner. It is possible that the effect of interferon on diacylglycerol concentration is mediated through the degradation of cell surface phospholipids, since both diacylglycerol and inositol monophosphate increase within 30 sec of incubation with human  $\beta$ -interferon (Fig. 2 and Table 4). No significant increase of inositol bis- and trisphosphate was detected, but the amount of radioactivity incorporated into these fractions in Daudi cells (Table 4) is too low to eliminate the possibility that such increases occur, because transient increases in the concentration of inositol bis- and trisphosphate are found in primary human fibroblasts exposed to types I and II human interferons (27). No significant changes in the concentration of phosphatidylinositol or phosphatidylinositol 4-phosphate and 4,5-bisphosphate were found in Daudi cells exposed to interferon. It is probable that the rise in diacylglycerol concentration activates protein kinase C in the interferon-treated cell, and that this in turn results in the phosphorylation of yet unidentified protein(s) that, when phosphorylated, are involved in effecting the biological action of interferon on cell division. This hypothesis becomes interesting in the light of the finding that 0.3–30 nM PMA can inhibit the division of Daudi cells in a dose-dependent manner (Table 3). PMA has been reported to mimic the effect of diacylglycerol by activating protein kinase C directly (28). Hamilton *et al.* (29) reported that  $\gamma$ -interferon stimulates protein kinase C activity in macrophages. These results suggest that protein kinase C may play a role in the action of interferon in cells, but the precise biochemical pathway remains unknown. These results also suggest that the action of interferon in Daudi cells probably does not involve an overall change in the concentration of cytosolic free Ca<sup>2+</sup> because no changes in cytosolic Ca<sup>2+</sup> were found during this time period (data not shown). However, it is not possible to rule out the possibility that critical changes of cytosolic free Ca<sup>2+</sup> in the cell may be too small to be detected. In any event, the data indicate that the degradation of phospholipids constitutes one of the early events of interferon action. The identification of the principal enzymes involved in this process is key to the elucidation of the biochemical pathway for the transduction of the interferon signal from the cell surface into the cell.

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