AN INTERFERON-INDUCED INCREASE IN CYCLIC AMP LEVELS PRECEDES THE ESTABLISHMENT OF THE ANTIVIRAL STATE

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SUMMARY: Mouse interferon induces an increase in cyclic AMP levels in interferon-sensitive mouse Ly cells but not in interferon-insensitive human KB-3 cells. The interferon-induced elevation in cyclic AMP precedes the induction of antiviral activity. Although interferon stimulation of the adenylate cyclase activity of Ly cell plasma membranes has not been detected, interferon is effective in stimulating this activity in plasma membranes from rat thyroid cells.

Recent reports (1-7) have suggested that the cell surface receptor for interferon is structurally similar to the cell surface receptor for cholera toxin and for each of the glycoprotein hormones: thyrotropin (TSH)*, human chorionic gonadotropin, luteinizing hormone, and follicle-stimulating hormone. In addition, these reports (1-7) have suggested that there are similarities in the mechanism by which each of these effectors transmits its message to the biochemical machinery of the cell. In this regard, it is well known that cholera toxin and the glycoprotein hormones can stimulate the adenylate cyclase activity of cell surface membranes and that resultant elevations in cellular cyclic AMP levels play an important role in linking the transcriptional and translational machinery of the cell to the informational input of the effector at the cell membrane (8-10).

In the present report we show that interferon stimulation of the adenylate cyclase system may also be important in transmitting the interferon message to the cell. We thus show that interferon can effect a rise in cellular cyclic AMP levels in interferon-sensitive cells, that the increase in cellular cyclic

* Abbreviations: TSH, thyrotropin; cyclic AMP, adenosine-3',5'-monophosphate.

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AMP levels does not occur in interferon-insensitive cells, and that the increase in cyclic AMP levels in the interferon-sensitive cells precedes the development of the antiviral state.

Although we have not been able to detect increased adenylate cyclase activity in the membranes of the interferon-sensitive Ly cells used in this report, mouse interferon can stimulate the adenylate cyclase activity of a membrane preparation derived from rat thyroid cells. The mechanism by which interferon induces the antiviral state and a potential pathophysiological role for interferon in Graves' disease are also discussed.

MATERIALS AND METHODS

Mouse Ly cells are an interferon-sensitive strain originally donated by Dr. J. Youngner, University of Pittsburgh School of Medicine; KB-3 cells are an interferon-insensitive human strain which do not respond to interferon, but can specifically bind mouse interferon to their surface (1, 2). Cells were grown as monolayers in Eagle's medium with 10% fetal calf serum and were treated with interferon in the same medium but without serum. To establish the efficacy of interferon treatment, cells were washed 5 times and infected with encephalomyocarditis virus at a virus/cell multiplicity of one (1, 2). Cells were then frozen and thawed at the times noted, and the culture fluids were assayed for virus yield by 10-fold serial dilutions in microtiter plates; the plates were observed for viral cytopathic effects until 72 hours after infection.

Mouse interferon was a preparation from the laboratory of Dr. Kurt Paucker, Medical College of Pennsylvania (11); its specific activity is at least 2×10^7 mouse reference international units per mg of protein, and its antiviral activity had the chemical and physical properties ascribed to interferon (12).

Cyclic AMP levels were measured in cells (1×10^7/flask) exposed to fresh medium containing no addition or containing 100 U/ml interferon, 0.5 mM 3-isobutyl-1-methyl xanthine, or 100 U/ml interferon plus 0.5 mM 3-isobutyl-1-methyl xanthine (13, 14). At various time intervals following the additions, the medium was removed by aspiration; the cells were rapidly washed with phosphate-buffered saline at pH 7.4, and 2 ml of ice-cold 6% trichloroacetic acid was added. The cells were removed from the flasks by scraping; the cell suspension was homogenized using a Potter Elvejhem appratus; and, the homogenates were centrifuged for 15 minutes at 14,000 x g. The cyclic AMP in the deproteinized supernatant was assayed using a binding protein assay (15) once the trichloroacetic acid was extracted with water-saturated diethyl ether (16). The sediment obtained from the centrifugation procedure was subjected to a colorometric protein analysis procedure (17) after treatment for 30 minutes at 90° in 0.1 M NaOH; 5 times recrystallized bovine serum albumin was the standard. Protein determinations were used to compensate for small variations (< 10%) in the number of cells in each flask; cyclic AMP levels are expressed per 1×10^7 cells.

Membrane preparations used for measurements of interferon-sensitive adenylate cyclase activity were made employing previously detailed procedures (18-20) with the exception that cell disruption and the washing and storage of membranes used an 0.01 M Tris-acetate buffer at pH 7.0 containing 1 mM EDTA, 0.1% mercaptoethanol, and 0.25 M sucrose. Adenylate cyclase activity was measured after

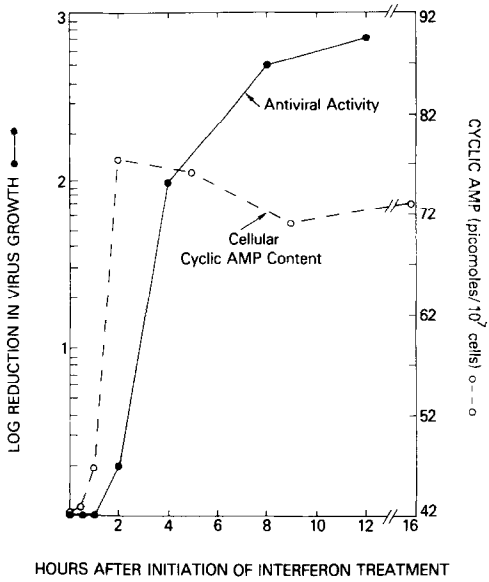

FIG. 1. Cyclic AMP levels and the antiviral activity of mouse interferon correlated in mouse Ly cells as a function of the time post-initiation of interferon treatment. Assay methods are presented in "Materials and Methods" and are analogous to those in Table I. Cyclic AMP measurements at each time point are the average of replicate determinations. In addition, duplicate analyses were performed on at least two different volumes of the trichloroacetic acid extract from each of the replicate flasks assayed at each time point in order to insure that cyclic AMP levels were estimated from linear portions of the control cyclic AMP binding protein displacement curves (15, 16). Components of the assay mixture did not interfere with the control cyclic AMP binding assay as determined from control displacement curves. Analogous data were obtained in four separate experiments.

13 minutes at 37° in a 50-µl assay volume containing the following components in addition to membranes and the appropriate effector: 3.2 mM ATP, 5.1 mM $MgCl_2$, 18.5 mM creatine phosphate, 26 mM Tris-chloride at pH 7.5, 6 mM theophyllin, 40 µg of creatine phosphokinase, and 50 µg of crystalline bovine serum albumin. Reactions were stopped by the addition of 50 µl of ice-cold 0.05 M acetate buffer at pH 4.0. Cyclic AMP was again assayed using the binding protein assay (15).

RESULTS

Mouse interferon induces an increase in the cyclic AMP level of interferon-sensitive mouse Ly cells (Fig. 1). The elevation is detectable within 30 minutes and is maximal by 2 hours after interferon exposure. The elevation in cyclic AMP precedes interferon-induced antiviral activity which is significant after 4 hours and is maximal after 7 hours (Fig. 1). KB-3 cells which are

TABLE I. Effect of mouse interferon on cyclic AMP levels in mouse Ly and human KB-3 cells as a function of interferon concentration and in the presence or absence of the phosphodiesterase inhibitor, 3-isobutyl-1-methyl xanthine

Treatment	Cellular cyclic AMP content [a]	
	Mouse Ly cells	Human KB-3 cells
	picomoles/flask of 1 x 10^7 cells	
None	40 ± 5	36
Interferon, 10 units/ml	46 ± 5	not done
Interferon, 30 units/ml	68 ± 5	not done
Interferon, 100 units/ml	81 ± 6	36 ± 5
Interferon, 300 units/ml	84 ± 5	not done
3-Isobutyl-1-methyl xanthine	74 ± 8	52 ± 5
Interferon, 100 units/ml, + 3-isobutyl-1-methyl xanthine	155 ± 10	55 ± 4

[a] Measured 4 hours after the addition of the noted agent in fresh medium, i.e., before the onset of detectable antiviral activity. As noted in the legend to Fig. 1, all values are the means of replicate analyses and were determined using several different volumes of the extracts to insure the validity of the measurements.

insensitive to mouse interferon do not exhibit cyclic AMP elevations when similarly exposed to interferon (Table I), despite the ability of KB-3 cells to bind mouse interferon to their cell membrane (2). The cyclic AMP elevation is thus correlated with the establishment of the antiviral state and is not simply a reflection of the ability of interferon to bind to the surface of the cell.

The cyclic AMP elevations induced by interferon do not appear to be simple linear expressions of the dose of interferon to which Ly cells are exposed (Table II). This phenomena is, however, not uncommon when intact cells or tissues are perturbed with hormonal effectors (10).

In the absence of interferon, 3-isobutyl-1-methyl xanthine increases measurable cyclic AMP levels in both KB-3 and Ly cells (Table I). In the presence of interferon plus 3-isobutyl-1-methyl xanthine, only the Ly cells exhibited cyclic AMP elevations above those induced by the phosphodiesterase inhibitor

TABLE II. Interferon stimulation of adenylate cyclase activity in rat thyroid cell membranes [a]

Incubation addition [b]	Adenylate cyclase activity
	picomoles cAMP/mg membrane protein [c]
None	190
Na F	605
TSH	300
Interferon, 200 units/ml	195
Interferon, 500 units/ml	220
Interferon, 1,000 units/ml	260
Interferon, 2,000 units/ml	275

[a] Rat thyroid membranes were prepared as described in "Materials and Methods" and references 13, 24, and 25, and stored at -80°.

[b] The concentrations of the different additions were as follows: Na F, 8 mM; TSH, 1 mg/ml; and interferon, as noted above.

[c] As noted in the legend to Fig. 1, these cyclic AMP values are the means of replicate determinations. No individual determination exceeded a 10% deviation from the mean, and adenylate cyclase assays were linear with time and protein under the assay conditions utilized.

alone (Table I). These results support the conclusion that elevations in cyclic AMP precede the establishment of the antiviral state in interferon-sensitive cells and suggest that absolute values of cyclic AMP measurable in cells treated with interferon are influenced by an active phosphodiesterase system.

Although attempts to directly measure interferon-stimulated adenylate cyclase activity in Ly cell membranes have thus far been unsuccessful (despite incubation additions of NAD, cell supernatant, or high concentrations of theophyllin), high concentrations of mouse interferon (1,000 units/ml) can stimulate the adenylate cyclase activity of rat thyroid cell membranes (Table II).

DISCUSSION

The current report demonstrates that mouse interferon can cause an increase in cyclic AMP levels in mouse Ly cells and that the increase precedes measurable detection of the antiviral state. These results support our hypothesis (3-7)

that interferon is similar in its message transmission mechanism to glycoprotein hormones such as TSH and to cholera toxin. These results should, however, not be interpreted to mean that cyclic AMP is the causal agent or second messenger in the induction of the antiviral state, since previous observations indicate that dibutyryl cyclic AMP can potentiate interferon action but does not alone induce antiviral activity (21).

Phosphorylated intermediates have previously been demonstrated in extracts from interferon-treated cells which had been incubated with double-stranded RNA (22). Although cyclic AMP could not substitute for double-stranded RNA in eliciting phosphorylation and did not increase the effect of the double-stranded RNA, it is possible that these intermediates are the result of an interferon-dependent activation of cAMP-dependent protein kinases (23). Since these phosphorylated intermediates may affect the transcriptional-translational processes induced by interferon (24), we believe the current observations may allow the further definition of the relationship between membrane changes and the ability of interferon to establish the antiviral state.

Although previous studies have been variable in their ability to clearly demonstrate cyclic AMP elevations as a consequence of interferon action (25-27), at least one report (27) has noted similar values to those presented in this report. The difficulties previously encountered may well relate to the effect of 1-isobutyl-3-methyl xanthine in the current report, i.e., it caused higher measurable levels of cyclic AMP. That an active phosphodiesterase system (13) within a short time during manipulative procedures can hide elevations has its precedent in the effect that the washing of cells has on TSH-induced cyclic AMP levels in thyroid cell cultures (13). Washing in the present experiments was required to eliminate media blanks but was performed extremely rapidly. These and other possible considerations, such as a cofactor requirement similar to the NAD-cholera toxin phenomenon (9), are under investigation in our attempts to directly measure interferon-induced adenylate cyclase activity in membrane preparations.

It has long been noted that the active symptomology of Graves' disease may be "induced" by viral infections in patients under otherwise adequate therapeutic control and management, or in patients with no prior history of the disease (28). The ability of interferon to affect the cyclic AMP levels of rat thyroid membranes coupled with the similarities in the structure and function of receptors for TSH and interferon (3-6), raises the possibility that interferon might play a role in the pathogenesis of this effect, i.e., as a thyroid stimulatory agent which is the counterpart of a thyroid-stimulating immunoglobulin (29-32). In this regard, Baron and Isaacs have previously shown that human thyroid cells are sensitive to the antiviral activity of interferon (33), and we have recently shown that membranes from the thyroids of patients with Graves' disease have an abnormally sensitive adenylate cyclase activity and an abnormal ganglioside composition in their membranes (34).

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