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INHIBITION OF G1 CYCLIN EXPRESSION AND G1 CYCLIN-DEPENDENT PROTEIN KINASES BY cAMP IN AN ASTROCYTIC CELL LINE

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Summary : The effects of cAMP on cell cycle progression were examined using an astrocytic cell line. We show that forskolin and 8-bromo-cAMP block the basic Fibroblast Growth Factor-induced DNA synthesis, do not inhibit mitogen activated protein kinase activation whereas they reduce G1 cyclin (E and D1) expression without modification of cyclin A level. Furthermore, they inhibit the activation of cyclin A- and cyclin E-dependent histone H1 kinases. These results suggest that cAMP may exert its antiproliferative effects through the regulation of cyclin synthesis and cyclin-dependent kinase activation. © 1994 Academic Press, Inc.

The decision of cells to growth is determined by a series of factor-induced signals that drive the progression of cell cycle. Cyclins and associated cyclin-dependent kinases (cdks) are key points in the regulatory events of cell cycle (1-2). The negative control of cell growth by agents such as Transforming Growth Factor βs (TGFβs), Interferons (IFNs), Tumor Necrosis Factor α (TNFα) as well as by contact inhibition or cell adhesion often modify the cyclin and cdk levels (3-13). cAMP also produces antiproliferative effects in certain cell types but the molecular mechanism of this action is not known. However, it has been reported that cAMP decreases the expression of D-type cyclins in human diploid fibroblasts (13,14), as well as that of CLN1 and CLN2, the yeast G1 cyclins (15,16), and also suppresses CYL1 gene expression, the murine homologue of cyclin D in macrophages treated by Colony Stimulating Factor-1 (CSF-1) (9). It also inhibits Mitogen Activated Protein kinase (MAP kinase) cascade in some mammalian cell types (17). To gain insight on the mechanism of cAMP-mediated growth inhibition, we examined its effects on G1 cyclin levels and the associated kinase activation by using an astrocytic cell line. Our results provide valuable clues to approach the mechanism by which cAMP leads the cells through proliferation or differentiation.

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MATERIALS AND METHODS

Cell culture: The astrocytic cell line C.LT.T.1.1. kindly provided by P. Rouget and C. Evrard (18) and obtained from mouse embryo cortex cells immortalized by polyoma virus large T gene was cultured as previously described (19). Cells used in this work were grown until confluence. They were maintained one week in contact-inhibited state in DMEM supplemented with 10% fetal calf serum. Culture medium was renewed before basic Fibroblast Growth Factor (bFGF) addition to a final concentration of 20 ng/ml, which suppressed contact inhibition.

DNA synthesis: For [^{3}H]-thymidine incorporation, cells were treated for the times indicated in the figures with bFGF +/- forskolin. 2 µCi/ml [^{3}H]-thymidine was added in culture medium for the last 3 hrs of incubation, and then cell cultures were treated as previously described (20). For immunocytochemical quantification of DNA synthesis, cells were treated for 30 hrs with bFGF +/- forskolin. 10 µM bromo deoxy uridine was added for the last 26 hrs. Cells were then fixed and permeabilized as described (21). Cells that synthesized DNA were revealed using a mouse anti-bromo deoxy uridine antibody mixed with DNAse and a fluorescein isothiocyanate labeled sheep anti mouse antibody (Amersham).

Immunoprecipitation: Cells were scraped in lysis buffer (80 mM β-glycerophosphate, 20 mM EGTA, 250 mM NaCl, 1 mM DTT, 15 mM $MgCl_2$, 0.1 % Triton X 100, pH 7.5) containing protease inhibitors (4 µg/ml antipain, 4 µg/ml leupeptin, 50 µg/ml aprotinin, 1 mM benzamidin, 1 µg/ml pepstatin, 1 µg/ml soybean trypsin inhibitor, 1 mM PMSF) and 1 mM sodium orthovanadate, and centrifugated 20 min. at 13,000 rpm. Supernatants were precleared with protein A-sepharose. Equal amounts of proteins of each precleared supernatant, were incubated one hr at 4°C with polyclonal rabbit anti cyclin A (Santa Cruz) or anti cyclin E (gift of Dr J.M. Roberts) antibody and for one hr with a 1/5 vol. of a 50% solution of protein A- sepharose beads. Beads were washed 3 times with lysis buffer and 3 times with incubation buffer (20 mM β-glycerophosphate, 25 mM Tris-HCl pH 7.5, 1 mM DTT, 10 mM $MgCl_2$ and 1 mM sodium orthovanadate), then tested for histone H1 kinase activity.

Western blot analysis: Cells were scraped in RIPA buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 % NP-40, 1 % sodium deoxycholate, 0.05 % SDS, 10 mM EDTA) containing protease inhibitors and 1 mM sodium orthovanadate. Equal amounts of proteins from each cell extract were subjected to SDS-PAGE (12 % gel) and transfered to nitrocellulose membrane by semidry transfer. Membranes were blocked with 5 % fat free milk. Blots were probed for 2 hrs with polyclonal rabbit anti cyclin A, anti cyclin E, anti cyclin D1 (gift of Dr D. Beach) or anti cdk2 antibody (UBI). The primary antibody was removed and immunoreactive bands were visualized using a horse-radish peroxidase-coupled anti rabbit antibody (Biosys) followed by ECL revelation system. For western blotting analysis of phosphotyrosine content of cell extracts, cells were treated as described (22) except ECL revelation system was used. In order to verify the migration pattern of MAP kinase p42/p44 isoforms, the membrane previously probed with antiphosphotyrosine antibody was washed with stripping buffer as described by Amersham and then was reprobed with anti-MAP kinase antibody as previously described (22).

Enzymatic assays: For histone H1 kinase activity, protein A-sepharose beads coupled to immunoprecipitated proteins were incubated 20 min at 30°C with 0.2 mg/ml histones H1, 40 µg/ml peptide inhibitor of cAMP-dependent protein kinases, 0.1 µCi/µl [γ^{32}P]-ATP and 30 µM ATP in incubation buffer. Incubations were stopped with 1/2 vol of 3x Laemmli buffer, samples were heated 30 min at 60°C, boiled 5 min and analysed on SDS-PAGE (12 %). Radiolabeled histone H1 bands were cut and counted.

MAP kinase activity was assayed on peptide consensus sequence for MAP kinase as described earlier (23).

RESULTS AND DISCUSSION

The astrocytic cell line C.LT.T.1.1. shows a growth arrest by contact inhibition at the confluence. However, bFGF can release cells from this state and synchronically reinitiate DNA synthesis as shown by [^{3}H] thymidine incorporation (fig. 1a) and immunocytochemistry to detect cells incorporating Bromo Deoxy Uridine (fig. 1b). Forskolin, the adenylate cyclase activator which increases intracellular levels of cAMP prevented bFGF to reinitiate DNA synthesis (fig. 1a

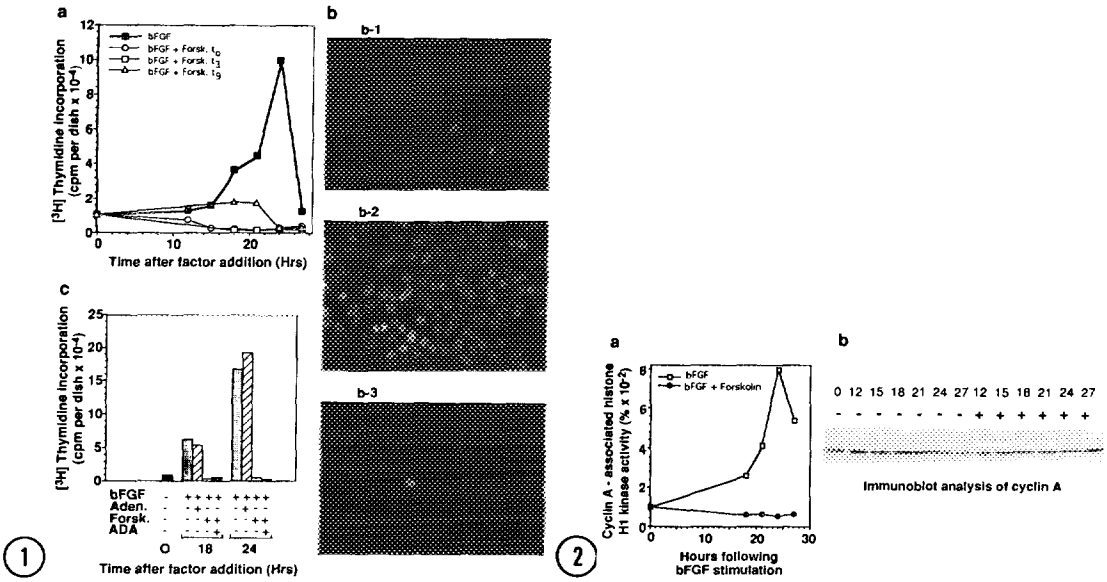

Figure 1. Inhibition of DNA synthesis by cAMP. (a) [^{3}H]-thymidine incorporation in cells induced by bFGF in the presence or in the absence of 10 μM forskolin, added at the same time (t_0), 3 hrs (t_3) or 9 hrs (t_9) after bFGF. (b) Immunocytochemical quantification of DNA synthesis by BrdU labeling in control cells (b-1) and bFGF-induced cells in the absence (b-2) or in the presence (b-3) of 10 μM forskolin. (c) Effect of adenosine on DNA synthesis and effect of adenosine deaminase (ADA) on cAMP-mediated inhibition of DNA synthesis. [^{3}H]-thymidine incorporation was measured after 0 hrs, 18 hrs or 24 hrs incubation with bFGF, bFGF + 0.1 mM adenosine (Aden.), bFGF + 10 μM forskolin (Forsk.), bFGF + 10 μM forskolin + 1 μg/ml ADA. Experiments presented here are representative of at least 3 independent experiments.

Figure 2. cAMP-mediated inhibition of Cyclin A-associated histone H1 kinase activity without change of cyclin A expression. (a) Histone H1 kinase activity associated to cyclin A immunoprecipitated from cell cultures. After addition of bFGF or bFGF + 10 μM forskolin cell cultures were stopped at 0, 18, 21, 24 and 27 hrs. The results are expressed as % of histone H1 kinase activity of untreated cells. (b) Cyclin A expression in cell extracts from cultures treated by bFGF with (+) or without (-) 10 μM forskolin. Cell cultures were stopped at 0, 12, 15, 18, 21, 24 or 27 hrs after growth factor addition. These figures are representative of at least 3 independent experiments.

and 1b). Similar results were obtained with 8-bromo-cAMP, the cell-permeable cAMP analogue (not shown).These results were shown when these factors were added between 0-9 hrs after bFGF addition (fig. 1a). Most of the experiments were performed with 10 μM forskolin or 1 mM 8-bromo-cAMP but complete inhibition was also observed with 2 μM forskolin or 0.2 mM 8-bromo-cAMP. Inhibition was not mimicked by adenosine (fig. 1c) which is usually released when cells are incubated with forskolin or 8-bromo-cAMP. In the same way, adenosine deaminase did not suppress the inhibition promoted by forskolin (fig. 1c).

Cyclin A synthetised in G1 can associate with cdk2, in early S phase, to form complexes which are active as histone H1 kinases. These activities participate in the control of G1/S boundary and contribute to promote the start of DNA synthesis and its progression. Cyclin A-associated histone H1 kinase activity contained in cell extracts immunoprecipitated by anti-cyclin A antibody was low in contact-inhibited cells. It increased after bFGF addition, mainly after a 18-hrs period to reach a maximal activity at 24 hrs (fig. 2a). Addition of forskolin completely

prevented histone H1 kinase increase (fig. 2a). As cyclin A-dependent histone H1 kinase activation is often preceded by induction of cyclin A accumulation (1,2), we speculated that the bFGF-induced increase of this activity and its inhibition by cAMP were due to modulations of cyclin A expression. Surprisingly, cyclin A expression was already high in contact-inhibited cells, was not increased during G1 and S phases and was not inhibited by cAMP (fig. 2b). Thus, the observed variations of cyclin A-dependent histone H1 kinase activity were independent of cyclin A expression. These observations prompted us to test other hypotheses to explain cAMP action on DNA synthesis. We studied its effects on other cyclin expression such as cyclin E and cyclin D1 which are generally required before cyclin A during cell cycle (1,2), and on the cdk2 activity associated with cyclins A and E. MAP kinase activation by growth factor can be required for entry in the cell cycle and is inhibited by cAMP in some cell types (17). We also studied the effects of cAMP on the activation of this enzyme.

In fig. 3b, an immunoblotting with anti-cyclin E antibody is shown. Cyclin E expression progressively increased between 12-15 hrs after exposure to bFGF, and it reached a maximum at 24 hrs. Forskolin greatly prevented its accumulation (fig. 3b). Cyclin E-dependent histone H1 kinase was activated in cells treated by bFGF. High activation was visible at 12 hrs and was maintained up to 21 hrs after bFGF addition. Forskolin strongly inhibited this activation (fig. 3a). In contrast to cyclin A-dependent histone H1 kinase, cyclin E-dependent histone H1 kinase activity was correlated with the modulations of cyclin E expression. Cdk2 is an important cdk which controls DNA synthesis. It can bind cyclin E in the middle of G1 and cyclin A after the start of the S phase (1,2). We showed that its expression was slightly increased when cells were stimulated by bFGF. This slight increase was apparently suppressed by forskolin (fig. 3c). Amplitude of the changes in cdk2 expression could not likely explain the changes in protein kinase activity observed for cyclin A- and E-dependent histone H1 kinases (fig. 2a and 3a). Fig. 3d shows that cyclin D1 expression was low in contact-inhibited cells. It was potently increased by bFGF exposure, earlier than that of cyclin E, and then it varied slightly through the cell cycle. Forskolin did not affect its level in early G1 (6 hrs), but severely reduced it in late G1 (between 13-19 hrs) and early S phases (21 hrs).

Kinetic of MAP kinase activation is shown in fig. 4a. Addition of bFGF promoted a rapid increase of MAP kinase activity measured on Myelin Basic Protein or peptide containing consensus sequence for MAP kinase substrates. Activity peaked at 10 min. and then progressively decreased. Addition of forskolin did not reduce this activation but rather increased it. Forskolin alone promoted a small activation of MAP kinase. Activation of this enzyme after bFGF addition was also followed by tyrosine phosphorylation on p42 and p44 proteins (fig. 4b) corresponding to isoforms of MAP kinase as confirmed by revelation with anti-MAP kinase antibody (fig. 4c). Stimulation of MAP kinase in bFGF-treated cells was associated with a shift of p42 isoform corresponding to reduced electrophoretic mobility (fig. 4c) as described in various systems. Forskolin neither reduced phosphotyrosine content of p42 and p44 isoforms (fig. 4b) nor suppressed the shift of p42 (fig. 4c), visible at all times where MAP kinase was activated (not shown).

This study shows, for the first time to our knowledge, that cAMP acting as an antiproliferative agent, can inhibit cyclin A- and cyclin E- dependent histone H1 kinase activation

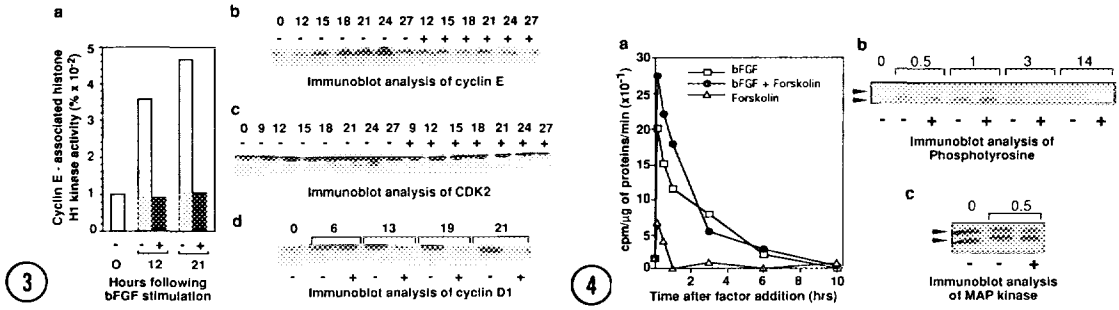

Figure 3. Inhibition by cAMP of cyclin E expression, cyclin E - associated histone H1 kinase activity and cyclin D1 expression. (a) Histone H1 kinase activity associated to cyclin E immunoprecipitated from cell cultures treated by bFGF with (+) or without (-) 10 µM forskolin. Cell cultures were stopped at 0, 12 and 21 hrs after exposure to factors and cyclin E-associated H1 kinase was immunoprecipitated. Results are expressed as % of H1 kinase activity of untreated cells. (b) Cyclin E expression in cell extracts from cultures treated by bFGF with (+) or without (-) 10 µM forskolin. Cell cultures were stopped at 0, 12, 15, 18, 21, 24, 27 hrs after factor addition. Note that the antibody recognizes two other bands (one minor at 56 kDa and one major at 85 kDa which is not regulated by growth factors and cAMP). (c) Cdk2 expression in cell extracts from cultures treated by bFGF with (+) or without (-) 10 µM forskolin. Cell cultures were stopped at 0, 9, 12, 15, 18, 21, 24, 27 hrs after factor addition. The antibody also detected additional minor bands, migrating at 28, 35, 45 and 80 kDa.(d) Cyclin D1 expression in cell extracts from cultures treated by bFGF with (+) or without (-) 10 µM forskolin. Cell cultures were stopped at 0, 6, 13, 19, 21 hrs after factor addition. These figures are representative of at least 3 independent experiments.

Figure 4. Absence of inhibition by cAMP of MAP kinase activation in cell cultures treated by bFGF. (a) Effect of cAMP on MAP kinase activity assayed on peptide consensus sequence for MAP kinase. Cultures of cells stimulated by bFGF, bFGF + 10 µM forskolin or 10 µM forskolin alone were stopped at 0, 10 or 30 min, 1, 3, 6, 10 hrs after factor addition. (b) Effect of cAMP on phosphotyrosine content of isoforms (p42/p44) of MAP kinase visualized by western blotting analysis. Cultures of cells treated by bFGF with (+) or without (-) 10 µM forskolin were stopped at 0, 30 min, 1, 3 and 14 hrs after factor addition. (c) Effect of cAMP on migration of isoforms (p42/p44) of MAP kinase visualized by western blotting analysis. Cell cultures treated by bFGF with (+) or without (-) 10 µM forskolin were stopped at 0 or 30 min after factor addition. Arrowheads in figs 4b and 4c show p42 and p44 MAP kinase isoforms.

during the cell cycle. It demonstrates that in C.LT.T.1.1. cells cyclin A-dependent histone H1 kinase activation is abolished without variation of cyclin A expression. This is opposite to cyclin E-dependent kinase, the activation of which is suppressed when cyclin E expression is reduced by cAMP. The cyclin D1 expression is also inhibited by cAMP, according to other observations (12-16). These results suggest that inhibition of cyclin D expression by cAMP is perhaps a general phenomenon. cAMP also suppresses the slight increase of cdk2 expression observed in bFGF-stimulated cells.

The mechanism of antiproliferative effect and changes in cyclin expression and cdk2 activity promoted by cAMP in C.LT.T.1.1. cells cannot be due to inhibition of MAP kinase. Indeed in these cells, cAMP moderately potentiates the effect of bFGF on this enzyme. It has also been recently reported that in PC12 cells cAMP activates MAP kinase (24), and in CCL39 cells, Prostaglandin E1 elevating cAMP levels, does not affect thrombin- or bFGF-stimulated MAP kinase activity while it strongly inhibits cell proliferation (25). It is possible that cAMP does not block early steps of bFGF-transduction in the astrocytic cell line, since we notice it is able to completely inhibit DNA synthesis even though if it is added 9 hrs after bFGF. cAMP must regulate cyclin D1 and E expressions through protein phosphorylation by cAMP-dependent

Protein Kinase (PKA). Cyclin D1 itself is a substrate for PKA (26). Its phosphorylation within cyclin box by PKA can obviously play a role in the antiproliferative action of cAMP. As it has been shown that the cyclin box mediates the interaction of cyclins with their respective cdk(s) (26), we can speculate that the post translational regulation of cyclin A by cAMP explain at least partially the inhibition of cyclin A-dependent protein kinase activity without modification of cyclin A level in C.LT.T.1.1. cells. The hypothesis of a PKA-dependent regulation could also involve cAMP Response Element-Binding (CREB) proteins which appear to be required for activation and repression of transcription (27). cAMP might regulate cyclin D1 and E expressions through phosphorylation by PKA of proteins of the CREB family. To progress in our understanding of cAMP action on cyclin-dependent protein kinases, it would be also interesting to know if recently identified cdk inhibitors (28) can be induced by cAMP. We cannot exclude that other regulators of cdks such as wee1 protein kinase, Cdk Activating Kinase (CAK), or protein phosphatases can be also affected by cAMP. Studies of the effects of cAMP on cyclins and cyclin-dependent protein kinases must be obviously brought together with the results using other inhibitors of DNA synthesis such as TGFβ, IFNs, TNFα known to modulate cyclin/cdks activities (3-13). Effects of TGFβ have been particularly well documented. It inhibits expression of some cyclins (4,7) and cdk activities (3, 5-7) and also prevents the formation of cyclin E-cdk2 complex (5). Furthermore, TGFβ provokes G1 arrest through $p27^{KIP1}$, a cyclin-cdk inhibitor (11) and induces expression of $p15^{INK1}$, a member of the $p16^{INK4}$ family, a cdk4 inhibitor (29). However, the transduction mechanisms of this growth factor are very little understood. It seems perhaps easier to progress in the knowledge of the molecular mechanisms of cAMP action since the primary intracellular target of cAMP is the well known cAMP-dependent protein kinase.

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