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Phospholipase C and phospholipase A_2 are involved in the antiviral activity of human interferon-α

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Treatment of human amniotic cells (UAC) with human interferon-α (Hu-IFNα) or phorbol myristate acetate (PMA) resulted in translocation of protein kinase C (PK-C) activity from the cytosol fraction to that of the membranes. Analysis of ^{32}P incorporation into phospholipid fractions and studies of alterations in fatty acid content for the major phospholipids of IFN-treated cells suggest that phospholipases C and A_2 are activated by Hu-IFNα . Addition of neomycin (an inhibitor of phospholipase C), as well as mepacrine (an inhibitor of phospholipase A_2) to IFN-treated cells inhibited the antiviral activity of Hu-IFNα in the vesicular stomatitis virus (VSV)-UAC system used. These observations indicate that (i) activation of PK-C and (ii) diacylglycerol formation, arachidonic acid and/or lysophosphatidylcholine release are important steps in the mechanism of action of IFN.

Interferon action; Protein kinase C; Phospholipase C; Phospholipase A_2; Neomycin; Mepacrine

1. INTRODUCTION

It has been suggested that the antiviral action of IFN may be mediated through its effect on PK-C [1]. Observations that different IFNs are capable of inducing an increase in the concentration of diacylglycerol in human fibroblasts [2] and Daudi cells [3,4] support this hypothesis. In addition, a substantial increase in activity of PK-C was demonstrated in macrophages treated with recombinant IFN-γ [5]; intracellular translocation of PK-C and blocking of IFN-γ-induced mRNA synthesis by PK-C inhibitors have also been reported [6]. Synthetic diacylglycerols were shown to be able to induce an IFN-like antiviral state [7].

Although it is generally accepted that tumor-promoting phorbol esters act by substituting for diacylglycerol, reducing the calcium requirement for PK-C [8], it has also been shown that unsaturated fatty acids (oleic acid and arachidonic acid) can activate PK-C independently of Ca^{2+} and phospholipids [9]. Since certain lysophospholipids, particularly lysophosphatidylcholine, are effective stimulators or inhibitors of PK-C [10], it has been proposed that PK-C is dually regulated [11]. One route is mediated by phospholipase C activation leading to diacylglycerol elevation, and the other by phospholipase A_2 activation leading to elevation of *cis* fatty acids or lysophospholipids.

Thus, it was of interest to study the role of PK-C and phospholipases C and phospholipase A_2 in the development of the Hu-IFN α-induced antiviral state.

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2. MATERIALS AND METHODS

Human amniotic cells (from V. Sorrentino, University of Rome) were grown in monolayer cultures using Parker's 199 medium or in suspension cultures using Eagle's medium both containing 10% fetal calf serum.

Hu-IFNα (spec. act. 1.23×10^7 IU/mg protein) was a product of EGIS Pharmacochemical Works (Budapest). 2 mM PMA (4-phorbol 12-myristate 13-acetate, Sigma) in 0.2-ml portions was stored in dimethyl sulfoxide at −20°C and diluted in Dulbecco's phosphate-buffered saline (PBS) before use.

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Neomycin B sulphate was purchased from SIFA and mepacrine (quinacrine) from Sigma.

The Indiana strain of vesicular stomatitis virus (VSV) was plaque-purified and passaged at low multiplicities in UAC cells.

Phospholipid-dependent protein kinase C activity was measured in the cytosol and in detergent-solubilized membrane fraction according to Kikkawa et al. [12] as detailed in [13].

Total lipids were extracted according to Folch et al. [14]. To determine radioactivity incorporated into polyphosphatidylinositols, total lipids were separated on silica gel G plates (20 × 20, Merck) using firstly chloroform/methanol/ammonia (4.3 M) (36:26:8, v/v) then *n*-propanol/ammonia (4.3 M) (6:3, v/v) in the same direction [15]. Detection of spots was performed by spraying with 0.05% 1-aminonaphthalene-8-sulphonic acid in 50% methanol under UV light. Phosphatidylinositol, di- and triphosphatidylinositols were then placed in liquid scintillation vials and counted. For determination of radioactivity in other phospholipids, aliquots of total lipid extract were spotted onto silica gel G plates containing 1.5% magnesium acetate and developed according to Rauser et al. [16] using chloroform/methanol/ammonium hydroxide (65:25:5, v/v) in the first dimension and chloroform/methanol/acetone/acetic acid/water (30:10:40:10:5, v/v) in the second. Lipid fractions were then removed and counted.

For analysis of the fatty acid content of phospholipids, total lipids were separated as described [16], then lipid fractions were transferred to screw-capped vials and transmethylated in the presence of 5% HCl in absolute methanol at 80°C for 3 h. GLC of fatty acid methyl esters was performed on a Hitachi model 263-80 gas chromatograph connected to a Hitachi M263 data processor. Separation was performed on 10% Carbowax 20M coated onto 100–200 mesh Supelcoport (Supelco, Bellefonte, CA).

For virus replication assay, UAC cells were grown in

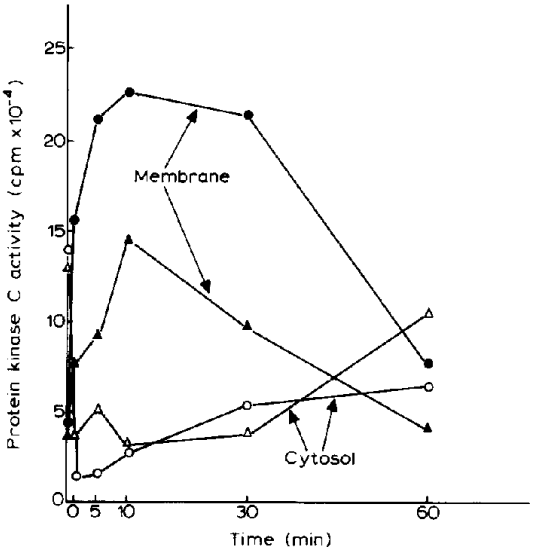

Fig.1. Time course of association of PK-C activity with plasma membrane. Suspension cultures of UAC cells in Eagle's MEM medium containing 10% fetal calf serum were washed in phosphate-buffered saline (PBS). Cells (2×10^7) were then suspended in Eppendorf tubes containing 1 ml PBS and stimulated with either PMA (final concentration 20 nM) or Hu-IFNα (final concentration 1000 U/ml). PMA (●,○), Hu-IFNα (▲, △). Cells were maintained in a water bath at 37°C, removed at the intervals indicated and the PK-C activity of samples assayed as detailed in [13]. PK-C activity was defined as the value observed in the presence of $CaCl_2$ and lipids minus that in the presence of EGTA, being expressed in units of cpm ^{32}P incorporated/2 min per total volume of each cytosolic and membrane preparation.

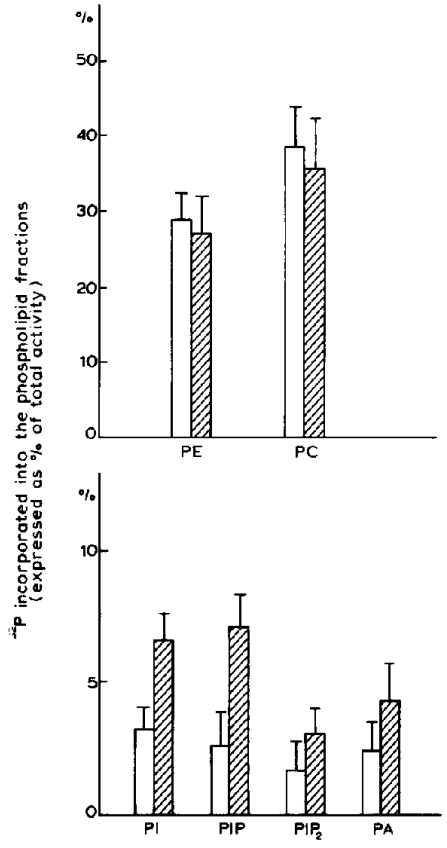

Fig.2. Effect of IFNα on ^{32}P incorporation into phospholipids of UAC cells. Samples of monolayer cultures of UAC cells (2×10^7 cells/tissue culture flask) in Parker's 199 medium containing 2% fetal calf serum were stimulated with 1000 U/ml Hu-IFNα. After 12 h incubation, cells were washed in PBS and labelled with [^{32}P]orthophosphate (40 kBq/ml) for 3 h in Parker's 199 medium containing 2% fetal calf serum. Cells were then harvested and lipid extracts chromatographed as set out in section 2. Phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidic acid (PA) and phosphatidylinositol bands (PI, PIP, PIP_2) were excised from the thin-layer plates and the radioactivity determined by liquid scintillation counting. (Unfilled bars) Control samples, (hatched bars) IFN-treated samples. Mean of three experiments.

monolayer cultures and pretreated for 1 h with 1 mM neomycin or 5 μM mepacrine. After pretreatment, the medium was changed and cells were exposed for 18 h to 1000 IU/ml Hu-IFNα (0.2 ml) as well as to the initial concentrations of neomycin or mepacrine. Cultures were then thoroughly washed in PBS and VSV reproduction capacity examined by studying the cytopathic effect exerted on monolayer cultures of UAC cells in Microtest TC plates (Falcon) 18 h after virus infection, the results being expressed in 50% tissue culture infectious doses ($TCID_{50}$).

3. RESULTS AND DISCUSSION

Fig.1 shows that both Hu-IFNα and PMA induce translocation of PK-C activity from the cytosol to the membrane fraction. It can also be seen that PMA-stimulated membrane association of PK-C is more rapid and of a higher intensity than that with IFN. These findings are consistent with our previous studies demonstrating an IFN-like antiviral effect of PMA [1].

Since PK-C is activated by diacylglycerol generated through the signal-dependent breakdown of inositol phospholipids induced by phospholipase C, the effect of Hu-IFNα on ^{32}P incorporation into the phospholipids was studied. Fig.2 shows that stimulation of UAC cells with Hu-IFNα significantly increases ^{32}P incorporation into phosphatidylinositol fractions and phosphatidic acid. In contrast, ^{32}P incorporation into the phosphatidylcholine and phosphatidylethanolamine fractions was not altered. Thus, it can be concluded that IFN treatment has no effect on [^{32}P]orthophosphate uptake but does increase the turnover of inositol phospholipids by activating phospholipase C.

The effects of IFN on fatty acid content of all major phospholipids were also studied (table 1). IFN induced significant alterations in the fatty acid composition of phosphatidylinositol. These changes, in accordance with the results in fig.2, also support the conclusion that IFN treatment activates phospholipase C. No significant changes occurred in the major fatty acids of other phospholipids except for the altered arachidonic acid (20:4) content of phosphatidylcholine. This alteration cannot be explained on the basis of an increase in the activity of phospholipase C but indicates activation of phospholipase A_2.

To determine whether activation of phospholipases C and A_2 is indeed involved in the generation of the antiviral state induced by Hu-IFNα, inhibitors of these enzymes were employed. Neomycin is known to block the hydrolysis of phosphoinositides [17,18] and mepacrine has been reported to inhibit phospholipase A_2 without affecting phospholipase C [19,20]. Fig.3 shows that neomycin inhibits the antiviral effect of IFN in a

Table 1

Fatty acid composition of phospholipids of control and IFN-treated human amnion cells

Phospholipid	Treatment	Fatty acid (% of total)										
		14:0	i.16:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4	20:5	22:6
Total phospholipid	control	2.3	3.0	18.8	4.3	3.7	40.2	2.3	0.7	9.4	0.8	0.5
	IFN-treated	2.3	3.7	21.2	4.7	4.4	41.7	1.9	0.7	9.0	0.5	–
Phosphatidylcholine	control	3.6	1.0	28.8	5.0	0.6	47.5	1.3	0.3	3.5	0.5	0.7
	IFN-treated	4.0	1.2	30.9	6.0	0.4	46.4	1.3	0.3	1.7	0.3	0.3
Phosphatidylethanolamine	control	1.8	8.6	7.7	2.3	4.5	35.0	1.3	–	19.8	2.4	2.8
	IFN-treated	2.1	8.0	7.5	2.3	3.9	36.5	1.4	0.2	20.0	2.6	2.8
Phosphatidylserine	control	–	–	4.0	2.3	22.3	35.8	1.2	0.3	10.7	1.9	1.2
	IFN-treated	–	–	4.7	3.3	24.7	40.6	1.4	–	11.9	2.4	0.6
Phosphatidylinositol	control	–	–	17.3	6.6	4.0	20.2	0.8	–	7.0	0.5	0.6
	IFN-treated	–	–	9.3	2.3	2.1	40.4	1.8	0.3	16.2	2.4	4.0

UAC cells (10^6/ml) suspended in Eagle's MEM containing 10% fetal calf serum were grown overnight in magnetically stirred vessels. The medium was then replaced by Eagle's MEM containing 2% fetal calf serum; cell samples were then treated with 1000 IU/ml Hu-IFNα for 12 h and, after centrifugation at 2000 × g for 10 min, cells (2×10^8/sample) were processed for lipid analysis (see section 2). Identification of peaks was performed using authentic standards. Errors in determinations were <2% and 5% for the major and minor fatty acids, respectively. Integrated peak areas of all fatty acids on the chromatogram were taken as the total (100%). Experiment performed 3 times, with the data being from a representative experiment

dose-dependent manner. This result is in good agreement with findings that the antiviral activity of human IFNα and β in human embryo fibroblasts can be also blocked with neomycin [7]. A similar phenomenon was observed when mepacrine was used (fig.3). Fig.3 depicts the results obtained in experiments where the effect of inhibitors proved to be maximal. It should be stressed, however, that the inhibitory effects are dependent on the actual concentration of IFN used. Neomycin and mepacrine were also effective when added 1 h after IFN (not shown), indicating that these agents do not act by blocking adsorption of IFN to cell surface receptors.

Apparently, the data obtained are in support of the hypothesis that activation of PK-C is involved in generating the effects of IFN [21,22]. It was also demonstrated that treatment of UAC cells with IFNα resulted in alterations in fatty acid content of phospholipids similar to those in the mouse IFNβ-S-180 mouse sarcoma cell system [23]. Since addition of neomycin and mepacrine partially inhibits the antiviral activity of IFN, it is reasonable to conclude that activation of phospholipases C and A_2 is involved in the action of IFN. Since these enzymes are associated with the formation of diacylglycerol and release of arachidonic acid and/or lysophosphatidylcholine, the participation of the latter mechanisms in the action of IFN, as supposed in previous studies [2–4,24], can be considered to be most likely.

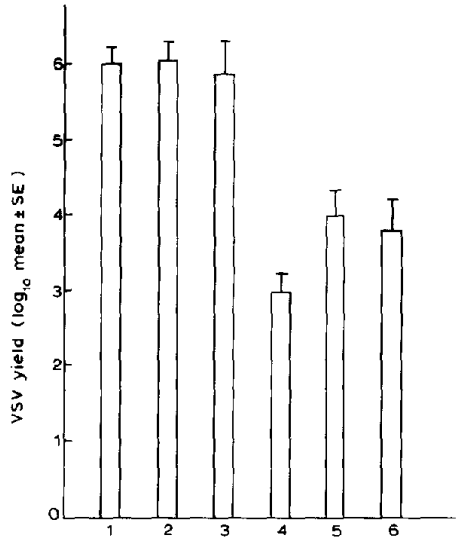

Fig.3. Effect of neomycin and mepacrine on the antiviral activity of Hu-IFNα. Results are presented as means of VSV yield/0.1 ml $\log_{10}$ ± SE for three experiments as measured by the cytopathic effect (see section 2). (1) Control, VSV-infected UAC cells. VSV-infected cells pretreated with: (2) 1 mM neomycin; (3) 5 μM mepacrine; (4) 1000 IU/ml Hu-IFNα; (5) 1000 IU/ml Hu-IFNα and 1 mM neomycin; (6) 1000 IU/ml Hu-IFNα and 5 μM mepacrine.

REFERENCES

[1] Premecz, G., Markovits, A. and Földes, I. (1985) FEBS Lett. 180, 300–302.
[2] Yap, W.H., Teo, T.S. and Tan, Y.H. (1986) Science 234, 355–358.
[3] Yap, W.H., Teo, T.S., McCoy, E. and Tan, Y.H. (1986) Proc. Natl. Acad. Sci. USA 83, 7765–7769.
[4] McCoy, E. and Strynadka, K. (1987) Progress in Clin. and Biol. Res., vol.246, Oncology and Immunology of Down Syndrome, pp.221–230, A.R. Liss, New York.
[5] Hamilton, T.A., Becton, D.L., Somers, S.D., Gray, P.W. and Adams, D.O. (1985) J. Biol. Chem. 260, 1378–1381.
[6] Xue-Dong, F., Goldberg, M. and Bloom, B.R. (1988) Proc. Natl. Acad. Sci. USA 95, 5122–5125.
[7] Cernescu, C., Constantinescu, St.N., Balta, F., Popescu, L.M. and Cajal, N. (1988) Biochem. Biophys. Res. Commun. 151, 402–407.
[8] Nishizuka, Y. (1984) Nature 308, 693–698.
[9] Murakami, K. and Routtenberg, A. (1985) FEBS Lett. 192, 189–193.
[10] Oishi, K., Raynor, R.L., Charp, P.A. and Kuo, J.F. (1988) J. Biol. Chem. 263, 6865–6871.
[11] Routtenberg, A. (1987) J. Neural. Transm. 24, 239–245.
[12] Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S. and Nishizuka, Y. (1982) J. Biol. Chem. 257, 13341–13348.
[13] Premecz, G., Markovits, A., Bagi, G., Farkas, T. and Földes, I. (1987) FEBS Lett. 226, 13–16.
[14] Folch, J., Lees, M. and Sloane-Stanley, G.H. (1961) J. Biol. Chem. 226, 497–509.
[15] Christopher, L.K., Bailey, J., Schoenbraun, L. and Kinsey, W. (1985) Lipids 20, 350–356.
[16] Rouser, G., Fleisher, S. and Yamamoto, A. (1970) Lipids 5, 494–496.
[17] Downes, C.P. and Michell, R.H. (1981) Biochem. J. 198, 133–140.
[18] Carney, D.H., Scott, D.L., Gordon, E.A. and LaBelle, E.F. (1985) Cell 42, 479–488.
[19] Lapetina, E.G., Billah, M.M. and Cuatrecasas, P. (1981) J. Biol. Chem. 256, 5037–5040.
[20] McGivney, A., Morita, Y., Crew, F.T., Hirata, F., Axerrod, J. and Siraganian, R.P. (1981) Arch. Biochem. Biophys. 212, 572–580.
[21] Tamm, I., Lin, S.L., Pfeffer, L.M. and Sehgal, P.B. (1987) in: Interferon 9 (Gresser, I. ed.) pp.13–74, Academic Press, London.
[22] Premecz, G., Markovits, A. and Földes, I. (1987) Acta Microbiol. Hung. 34, 45–55.
[23] Chandrabose, K. and Cuatrecasas, P. (1981) Biochem. Biophys. Res. Commun. 98, 661–668.
[24] Fuse, A., Mahmud, I. and Kuwata, T. (1982) Cancer Res. 42, 3209–3214.