Increase of interferon antiviral activity by exogenous cyclic adenosine-3′:5′-monophosphate (cAMP)[1]

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Summary. Some effects of cAMP on replication of Semliki Forest Virus in chick embryo fibroblast cell cultures are described. Depending on concentration, the incorporation of [^{3}H]-uridine into viral RNA or the formation of plaque-forming units is inhibited; the highest concentration tested was 8 mM. Cyclic AMP has an effect of its own and increases the Interferon action in the lower concentration ranges of Interferon (up to 1 unit/ml). The effect of cyclic AMP is fast, needs no induction and is also visible in late phases of viral replication. However, these experiments do not establish a causal relation between cAMP and Interferon.

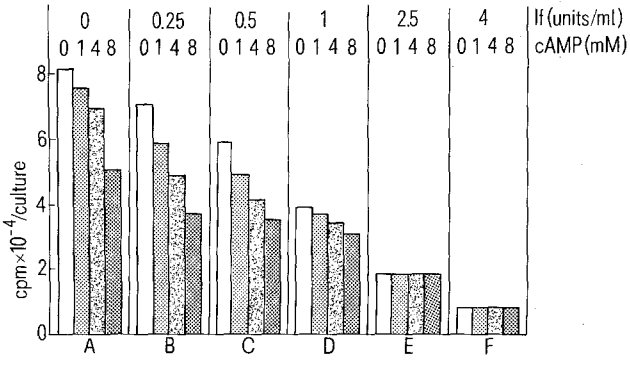
Recent reports have suggested that the cell surface receptor for Interferon is structurally similar to that for cholera toxin and for the glycoprotein hormones[4–6]. These latter effectors can stimulate the adenylate cyclase activity of cell surface membranes; elevations in cellular cAMP levels result. Interferon can effect a rise in cellular cAMP levels in Interferon-sensitive cells[7,8], which precedes the development of the antiviral state[9]. Human KB-3 cells are insensitive to mouse Interferon and do not exhibit cAMP elevations when in contact with this Interferon, despite the fact that they bind it. The cAMP elevation therefore does not simply reflect the binding of Interferon to the cell surface[9]. In this work the action of cAMP itself was studied.

Materials and methods. Cell cultures, media and infection with Semliki Forest Virus (SFV), Zürich strain, were as described[10]. Interferon preparations were those used earlier (Interferon 14: 50 units/mg protein; Interferon 10/1: 20,000 units/mg[11]). 1 unit is defined as that amount of protein which reduces plaque counts[12] or uridine incorporation into viral RNA[10] to 50% of the control value. Antiviral activities were assayed either by measuring the incorporation of [^{3}H]-uridine[13] into acid-precipitable material of SFV-infected chick embryo fibroblast (CEF) monolayers treated with actinomycin D (RNA-test[10]) or with the plaque test[12]. Times of additions, all relative to each other, are given in the legend. Because of equivalence of results, only those after use of Interferon 14 are shown. Nucleosides and nucleotides were prepared as described[14]. The following terms are used: 'presence' of Interferon or cAMP or both means that these compounds are *in* the culture fluid; 'induction period' is the time between addition of the compounds tested and the addition of actinomycin D and may be shorter or longer than, or equal to, the time of presence. All experiments were done at 37 °C.

Results and discussion. In a first experiment, times of presence and induction periods were constant, but the concentrations of Interferon and cAMP were variable. The figure shows that resistance levels with Interferon alone (cAMP: 0 mM) have developed normally (bars A to F). In our system cAMP *alone* inhibits also the uridine incorporation into viral RNA (bars A); there is almost a linear dose-response relationship within the concentration range used. In later experiments, 8 mM cAMP was used regularly. In addition, cAMP increases the Interferon action in the lower concentration ranges of Interferon used (bars B–D). Again, 8 mM cAMP results in the strongest additional effect. With the highest Interferon concentration (2.5–4 units/ml), there is no further increase of the resistance level (bars E and F). In order to make sure that no artifact was measured (for example, changes in RNA precursor pools) production of infectious units in a similar experiment was assayed. The results were the same. In another experiment, cAMP was given *alone* 1 h *after* infection, and culture fluids were examined for plaque-forming units (PFU) after 9 h of infection. PFU/ml were: without cAMP, 1.7×10^9 PFU/ml; with 1 mM cAMP, 1.5×10^9 PFU/ml; with 3 mM, 1.3×10^9 PFU/ml; with 5 mM, 0.9×10^9 PFU/ml; with 8 mM, 0.8×10^9 PFU/ml. Other experiments have shown similar effects of dbcAMP[13], but no effects of 2′:3′ cAMP, adenosine, 5′ AMP, cytidine or uridine (plaque tests).

In another experiment, cAMP was given at the time of the full resistance level induced with Interferon; 0.25–1 units/ml of Interferon were added to CEF-cultures over night. This would still allow a further increase of the antiviral state. 2 h before infection, cultures received 8 mM cAMP and then the routine RNA-test procedure was used. At no Interferon concentration was an additional protection enforced. We conclude that cAMP has to be present during the induction period of Interferon to yield an effect.

Other experiments showed that the inhibition of viral reproduction by cAMP is fully established if it is in contact with the cells for 0.5 h before infection. Longer presence times do not increase the effect. This favours the idea that cAMP has no inducing activity per se, but either acts directly or via *activation* of preexisting inhibitory molecules. The same conclusion was reached in another experiment, where cAMP alone was given to CEF-monolayers 4 h before infection. Actinomycin was added at 4, 3, 2 and 1 h before infection, to interrupt possible induction phenomena at different levels of development. Again, the inhibitory effect was seen, but it did not increase, in contrast to what is seen in the case of Interferon. Finally, if the above conclusions are reasonable, an action of cAMP



Development of resistance levels; times of presence and induction periods are constant, concentrations are variable (RNA-test). 72 cell cultures were divided into sets A–F. Interferon, if added, was given 2 h before infection in a concentration range from 0.25 units to 4 units/ml; cAMP, if added, was given simultaneously (1, 4, 8 mM) to each set so that subsets of 3 dishes contained the same amount of cAMP. After a presence of 2 h, cultures were washed and infected for 1 h with SFV (m.o.i. = 1). 90 min after removing the virus, 1 μg/ml actinomycin D was added; 1 h after actinomycin, [^{3}H]-uridine (3 μCi/culture) was given, and 5.5 h later cells were harvested and radioactivity in acid-precipitable material was determined. Thus, induction periods lasted always 4.5 h and each experiment lasted 11 h. Controls were mock-infected or received mock-Interferon. Bars represent means of 4 experiments, each with 3 dishes per bar.

alone might be visible even in later stages of the viral replication. Experiments showed an effect of cAMP at all times, mostly pronounced during maximal speed of replication around the 7th to 9th h of infection.

Our findings extend the few observations made by others. Replication of adenovirus, type 2, is inhibited by dbcAMP; the cell line itself has an effect[15]. An increased antiviral activity of Interferon in CEF infected with SFV was mentioned[16]. However, the following findings are new: a) cAMP has an antiviral activity of its own in our system; b) cAMP acts also in the late phases of replication of SFV; c) cAMP has probably no inducing activity in respect to antiviral resistance development. In the figures and tables of Weber and Stewart[8], showing cAMP potentiation of Interferon, marginal effects of cAMP alone - reducing vesicular stomatitis plaque numbers in L-cells to 80% at maximum -, can be seen.

With respect to the cooperative effects of Interferon and cAMP, however, there is a clear difference between our results and theirs[8]: they show a synergistic effect on plaque reduction of exogenously added Interferon and cAMP, whereas we find an at most additive effect, disappearing at higher concentrations of Interferon. Virus-host systems might play a role.

Interestingly enough, our cAMP effects are in contrast to those in uninfected CEF[14], where the biosynthesis of all RNA types is stimulated after an induction time of about 3 h. Our results are compatible with the view that Interferon acts at the cell membrane at a site where the adenylate-cyclase is influenced; however, they do not prove a causal relation between Interferon and cAMP. An influence of cAMP on Interferon synthesis after the challenge with the test virus is a remote possibility[17-19].

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13 Reagents: Actinomycin D (Calbiochem, Switzerland); Medium Eagle MEM is minimal essential medium-Hanks (BBL, USA); Medium 199-Hanks (Difco, USA); Fetal calf serum (Flow, Scotland); [^{3}H]-uridine, uniformly labeled, 3.5–6.3 Ci/mM (Amersham, England); 3′:5′ cAMP; 2′:3′ cAMP; N^6, $O^{2'}$-dibutyryl-3′:5′ cAMP (dbcAMP) and other nucleosides and nucleotides, all from Calbiochem.
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Altered or increased transfer-RNA methylation in the course of Interferon action on cells in culture?[1]

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Summary. The induction of the antiviral state by Interferon might reflect the decrease of the rate of biosynthesis, the degradation or the alteration of one or several tRNAs. This could result in rate-limiting concentrations for codons common in viral RNA but rare in host mRNA. Altered methylation of tRNA could be the basis of such a phenomenon. However, we could not find an altered extent of methylation of total tRNA or an altered pattern of methylation, if mixed tRNAs were chromatographed on MAK- or BD-cellulose columns, despite a large range of conditions of pretreatment of chick embryo fibroblast cultures with interferon.

The regulation of protein biosynthesis with rate-limiting function or concentration of certain tRNA species has been described[4,5]. It is therefore tempting to speculate that cells could be protected against viral infections by lowering function or concentration of tRNAs serving codons, which are common in viral RNA in comparison to host RNA. Interferon could be a substance which induces such a type of regulation. Some papers indicate a connection between Interferon and tRNA[6-12]. A possible explanation for these observations is offered by the finding that Interferon stimulates tRNA-methylase enzymes[13] assayed in vitro. There is evidence that the distribution of methylgroups in tRNA governs the efficiency with which these molecules accept aminoacids and act as donors[14,15]. The main effects were found if non-homologous tRNAs were used as acceptors in this assay in vitro[13]. Therefore we wanted to know more about the methylation of tRNA in vivo (with homologous tRNAs) after treatment with Interferon.

Materials and methods. Cell cultures, media and infection with Semliki Forest Virus (SFV), Zurich strain, were as described[16,17]. Interferon preparations were those used earlier[18]. Antiviral activities were assayed as described[16,17].

Extent of tRNA-methylation: In the evening before the start, chick embryo fibroblast (CEF) cultures received Eagle's medium with $^1/_{10}$ of the normal aminoacid concentration ($^1/_{10}$ Eagle) to deplete the 1C-fragment pool, 10^{-4} M