STIMULATION OF VIRUS-INDUCED FUSION OF EHRLICH ASCITES TUMOR CELLS BY 3',5'-CYCLIC AMP

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SUMMARY: HVJ(Sendai virus)-induced fusion of Ehrlich ascites tumor cells was found to be stimulated by treatments which increase the intracellular level of cyclic AMP. This stimulation was optimal at an external concentration of Ca^{++} of about 0.5 mM. During the process of cell fusion, the intracellular concentration of cyclic AMP was increased with a maximum at 2 min after the initiation of the fusion reaction.

Evidence is also presented which suggests that the increase of the cyclic nucleotide is a part of control mechanism of HVJ-induced fusion of eukariotic cells. Thus, this cyclic AMP-stimulated process could be one of the step(s) requiring ATP and Ca^{++}, both of which are necessary for the overall fusion process of the tumor cells.

Membrane fusion is an important physiological process which involved in a wide variety of life phenomena (1, 2) and has recently been extensively studied (1). However, the mechanisms regulating membrane fusion are not yet well understood. For studies of such control mechanisms, HVJ*(Sendai virus)-induced fusion of Ehrlich ascites tumor cells, first described by Okada (3), provides an excellent experimental system because of its high efficiency, the ease with which it can be assayed, and the wealth of accumulated information on it. This virus-induced cell fusion has been shown to require both ATP and Ca^{++}(4, 5) and similar requirements for ATP and/or Ca^{++} have been reported for other membrane fusion processes (6). It is well established, on the other hand, that 3',5'-cyclic AMP acts as an intracellular mediator of membrane-linked events (6) and that in some cases cell excitation is accompanied by increases in both cyclic AMP

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* Abbreviations: HVJ, hemagglutinating virus of Japan; BSS, balanced salt solution; HAU, hemagglutination unit; IMX, 3-isobutyl-1-methylxanthine

synthesis from ATP and cellular uptake of Ca^{++}(6). It is also well known that the regulatory function of cyclic AMP is expressed through the ATP-linked phosphorylation of cellular proteins(7). These findings seemed to us to suggest the possibility that cyclic AMP plays a role in the process of cell fusion. This communication reports that reagents known to increase the intracellular level of cyclic AMP enhance HVJ-induced cell fusion and that extracellular Ca^{++} is required for this stimulation.

MATERIALS AND METHODS: Ehrlich ascites tumor cells were grown in ddo mice, harvested, and washed as described (8). The washed cells were suspended in a balanced salt solution (BSS) consisting of 0.14 M NaCl. 54 mM KCl, 0.34 mM Na_2HPO_4, and 0.44 mM KH_2PO_4 and 10 mM Tris-HCl buffer (pH 7.6). HVJ, Z strain, was cultivated in embryonated eggs, purified and suspended in BSS. The dose of the virus was expressed in terms of its hemagglutination unit(HAU) which was determined by Salk's pattern method (8). Cell fusion was measured as follows. A desired dose of the virus was added to the tumor cells suspended (to a desired density) in BSS containing 2 mM $CaCl_2$ (final volume, 1 ml) at 0°C and the mixture was allowed to stand for 15 min in ice to complete the virus-induced aggregation of the cells. The fusion reaction was then started by raising the temperature to 37°C and terminated after 15 min of incubation by cooling rapidly in an ice bath. The number of cells was counted before and after the experiment by the method of Okada and Tadokoro (9). Since one fusion event results in the decrease of cell number by one, the efficiency of cell fusion was expressed as "fusion frequency", i. e. percent decrease in cell number. For determination of the intracellular level of cyclic AMP, the cells were sedimented from 2 ml of suspension after rapid chilling and extracted with 0.4 ml of 10 % $HClO_4$. The extract was neutralized with 3 M K_2CO_3 and the resulting $KClO_4$ precipitate was removed by centrifugation. The cyclic AMP content in the supernatant was determined by competitive binding method (10) using [^{3}H]-cyclic AMP as competitor. A bovine adrenal extract prepared as described by Brown et al.(11) was used as a cyclic AMP-binding protein preparation.

RESULTS AND DISCUSSION: As shown in Fig. 1, virus-induced fusion of Ehrlich ascites tumor cells was enhanced by theophylline, a well-known inhibitor of cyclic AMP phosphodiesterase (12), at concentrations similar to those used for studies of other cyclic AMP-dependent cellular events(13). Brief preincubation of the cells with theophylline (5 min at 4°C) was needed to obtain maximal activation. Similar stimulation was observed with several other reagents known to elevate the intracellular content of cyclic AMP. 3-Isobutyl-1-methylxanthine stimulated cell fusion at much lower concentrations (such as 0.5 mM) than theophylline, as expected from the fact that the former is a far more effective inhibitor of cyclic AMP phosphodiesterase(14). Although these

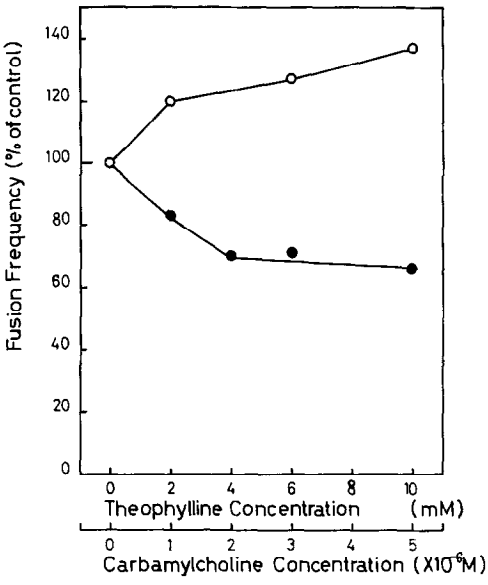

Fig. 1 Effect of theophylline and carbamylcholine on HVJ-induced fusion of Ehrlich ascites tumor cells. Cells suspended in BSS containing 2 mM Ca^{++} (1.84×10^7 cells in 1 ml) were preincubated with theophylline or carbamylcholine for 20 min then cooled before addition of 1,000 HAU of the virus. Fusion frequency was determined as described in "MATERIALS AND METHODS", except that theophyllnie or carbamylcholine was added as indicated. O—O, with theophylline, ●—●, with carbamylcholine.

inhibitors have been shown to inhibit the hydrolysis of cyclic GMP as well (12), it was unlikely that the increase in cyclic GMP was responsible for the observed stimulation because carbamylcholine, which is known to increase the cyclic GMP content(15), was inhibitory rather than stimulatory to cell fusion (Fig. 1). Dibutyrl cyclic AMP, which readily enters the cells, and prostaglandin E_2, which has been shown to activate adenyl cyclase in thyroid cells(13), were also found to enhance the virus-induced cell fusion. The stimulatory effects of these reagents were again concentration dependent.

To see if the stimulation was really caused by an increase in the intracellular concentration of cyclic AMP, the content of the nucleotide was measured during the course of cell fusion in the presence of 10 mM theophylline. As shown in Fig. 2, it was found that the cyclic AMP content was maximal 2min after the initiation of the fusion reaction by raising the temperature of the virus-cell aggregates to 37°C. This time

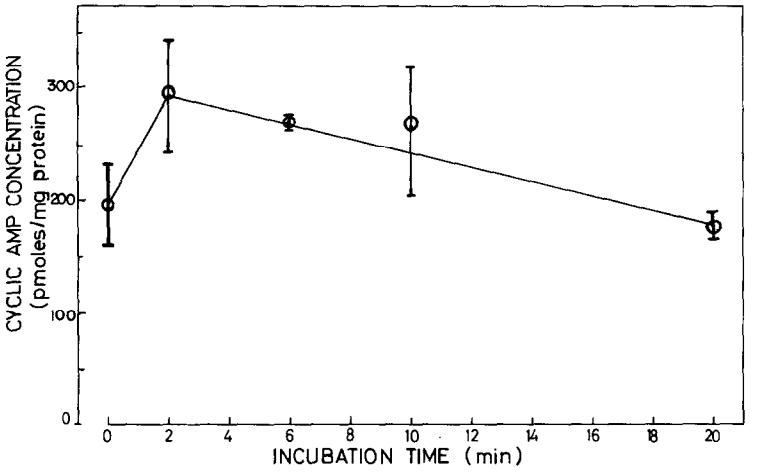

Fig. 2 Intracellular level of cyclic AMP during the cource of HVJ-induced fusion of Ehrlich ascites tumor cells. Preincubation and agglutination with HVJ were performed as described in the legend of Fig. 1 except that 10 mM theophylline was included. The fusion reaction was started by rapidly raising the temperature to 37°C, and the intracellular level of cyclic AMP was determined as described in "MATERIALS AND METHODS". Vertical bars in the figure indicate range of the nucleotide concentrations obtained from two separate runs, determinations were also made two times for each of the sample. Average of four determinations was shown as points. Cyclic AMP content of untreated cells in this paticular experiment was 56 picomoles/mg of cellular protein.

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point was therefore chosen to be used in experiments designed to assess the effects of several treatments on the cyclic AMP level. It was thus found that the increase of cyclic AMP at a fixed theophylline concentration was almost proportional to the amounts of the virus added, up to 2,000 HAU/ml. Theophylline, IMX and prostaglandin E_2 were all effective in elevating the intracellular cyclic AMP content during the fusion reaction.

Since extracellular Ca^{++} is required for several cyclic AMP-mediated processes (6), the effect of Ca^{++} concentration on the theophylline-induced stimulation of the cell fusion was examined. As shown in table I, theophylline caused no stimulation of cell fusion in the absence of added Ca^{++} and the stimulation was maximal at a Ca^{++} concentration of about 0.5 mM. At higher concentrations, such as 5 to 10 mM, the stimulatory effect of theophylline was again abolished. Since the intracellular concentration of cyclic AMP was almost equally elevated by theophyllnie regardless of the presence or absence

Table I

Effect of Ca^{++} concentrations on theophylline stimulation of HVJ-induced cell fusion

Ca^{++} concentration (mM)	Cyclic AMP (picomoles/mg. protein) + theophy.	- theophy.	Fusion frequency with theophylline (% of control)
0.0	250	133	95
0.2	-	-	111
0.5	283	83	169
2.0	251	125	131
10.0	267	133	100

Fusion was performed in Tricine buffered medium [135mM NaCl 5.4 mM KCl and 40 mM Tricine-NaOH buffer (pH 7.8)(24)] instead of BSS to avoid precipitate formation at higher Ca^{++} concentrations. The dose of HVJ was decreased to 400 HAU in these experiments, since HVJ at higher doses caused lysis of cells at low Ca^{++} concentrations. Fusion frequency differs at different Ca^{++} concentrations. Therefore, the effect of 10 mM theophylline on the fusion frequency was compared with that without it at the same Ca^{++} concentration. The fusion frequency without Ca^{++} addition was 46 % of that with 2 mM Ca^{++} in this particular experiment.

of Ca^{++}(Table I) in accordance with other reports (6, 16), it seems reasonable to assume that extracellular Ca^{++} participates in a partial step of the over all cell fusion process that is activated by cyclic AMP.

Although partial reactions of virus-induced cell fusion are not yet established, agglutination of the tumor cells by the virus is thought to represent the initial step of the overall cell fusion process. Since this step was not appreciably influenced by the addition of theophylline(measured turbidometrically), it is clear that cyclic AMP exhibits its action at a later step or steps of the whole process. In preliminary experiments we observed that HVJ-induced hemolysis and fusion of human erythrocytes was not affected by theophylline (K. Ohki, unpublished observation). These results were in accordance with the lack of a requirement for ATP in the fusion reaction of human erythrocytes (22, 23). Therefore, an increase of intracellular cyclic AMP may not be the initial signal for

the fusion event, but might stimulate a partial reaction of the fusion process of eukaryotic cells, and thus enhance the overall fusion process.

Recent studies of the fusion of phospholipid vesicles (18) and of protoplasts of liliaceous plant cells (19) strongly suggest that bare membranes of phospholipid bilayers possess the ability to fuse spontaneously to each other provided that the composition of the bilayer is appropriate and other requirements are fulfilled. Thus, the control mechanism of cell fusion could involve a process that affects molecular distribution on the surface of the cell membrane and modifies the membrane structure to a form in which contact of fusable parts of the phospholipid bilayer becomes possible. Some cytoplasmic components, such as microfilaments and microtubules (20, 21), may participate in these processes as in the case of cap formation. In this regard, recent findings that the same cell fusion system was inhibited by an inhibitor of microfilaments, cytochalasin D (22), and by several protease inhibitors (22), while it was enhanced by the addition of colchicine (22), are interesting and may indicate several possible points of cyclic AMP action. One of our next problems is to pinpoint the sites of action of cyclic AMP and Ca^{++} in this complex system.

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