Antiviral Research, 1 (1981) 185–192
Elsevier/North-Holland Biomedical Press

EFFECT OF INTERFERON ON CYCLIC ADENOSINE MONOPHOSPHATE LEVELS IN CELLS

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(Received 26 January 1981; accepted 15 June 1981)

Treatment of murine and human cells with homologous interferon (IFN) resulted in an elevation of the cellular cyclic adenosine monophosphate (cAMP) levels in a time- and dose-dependent manner. A similar elevation of cAMP levels could be produced by treatment of cells with isoproterenol, prostaglandin or methylxanthine. However, these agents did not produce an antiviral state. Pretreatment with an inhibitor of adenyl cyclase, *N*-ethylmaleimide, prevented the effect of IFN on cAMP levels, but did not influence its antiviral activity.

interferon antiviral activity cyclic AMP

The establishment of an antiviral state in cells by interferon (IFN) treatment requires binding of IFN to plasma membrane receptors [6, 20, 21]; this process is followed by alterations of the cell surface and membrane itself [7]. It has not been convincingly demonstrated that IFN actually enters the cells, and it may be that contact with the cell membrane is a sufficient stimulus for development of the antiviral state [2], in which case the IFN-produced membrane alterations might be expected to trigger a second messenger or messenger system.

It has been proposed that cyclic 3′,5′-adenosine monophosphate (cAMP), through activation of the adenyl-cyclase system, could be involved in such a secondary messenger system. Addition of the cAMP analog, dibutyl-cAMP enhanced the antiviral activity induced by IFN [1, 8, 13, 22] and IFN treatment of sensitive cells resulted in an increase in intracellular cAMP levels, which preceded the development of the antiviral state [16, 17]. In IFN-insensitive heterologous cells neither cAMP level nor sensitivity to virus were altered by IFN treatment [17].

In the present study, we confirmed the observation that cAMP levels are altered following IFN treatment and have attempted to evaluate the role of this phenomenon in the development of the antiviral state.

Cells were grown in monolayers with Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum. L-929 cells (an IFN-sensitive cell line) and KB cells (a line from a human epidermoid carcinoma) were obtained from the American

Type Culture Collection (Rockville, MD). A line of murine osteogenic sarcoma cells (OGS) was established in C57B1/6 mice in our laboratory with ^{237}Pu irradiation of mice [9]. Diploid human foreskin fibroblasts (HFF) were derived in our laboratory, and used in their 20th–30th in vitro passage.

Isoproterenol (IP), prostaglandin E_1 (PG), 3-isobutyl-1-methylxanthine (MX), *N*-ethylmaleimide (N-em) and cyclic 3′,5′-adenosine monophosphate (cAMP) were purchased from Sigma Chemical Company (St. Louis, MO). For each experiment fresh working solutions were made in MEM.

Murine type I IFN, produced by Dr. K. Paucker (Medical College of Pennsylvania, Philadelphia, PA), was received from the Antiviral Program of the National Institutes of Allergy and Infectious Diseases, Bethesda, MD. The specific activity of this preparation was 1.3×10^7 units/mg protein. Partially purified human leukocyte interferon (HuIFN-α) was generously given to us by Dr. K. Cantell (State Public Health Institute, Helsinki, Finland). The specific activity of this preparation was 1×10^6 units/mg protein. The antiviral activity of these preparations against vesicular stomatitis virus (VSV) was assayed by the plaque reduction method [12] or by an infectivity inhibition microtest [4] in L-929 cells and in HFF cells, respectively.

To assay the IFN activity on virus multiplication the effect on virus yield was also tested. Cells were treated overnight with IFN preparations, washed and inoculated with 1000 plaque-forming units of VSV. After 18 h incubation, the cells were frozen and thawed and the total infectious virus yield was assayed by an end-point infectivity microtest in L-929 cell monolayers with 5–6 parallels for each dilution. The 50% infectivity doses were calculated by the Reed and Münch method.

For cAMP assay, cells were grown as monolayers in polystyrene Petri dishes, either individual dishes of 6 cm diameter or 6-well dishes of 3 cm diameter (Linbro Chemicals, Co., New Haven, CT). The monolayers were treated with IFN or with the various agents at 37°C for the times indicated. After incubation the cells were scraped off by a rubber policeman. Parallel cultures were trypsinized for determination of the cell counts. The cells were then washed and resuspended in MEM to which Hepes buffer had been added to a concentration of 15 mM, pH 7.4. Cell suspensions were boiled for 2 s [11], sonicated for 15 s and filtered through membrane filters of 0.45 μm pore size. Alternatively, after sonication the suspensions were centrifuged for 20 min at $3000 \times g$ at 4°C to remove cellular debris. The cAMP content of the suspensions from the supernatants of ca. 10^6 cells were assayed by a radioimmunoassay, employing ^{125}I-labelled cAMP (New England Nuclear, Boston, MA). The values were expressed in picomoles (pmol) per 10^6 cells, representing the mean of four or six determinations, representing two or three parallel samples, each tested twice in parallel.

Fig. 1 shows the result of an experiment which confirmed that treatment of L-929 cells with mouse IFN resulted in an elevation of the intracellular cAMP levels; this effect was time-dependent. Treatment with 500 units/ml of mouse IFN gave a slight increase after 30 min of incubation. The increase was found to be maximal after 1 h of treatment, and the levels of cAMP remained elevated for at least 24 h. The development of the

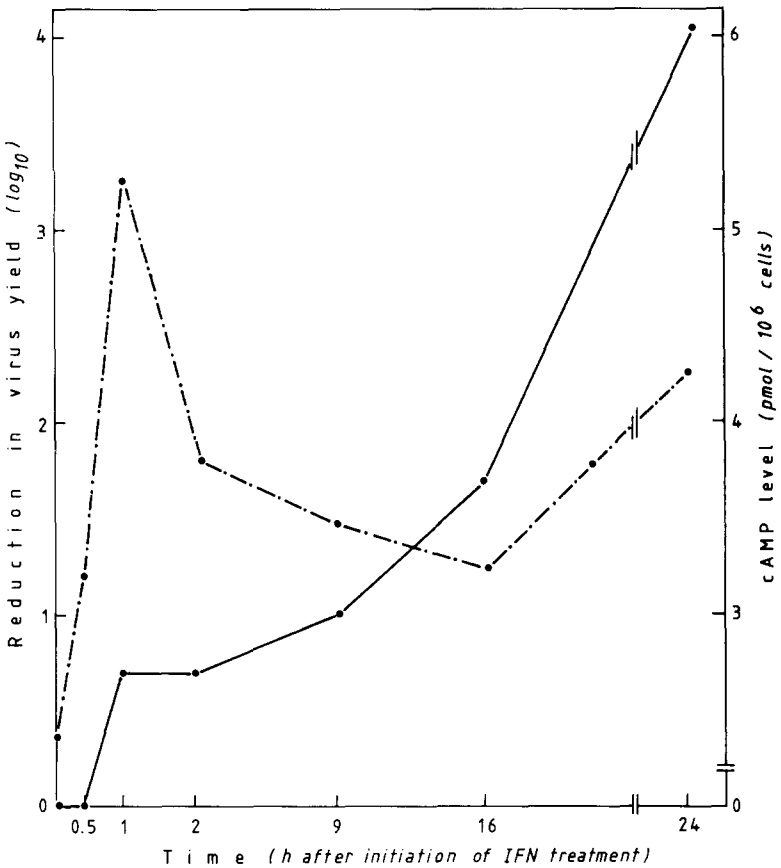

Fig. 1. Kinetics of the effect of IFN (500 units/ml) on cAMP levels (●–·–●) and resistance to virus infection (●——●) in L-929 cells.

antiviral state after the same treatment followed the changes in cAMP levels. In a second type of experiment different concentrations of IFN were used. As shown in Table 1, the effect of IFN on cAMP levels was concentration-dependent, but there was no direct linear correlation. In most experiments, more than 100 units/ml of IFN were necessary to produce a clear-cut stimulation. A similar relationship was found in experiments on murine OGS cells. Treatment of human HFF and KB cells with 100 units/ml of mouse IFN had no effect on cAMP levels. In some experiments (data not shown) treatment with higher concentrations ($> 10^3$ units/ml) of murine IFN (MuIFN) resulted in slightly elevated cAMP levels in human KB cells. The specificity of the effect of IFN on cAMP levels was further supported by the results of experiments employing HuIFN-α (Table 1). After 2 h of treatment, the cAMP levels were significantly elevated in human cells but not in murine cells.

In an attempt to correlate cAMP elevation to antiviral activity, L-929 cells were

TABLE 1

Effect of murine and human IFN on cAMP levels in homologous and heterologous cells

Expt. No.	IFN species	Cells	cAMP levels[a] after 2 h treatment with IFN at concentrations (units/ml)					
			0	5	50	100	500	1000
1	Murine (MuIFN)	L-929	3.75 (0.05;6)	4.25 (0.3;6)	4.0 (0.05;6)	–	4.95 (0.20;6)	9.0 (0.35;6)
		OGS	2.5 (0.15;4)	–	3.3 (0.4 ;4)	2.8 (0.05;4)	3.5 (0.01;4)	4.2 (0.15;4)
		KB	1.7 (0.15;4)	–	2.0 (0.20;4)	2.1 (0.15;4)	2.1 (0.05;4)	2.4 (0.25;4)
		HFF	3.8 (0.05;6)	–	3.3 (0.15;6)	2.8 (0.35;6)	3.5 (0.20;6)	4.2 (0.35;6)
2	Human (HuIFN-α)	L-929	3.1 (0.3 ;6)	–	–	–	2.7 (0.15;6)	–
		OGS	2.5 (0.35;4)	–	3.5 (0.55;4)	–	2.5 (0.25;4)	–
		KB	1.7 (0.1 ;4)	–	3.9 (0.25;4)	–	3.9 (0.15;4)	–
		HFF	4.7 (0.3 ;6)	–	–	–	7.4 (0.25;6)	–

[a] Mean pmol/10^6 cells (S.D.; number of determinations).

treated with cAMP-elevating agents alone and in combination with homologous or heterologous IFN. Table 2 shows that cAMP levels were elevated by 25–40% after treatment with each of the three agents, which was comparable to the effect of 100 units/ml of homologous IFN. Addition of MuIFN resulted in a further slight elevation of cAMP levels in combination with the phosphodiesterase inhibitor MX, but not in combinations with the other agents. HuIFN-α had no effect whether used alone or in combination with any of the agents. Table 3 shows the results of the same treatment on virus replication: 100 units/ml of homologous IFN reduced the infectious virus yields ca. 100-fold. IP had a slight but consistent depressing effect on virus replication. Neither MX, PG nor exogenously added cAMP influenced the VSV yields. However, the antiviral activity of MuIFN on L-929 cells was slightly potentiated when used in combination with MX, PG or cAMP. Identical results (data not shown) were obtained in experiments on OGS and HFF cells.

Pretreatment of the cells with an inhibitor of the adenyl cyclase system, N-em, for 2 h prior to addition of either IFN or the other cAMP-stimulating agents, prevented the rise of the cellular cAMP levels (Tables 2 and 4), although N-em alone had no significant effect on the cAMP levels. Pretreatment of L-929 cells with N-em did not influence the multiplication of VSV, nor the antiviral effect of MuIFN (Table 5), suggesting that an increase in the intracellular level of cAMP is not essential for the establishment of the antiviral effect of interferon.

The present observations confirm the earlier reports [16, 17] that treatment of sensitive cells with homologous IFN is followed by an elevation of cellular cAMP levels. This effect seems to be species-specific, to some extent dose-dependent, and is followed by the development of the antiviral state in the cells. Although conflicting results have been reported [19], it has been shown convincingly by several groups of investigators [3, 5, 14, 15, 18] that IFNs are adsorbed to both homologous and heterologous cells. It seems likely therefore that the species specificity of cAMP stimulation resides in a step that

TABLE 2

Effect of IP, PG and MX on cAMP levels in L-929 cells in the presence and absence of IFN or N-em

First component of treatment mixture	Second component of treatment mixture[a]				
	Medium	IP (10^{-6} M)	PG (0.2 μg/ml)	MX (10^{-4} M)	Mouse IFN[b]
Medium	2.3(0.1)[c]	3.7(0.15)	3.2(0.15)	3.0(0.0)	–
Mouse IFN[b]	3.1(0.2)	3.7(0.1)	3.2(0.2)	3.8(0.15)	–
Human IFN[b]	2.0(0.3)	3.5(0.15)	3.1(0.15)	3.1(0.35)	–
N-em (10^{-8} M)	2.2(0.25)	2.5(0.20)	2.2(0.15)	2.25(0.15)	2.25(0.1)

[a] All agents were added simultaneously to the cells and incubated for 2 h before assaying for cAMP.
[b] 100 units/ml.
[c] mean pmol/10^6 cells (S.D.; four determinations).

TABLE 3

Effect of IP, PG, MX and exogenous cAMP on the antiviral activity of IFN in homologous and heterologous cells

Cells	First component of treatment mixture	Second component of treatment mixture[a]					
		Medium	IP (10^{-6} M)	PG (0.2 μg/ml)	MX (10^{-4} M)	cAMP (1 mM)	Human IFN[b]
L-929	Medium	0[c]	0.4	0	0	0	–[d]
L-929	Mouse IFN[b]	2.2	2.0	2.65	2.7	3.0	–
KB	Medium	0.2	0.65	–	–	–	2.0
KB	Mouse IFN[b]	0.35	0.55	–	–	–	2.0

[a] All agents were added simultaneously to the cells and incubated for 4 h before challenge with VSV.
[b] 100 units/ml.
[c] Log_{10} reduction in virus yield, 16 h after challenge with VSV.
[d] Not tested.

TABLE 4

Effect of the adenyl cyclase inhibitor, N-em, on the cAMP-stimulating activity of mouse IFN in L-929 cells

Dose of N-em pretreatment[a]	cAMP levels[b] after treatment with IFN at concentrations (units/ml)		
	0	100	1000
0	2.1 (0.0 ;6)	3.3 (0.1 ;4)	3.6 (0.05;4)
10^{-6} M[c]	1.3 (0.3 ;4)	0.9 (0.25;4)	1.6 (0.15;4)
10^{-7} M	2.1 (0.15;4)	1.6 (0.05;4)	1.9 (0.1 ;4)
10^{-8} M	2.1 (0.3 ;6)	2.9 (0.05;6)	3.1 (0.15;6)

[a] 2 h incubation before addition of IFN. N-em was removed before addition of IFN.
[b] Mean pmol/10^6 cells (S.D.; number of determinations)
[c] 10^{-6} M of N-em was toxic on the cells after incubation for 18 h.

TABLE 5

Effect of the adenyl cyclase inhibitor, N-em, on the antiviral activity of mouse IFN in L-929 cells

Dose of N-em pretreatment[a]	Resistance to viral infection[b] after treatment with IFN at concentrations (units/ml)		
	0	100	1000
0	0	1.2	3.7
10^{-7} M	0	1.5	3.6
10^{-8} M	0	1.7	3.4

[a] 2 h incubation before addition of IFN. N-em was removed before addition of IFN.
[b] Log_{10} reduction in virus yield 16 h after challenge with VSV.

occurs past the primary IFN-binding process, and is similar to that responsible for species specificity of the antiviral activity.

In the present study, we have further analysed the relationship between cAMP levels and the antiviral effect and found that they are not correlated, as in a recently published study [10]. Thus cAMP-elevating agents had no significant antiviral effect (with the possible exception of IP) and N-em, which inhibits the increase in intracellular levels of cAMP seen in IFN-treated cells, did not affect the antiviral activity of IFN. In view of these results we conclude that the stimulation of adenyl cyclase and elevation of cellular cAMP levels by IFN, although a specific effect, is not an essential mediating mechanism of the antiviral activity.

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