Volume 248, number 1,2, 73–77 FEB 07110 May 1989

The roles of protein kinase C and cyclic nucleotide dependent kinase in signal transduction in human interferon γ induction by poly I:poly C

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Received 13 March 1989

The signal transduction mechanisms involved in interferon (IFN) γ induction in human peripheral mononuclear lymphocyte nylon-nonadherent cells (NNA cells) by stimulation with poly(I):poly(C) are investigated. Significant enhancement of IFN γ production by poly(I):poly(C) is observed in the presence of the phorbol ester, 12-O-tetradecanoylphorbol 13-acetate (TPA, a protein kinase C (PKC) activator). Our study shows that in NNA cells, poly(I):poly(C) with or without TPA causes prolonged activation of cytosolic PKC of NNA cells for at least 120 min. The level of activation of PKC is quite remarkable in the case of the combined stimulation by poly(I):poly(C) and TPA as compared to poly(I):poly(C) alone. This demonstrates that prolonged activation of cytosolic PKC for at least 120 min is essential for high levels of production of IFN γ. Moreover, inhibition experiments using the PKC inhibitor H-7 and cAMP-dependent protein kinase inhibitor H-8 suggest that the mechanism of signal transduction with regard to PKC is involved in stimulation of IFN γ production in NNA cells by poly(I):poly(C) in the presence of TPA and that along with PKC, cAMP-dependent protein kinase is probably involved in induction of IFN γ by stimulation with poly(I):poly(C) alone.

Interferon γ; Poly(I):poly(C); Protein kinase C; cyclic AMP

1. INTRODUCTION

Two distinct major classes of intracellular signaling in response to extracellular stimulation have been established thus far. One type, mediated by activation of adenylate cyclase, results in an increase in cellular cAMP. cAMP leads to activation of cAMP-dependent protein kinase [1,2]. The other regulates PI turnover and diacylglycerol, a degradation product of PI, results in PKC activation [3,4].

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Abbreviations: cAMP, adenosine 3′,5′-cyclic monophosphate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; IFN, interferon; PKC, protein kinase C; NNA cells, nylon-nonadherent cells; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride; H-8, *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride; PI, phosphatidylinositol; PBML, peripheral blood mononuclear lymphocyte

Recently, it has been reported that some cytokines are induced in several cells accompanied by increases in intracellular cAMP levels [5,6]. Moreover, in some cells, a number of biological responses are evoked, correlated with activation of PKC due to various stimuli [7,8]. Concerning induction of IFN γ, the mechanisms of intracellular signaling involved in IFN γ gene expression remain unknown.

We have previously shown that human peripheral mononuclear lymphocyte NNA cells produce IFN γ on stimulation with poly(I):poly(C) [9]. It was proved that PKC activators, such as TPA, could greatly enhance IFN γ production by poly(I):poly(C) as in other cases of induction of IFN γ using lectins or mitogens [10,11].

For the purpose of investigating the signal-transducing mechanisms involved in induction of IFN γ by poly(I):poly(C), we performed inhibition experiments on IFN γ induction using the PKC inhibitor H-7 and cAMP-dependent protein kinase

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

inhibitor H-8, also examining changes in PKC activities and cAMP levels following stimulation of IFN γ induction. Here, we show that a somewhat longer plateau peak for activation of cytosolic PKC occurs in the case of poly(I):poly(C) stimulation, with or without TPA. We also provide evidence that the relative levels of activation of PKC closely agree with the relative magnitudes of the induced IFN γ titers. We also describe the rather gradual degradation pattern of intracellular cAMP levels occurring in the case of stimulation by poly(I):poly(C) alone as compared with that of a combination of poly(I):poly(C) and TPA, and demonstrate that different pathways of signal transduction may operate between stimulation with poly(I):poly(C) alone and with a combination of poly(I):poly(C) and TPA.

2. MATERIALS AND METHODS

2.1. *Cell separation*

Human peripheral blood mononuclear lymphocytes (PBML) were isolated from whole blood of healthy donors by Ficoll-Hypaque (Pharmacia LKB Biotechnology) gradient centrifugation. PBML at 2×10^6 cells/ml in RPMI 1640 medium supplemented with heat-inactivated fetal bovine serum (HyClone, Logan, UT) were incubated at 37°C for 60 min in tissue culture plastic dishes (Corning, 25010, NY), and nonadherent cells were carefully collected. NNA cells were separated by incubating cells not adhering to plastic dishes in a nylon column at 37°C for 60 min. After elution with the above medium, nonadherent cells were washed and collected for IFN γ induction.

Table 1

Effect of TPA on IFN γ induction by poly(I):poly(C)

Stimuli	IFN titer (U/ml)
None	<6
TPA (10 ng/ml)	
2 h	<6
24 h	90
Poly(I):poly(C) (200 μg/ml)	
2 h	1530
24 h	3200
Poly(I):poly(C) + TPA	
2 h	15600
24 h	17600

NNA cells were induced by using poly(I):poly(C), TPA or poly(I):poly(C) with TPA as indicated. In the case of 2 h induction, cells were washed three times, resuspended in fresh medium and incubated for a further 22 h

2.2. *IFN induction and titration*

NNA cells at 5×10^6 cells/ml in the above medium were treated with poly(I):poly(C) (PL Biochemicals, Milwaukee, WI) in the presence of TPA (Sigma) or various other test reagents. After incubation for 20–24 h at 37°C, supernatants were collected and assayed for IFN titer.

IFN assays were carried out in FL cells using Sindbis virus for challenging. IFN titers are expressed in international units (IU), based on reference standards for human IFN γ (G-023-901-530) supplied by NIH.

2.3. *Determination of cAMP*

Approx. 5×10^6 NNA cells were treated with stimulatory reagents for different periods at 37°C, followed by centrifugation and boiling of the cellular pellets in 50 mM sodium acetate buffer (pH 4.0) containing 0.2 mM isobutylmethylxanthine (IBMX). Supernatant fractions from these samples were then subjected to cAMP radioimmunoassay using a cAMP RI kit (DuPont-New England Nuclear) as in [12]. Forskolin (Sigma) was used as the control reagent for adenylate cyclase activation resulting in cAMP accumulation.

2.4. *Assay of PKC activities*

PKC activities were determined as described by Kikkawa et al. [13]. Briefly, 10^7 NNA cells were treated with test reagents for various periods at 37°C, and then washed three times with serum-free RPMI 1640/1 mM sucrose. Cellular pellets were lysed in 50 μl distilled water and added to 500 μl lysis buffer [20 mM Tris-HCl (pH 7.5)/2 mM EDTA/0.5 mM EGTA/10 μg/ml aprotinin/0.33 M sucrose/2 mM phenylmethylsulfonyl fluoride]. After sonication for 15 s at 4°C with lysis

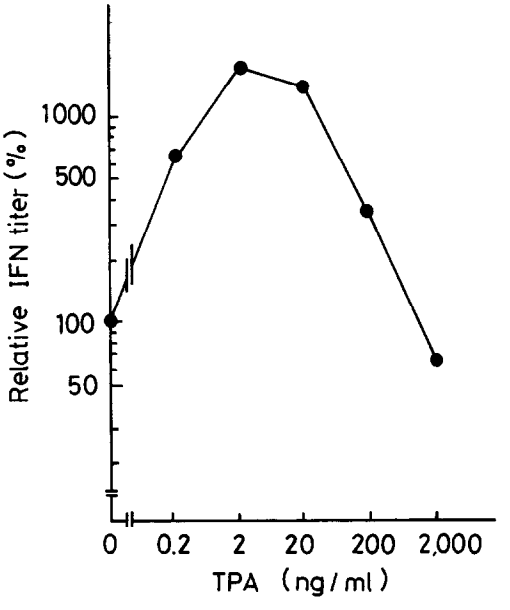

Fig.1. Effect of TPA on IFN γ induction of NNA cells by poly(I):poly(C) NNA cells were induced by poly(I):poly(C) (200 μg/ml) for 24 h in the presence of the indicated doses of TPA. Data are expressed as the percent of each titer.

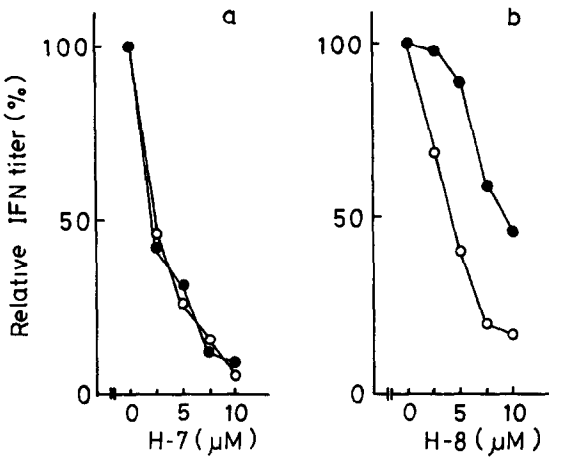

Fig.2. Dose responses of protein kinase inhibitors for inhibition of IFN γ induction. NNA cells were induced for 24 h by 200 μg/ml of poly(I):poly(C) (○) or poly(I):poly(C) with 10 ng/ml of TPA (●) in the presence of the indicated doses of H-7 (a) or H-8 (b). Data are expressed as the percent of each IFN titer.

buffer, cytosol and particulate membrane fractions were prepared by centrifugation at 100000 × g for 60 min. Centrifuged membrane fractions were homogenized using a glass rod in 1% Nonidet P-40 containing lysis buffer, gently rotated for 30 min at 4°C, and centrifuged at 12000 rpm for 15 min. Aliquots (50 μl) were assayed for PKC activities in a medium comprising 2 mM $CaCl_2$/10 mM Mg acetate/100 μM [γ-^{32}P]ATP (spec. act. 3000 Ci/mmol (New England Nuclear), 50 μg H1 histone/5 μg phosphatidylserine/1 μg diolein. The assay mixture was incubated for 10 min at 30°C and the reaction was stopped on ice by adding 10% trichloroacetic acid. The acid-insoluble materials were collected on GF/C and the radioactivity was measured by liquid scintillation counting.

3. RESULTS

The production of IFN γ by poly(I):poly(C) in NNA cells was markedly stimulated in the presence of TPA (10 ng/ml) (table 1). We also examined the effect of different doses of TPA on IFN γ production by poly(I):poly(C) (fig.1). In the presence of high doses of TPA, the production of IFN γ by poly(I):poly(C) was strongly inhibited (down-regulation of PKC) [14].

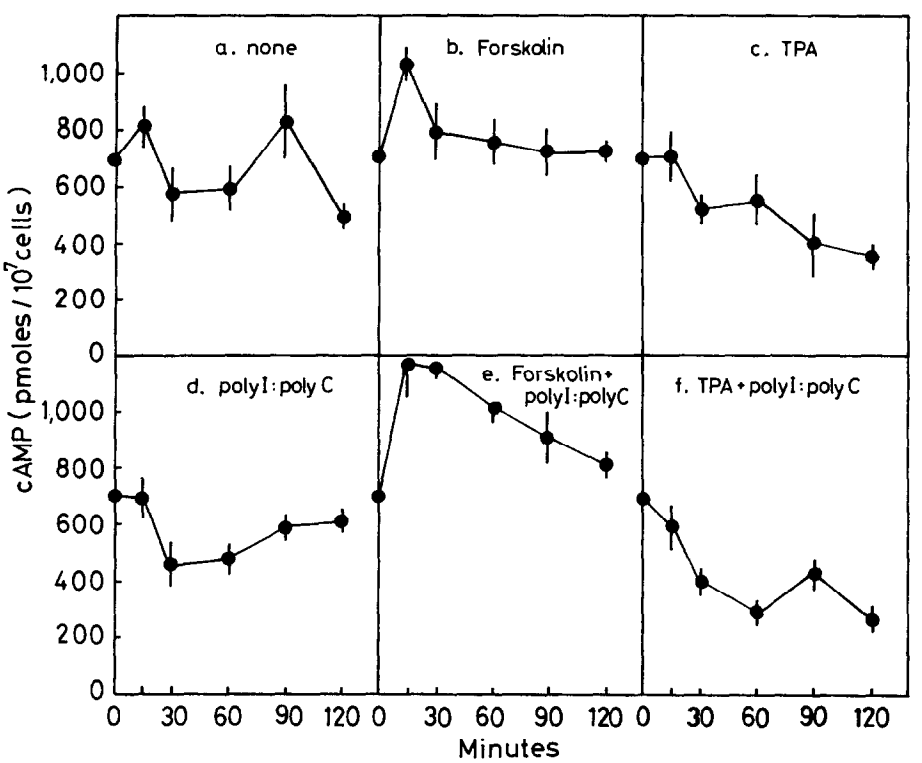

Fig.3. Intracellular cAMP accumulation of NNA cells after treatment with various stimuli. NNA cells were stimulated with forskolin (1 μg/ml), TPA (10 ng/ml), poly(I):poly(C) (200 μg/ml), forskolin with poly(I):poly(C) and TPA with poly(I):poly(C) for varying periods as indicated at 37°C. Intracellular cAMP accumulation was then quantitated as described.

The PKC inhibitor H-7 reduced IFN γ production by poly(I):poly(C) with or without TPA (fig.2a). Another protein kinase inhibitor, namely H-8, which is known to inhibit cyclic-nucleotide-dependent protein kinase, inhibited IFN γ production by poly(I):poly(C), however, the degree of inhibition was lower in the case of poly(I):poly(C) induction in the presence of TPA (fig.2b).

To determine whether a correlation exists between cellular cAMP levels and the efficiency of IFN γ induction, we determined cellular cAMP levels after treatment with test reagents (fig.3). In the case of stimulation by forskolin, used as a control for adenylate cyclase activation, a transient increase in cAMP levels was observed (fig.3b). Although no increase in cAMP levels was noted with stimulation by poly(I):poly(C) (fig.3d), combined stimulation by poly(I):poly(C) and forskolin resulted in cAMP levels increasing and decreasing rather slowly in comparison with stimulation by forskolin present alone (fig.3b,e). Moreover, the drop in cAMP levels is also less steep in the case of stimulation with poly(I):poly(C) present alone. TPA stimulation with or without poly(I):poly(C) did not elevate cAMP levels (fig.3c,f).

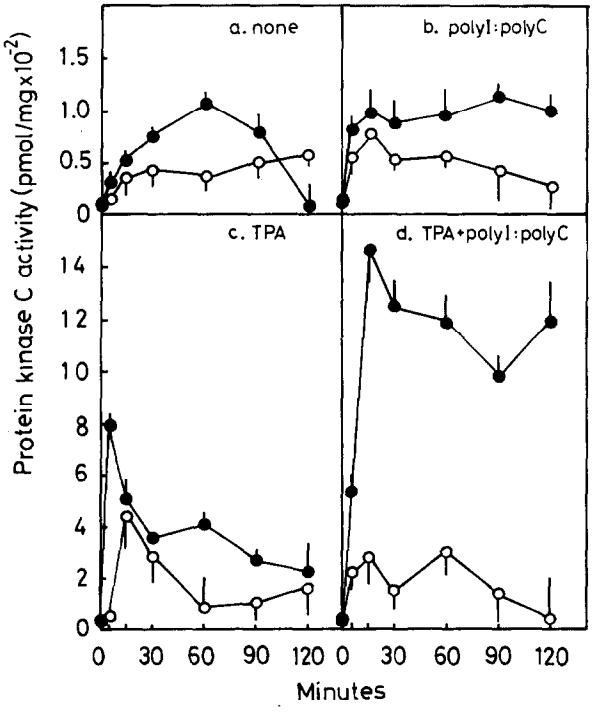

Fig.4. Protein kinase C activity in cytosolic and particulate fractions of NNA cells. NNA cells were treated with poly(I):poly(C) (200 μg/ml), TPA (10 ng/ml) and poly(I):poly(C) with TPA for different periods as indicated at 37°C. Cells were then harvested and separation of cytosolic (●) from particulate (○) fractions was performed. PKC activities were assayed as described.

Subsequently, we investigated the PKC activities in cytosolic and particulate fractions of NNA cells after treatment with stimulatory reagents (fig.4). Cytosolic PKC was activated 3–5 min after addition of poly(I):poly(C), TPA or a combination of both. The degree of activation was extraordinarily large on combined stimulation by poly(I):poly(C) and TPA (fig.4d). Although rapid reduction of cytosolic PKC activities was observed in the case of stimulation with TPA alone (fig.4c), continuous activation of PKC for at least 120 min was noted for combined stimulation by TPA and poly(I):poly(C). In the case of stimulation by poly(I):poly(C) present alone, the level of activation was much less than that of TPA, however continuous activation of cytosolic PKC was also observed for at least 120 min (fig.4b). We found no remarkable differences in the changes in PKC activities of particulate fractions responding to test reagents. Only in the case of stimulation by TPA was a transient increase in PKC activity seen.

4. DISCUSSION

We have demonstrated that poly(I):poly(C), with or without TPA, causes prolonged activation of cytosolic PKC for at least 120 min. The level of activation is approx. 10-times higher in the case of stimulation by a combination of poly(I):poly(C) and TPA compared to poly(I):poly(C) alone. The magnitude of this effect shows a good correlation between the relative IFN γ titers for stimulation by poly(I):poly(C) with TPA and by poly(I):poly(C) alone (table 1).

From the inhibition experiments showing that IFN γ induction by poly(I):poly(C) is markedly inhibited by the PKC inhibitor H-7 irrespective of whether TPA is present, the conclusion that PKC must be involved in signal-transducing mechanisms in IFN γ induction is demonstrated to be valid.

Conversely, cellular cAMP probably plays no role in the induction of IFN γ by poly(I):poly(C) in the presence of TPA, since no detectable increase

in cAMP was observed for such stimulation. Moreover, the cAMP-dependent protein kinase inhibitor H-8 has a much less inhibitory effect on IFN γ induction by poly(I):poly(C) in the presence of TPA.

On the other hand, in the case of stimulation by poly(I):poly(C) alone, PKC is surely involved in the signal-transducing mechanisms of IFN γ induction, however, the participation of cAMP dependent protein kinase cannot be ignored, since H-8 also inhibits IFN γ induction for the case of poly(I):poly(C) present alone. The evidence that decreases in cellular cAMP are gradual in stimulation by poly(I):poly(C) alone vs TPA alone or a combination of poly(I):poly(C) with TPA may be in support of the possible participation of cAMP-dependent protein kinase in the signal-transducing mechanisms for IFN γ induction by poly(I):poly(C).

Further evidence that PKC is involved in induction of IFN γ by poly(I):poly(C) is that a high dose (2 μg/ml) of TPA decreases IFN γ production by poly(I):poly(C). This effect is referred to as down-regulation of PKC by TPA.

The results in table 1 demonstrate that 2 h stimulation by poly(I):poly(C) with or without TPA is almost sufficient for IFN γ induction. Our data show that activation of cytosolic PKC is sustained in the presence of poly(I):poly(C) for at least 2 h so that, during this period, activated PKC probably reaches a level sufficient to stimulate IFN γ gene expression. Also, the level of activation of cytosolic PKC correlates well with the inducibility of IFN γ by each stimulus.

Acknowledgement: We thank Dr E. Tokunaga, a Director of the Japanese Red Cross, Central Blood Center, for his help in this research.

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