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Cyclic AMP inhibits mitogen-induced DNA synthesis in hamster fibroblasts, regardless of the signalling pathway involved

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Mitogen-induced initiation of DNA synthesis in quiescent Chinese hamster lung fibroblasts (CCL39) is strongly inhibited by 8-Br cAMP and cAMP-elevating agents (prostaglandin E_1, cholera toxin, isobutylmethylxanthine). This inhibition is reversible and occurs very early in G_0/G_1. As exponential growth is much less affected by increased cAMP, we propose that cAMP inhibits an early signal essential for the exit from G_0. CCL39 cells can be stimulated by α-thrombin, which activates phosphoinositide (PI) breakdown, as well as by mitogens (FGF or FGF+serotonin) which do not involve the PI pathway. Here we show that the action of both classes of mitogens is likewise inhibited by cAMP. Therefore, although PI breakdown is inhibited by cAMP in CCL39 cells, this effect cannot entirely account for the antimitogenic activity of cAMP. Other early steps of the mitogenic response must be also affected.

DNA synthesis; cyclic AMP; Growth factor; (Hamster fibroblast)

1. INTRODUCTION

The role of cAMP in growth control has been the subject of a large and controversial literature. While elevation of intracellular cAMP has been found to cause growth inhibition in a wide variety of cell types [1], it appears on the contrary to act as a mitogenic signal in some cells such as Swiss 3T3 cells [2]. The reasons for these discrepancies are still largely unknown, but one possibility is that the sensitivity to cAMP of the mitogenic signalling pathways varies from tissue to tissue. We have recently proposed that growth factors can be divided into two classes which act through distinct signal-transduction pathways [3]. Receptors for the first class possess a tyrosine kinase activity [4], whereas receptors for the second are coupled through GTP-binding proteins to various effectors including a phosphoinositide-specific phospholipase C [5]. Most interestingly, phosphoinositide hydrolysis is inhibited by cAMP in some cell types [6–9] but not in others [9–12]. Here, we have therefore addressed the question of a possible correlation between the inhibitory effects of cAMP on phosphoinositide breakdown and reinitiation of DNA synthesis, using a Chinese hamster lung fibroblast line (CCL39), which responds to either class of mitogens. DNA synthesis can be induced in these cells by thrombin [13], with stimulation of inositol lipid metabolism [14] or by FGF without any significant phosphoinositide hydrolysis [15]. Here we show that cAMP, which inhibits thrombin-induced inositol phosphate formation [6,16], strongly inhibits the mitogenic response to thrombin but also that to FGF, which indicates that the inhibitory activity of cAMP is not restricted to the inositol pathway.

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Abbreviations: FGF, fibroblast growth factor; IBMX, 3-isobutyl-1-methylxanthine

2. MATERIALS AND METHODS

2.1. *Materials*

Highly purified α-thrombin (2660 NIH units/mg) was generously provided by Dr J.W. Fenton ii (New York State Department of Health, Albany, NY). Recombinant basic FGF

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was a gift from Dr D. Gospodarowicz (University of California, Medical Center, San Francisco, CA). Prostaglandin E_1 (PGE_1), cholera toxin and serotonin were from Sigma. 8-Br cAMP was purchased from Boehringer Mannheim. [^{3}H]Thymidine was from Amersham.

2.2. *Cell culture*

The Chinese hamster lung fibroblast line (CCL39) was maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% (v/v) fetal calf serum, antibiotics (50 U penicillin/ml and 50 μg streptomycin/ml) in a humidified atmosphere of 5% CO_2/95% air.

2.3. *DNA synthesis*

Cells grown to confluence in 24-well culture plates were arrested in G_0 by 24 h incubation in serum-free medium (1 : 1 ratio of DMEM : Ham's F-12). Cells were then incubated with the mitogens and the different agents to be tested as indicated in the figure legends. Reinitiation of DNA synthesis was determined either by [^{3}H]thymidine (1 μCi/ml, 4.5 μM) accumulation over 24 h, or [^{3}H]thymidine (2 μCi/ml) incorporation during a 2 h pulse at different time points in the cell cycle. At the end of the incubation period, radioactivity in trichloroacetic acid-precipitable material was determined after solubilization of the cells in 0.1 N NaOH.

2.4. *Cell growth assay*

Cells were plated at an initial density of 2×10^4 cells per 35-mm culture dish in 2 ml DMEM plus 10% FCS. After 24 h incubation to allow for cell attachment, the medium was changed to DMEM plus 10% FCS and various agents as described in the figure legends. For determination of cell number, cells were removed from the wells with trypsin and counted in a Coulter counter.

3. RESULTS

Reinitiation of DNA synthesis can be induced in quiescent CCL39 cells by either thrombin [13] or FGF alone [15], or more efficiently by combinations of FGF and thrombin [17] or FGF and serotonin [18]. Fig.1 shows that whatever the mitogen or the combination of mitogens used, DNA synthesis is strongly inhibited by elevation of intracellular cAMP. A dose-dependent decrease in [^{3}H]thymidine incorporation was caused by activation of adenylate cyclase, whether receptor-mediated with PGE_1 (fig.1A) or nonreceptor-mediated with cholera toxin (fig.1B), as well as by addition of the cAMP derivative 8-Br cAMP (fig.1C). Half-maximal inhibition was observed around 3×10^{-8} M PGE_1, 0.2 ng/ml cholera toxin and 0.1–0.2 mM 8-Br cAMP. The response to FGF was completely abolished by the three inhibitors, whereas thrombin-induced DNA synthesis appeared slightly less sensitive to PGE_1 and cholera toxin, with maximal inhibition of 75 and 60%, respectively. Initiation of DNA synthesis in CCL39 cells was also strongly inhibited by IBMX, an inhibitor of cyclic nucleotide phosphodiesterase, with an IC_{50} of about 0.1 mM (not shown).

To determine at which stage of the cell cycle

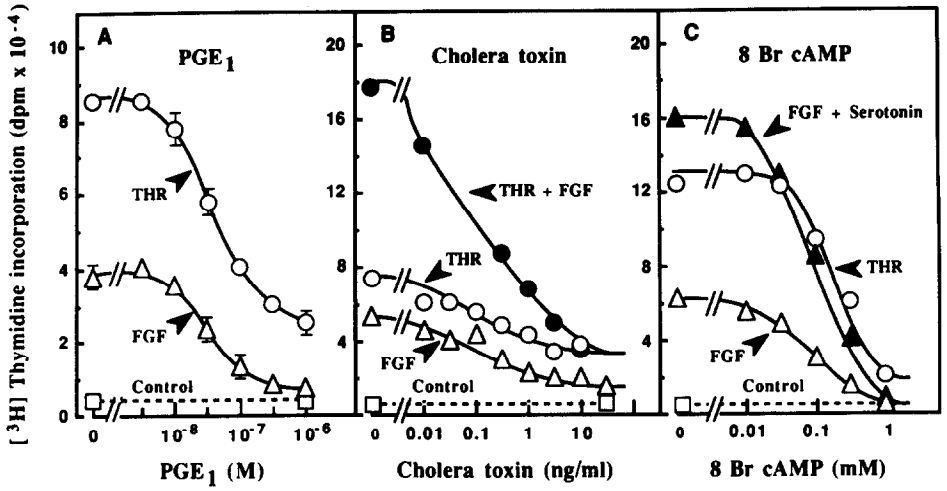

Fig.1. Inhibition of mitogen-induced DNA synthesis in CCL39 cells by PGE_1, cholera toxin or 8-Br cAMP. G_0-arrested CCL39 cells were incubated for 24 h with [^{3}H]thymidine (1 μCi/ml) in the absence (□) or presence of various mitogens: 1 U/ml thrombin (○) or 20 ng/ml FGF (△) in A; 0.1 U/ml thrombin (○), 20 ng/ml FGF (△) or 0.1 U/ml thrombin + 20 ng/ml FGF (●) in B; 1 U/ml thrombin (○), 20 ng/ml FGF (△) or 20 ng/ml FGF + 1 μM serotonin (▲) in C. PGE_1 (A), cholera toxin (B) or 8-Br cAMP (C) were added together with the mitogens at the indicated concentrations. Results are means of duplicate determinations.

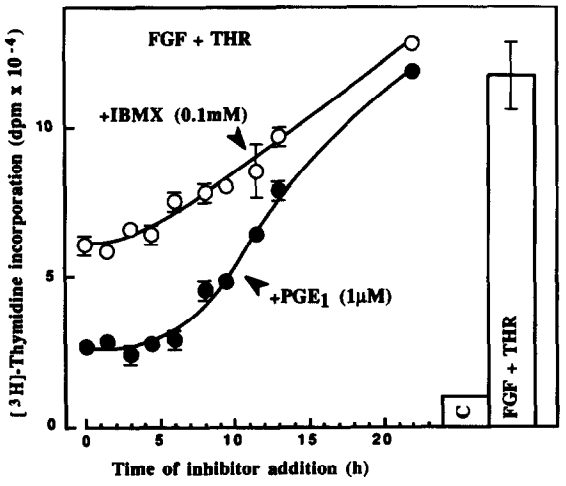

Fig.2. Time-dependent inhibition by cAMP of mitogen-induced DNA synthesis in CCL39 cells. G_0-arrested cells were incubated at time 0 with [^{3}H]thymidine (1 μCi/ml) and 20 ng/ml FGF + 10^{-3} U/ml thrombin. At the times indicated, cultures received either 1 μM PGE_1 (●) or 0.1 mM IBMX (○), and [^{3}H]thymidine incorporation was measured 24 h after growth factor addition. Bars represent [^{3}H]thymidine incorporation in the absence of inhibitor, in unstimulated control cells (C) or in stimulated cells (FGF + THR).

cAMP exerts its inhibitory effect, cAMP-elevating agents were added at various times after the mitogens. Fig.2 shows that the inhibition of DNA synthesis was progressively attenuated when the additions of PGE_1 or IBMX were delayed, with a 50% reduction after 13 h, regardless of the degree of the maximal inhibition (PGE_1 was used at a maximally inhibitory concentration whereas IBMX was used at an IC_{50}). Similar data were obtained with FGF alone (not shown). These results clearly indicate that the inhibitory activity of cAMP occurs very early in the G_0/G_1 phase of the cell cycle, irrespective of the mitogens used.

The reversibility of cAMP inhibition was investigated in fig.3. PGE_1 was chosen to induce a transient elevation of cAMP, because this compound can be readily washed out, as checked by the return of adenylate cyclase activity to basal level (not shown). After 4 h exposure to 1 μM PGE_1, thrombin- or FGF-stimulated CCL39 cells entered the S phase but with a marked delay, which at least in thrombin-stimulated cells roughly corresponded to the length of PGE_1 treatment. Thus, elevation of cAMP at the moment of mitogen ad-

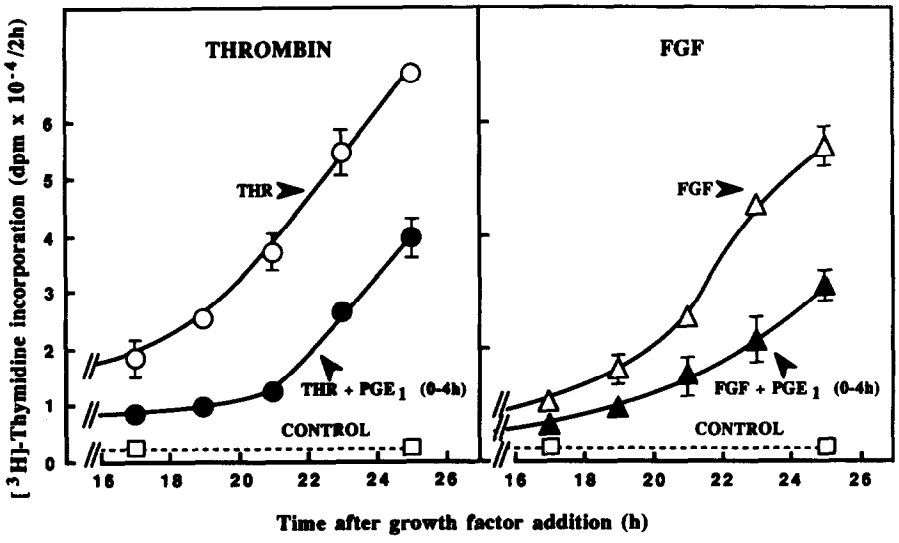

Fig.3. Effect of short exposure to PGE_1 on thrombin- and FGF-induced DNA synthesis in CCL39 cells. G_0-arrested cells were incubated at time 0 with (●, ▲) or without (○, △, □) 1 μM PGE_1 in the presence of 1 U/ml thrombin (○,●), 20 ng/ml FGF (△, ▲) or without growth factor (□). After 4 h the medium was removed and cultures were washed four times to eliminate PGE_1 (control cultures without PGE_1 were treated in the same way). Then mitogens were added back in fresh medium and incubations continued. The incorporation of [^{3}H]thymidine (2 μCi/ml) was monitored at 2-h intervals from 16 to 24 h after the stimulation by mitogens. Values are plotted at the times corresponding to the middle of the pulses and represent means ± range of duplicate determinations.

dition appears to block reversibly cells at a point in G_0 or early G_1.

To determine whether exponentially growing cells are as sensitive to cAMP as G_0-arrested cells, serum-induced proliferation was measured in the presence of 1 μM PGE_1 or 1 mM 8-Br cAMP (fig.4). Clearly cell growth was much less affected by cAMP than reinitiation of DNA synthesis in quiescent cells, since 1 μM PGE_1 did not change the growth rate whereas it reduced serum-induced DNA synthesis by 60% in quiescent cells (fig.4, inset). Significant inhibition of growth (by about 50%) was caused by 1 mM 8-Br cAMP but it should be noted that this concentration inhibited the reinitiation of DNA synthesis by ~90% in quiescent cells (fig.4, inset). A lower concentration (0.2 mM), which gave half-maximal inhibition of serum-induced DNA synthesis in G_0-arrested cells, did not affect growth of cycling cells (not shown).

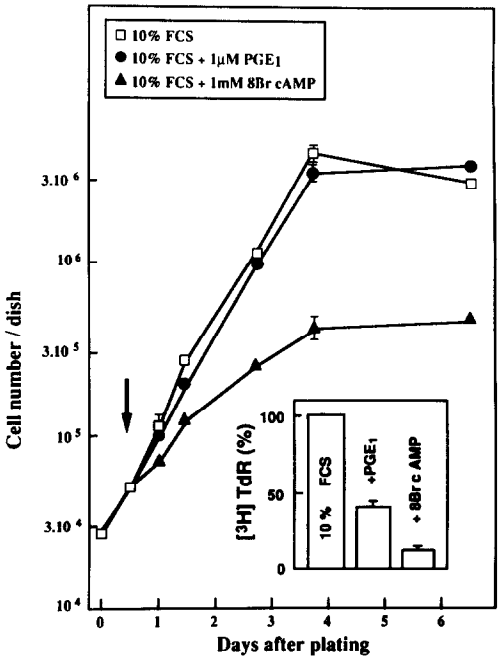

Fig.4. Effect of PGE_1 and 8-Br cAMP on growth of CCL39 cells. Cells were plated as indicated in the text and at the time indicated by the arrow received fresh medium containing 10% FCS with 1 μM PGE_1 (●), 1 mM 8-Br cAMP (▲) or no addition (□). Medium was replaced daily and cell number was determined at the times indicated. (Inset) G_0-arrested cells were incubated for 24 h with [^{3}H]thymidine (1 μCi/ml) and 10% FCS (first bar), 10% FCS + 1 μM PGE_1 (second bar) or 10% FCS + 1 mM 8-Br cAMP (third bar). Results are means of triplicate determinations and are expressed relative to [^{3}H]thymidine incorporation in control cells (100% = 2 × 10^5 dpm/well).

4. DISCUSSION

We report here that reinitiation of DNA synthesis by mitogens in G_0-arrested CCL39 cells is strongly inhibited by elevation of intracellular cAMP, irrespective of the method used to increase cAMP. Similar results were obtained with PGE_1, cholera toxin, IBMX and 8-Br cAMP. The inhibition is reversible, since it is suppressed by wash-out of cAMP-elevating agents, and it occurs at a very early stage in the progression from G_0 to S phase. This observation is in agreement with studies in other cell types [1,19,20] but in contrast with other reports describing an inhibitory effect of cAMP in late G_1 [21,22]. Proliferation of CCL39 cells in the presence of 10% fetal calf serum is markedly less sensitive to elevated cAMP. This is not due to the presence in serum of growth factors which allow the cells to by-pass the cAMP-sensitive step, because serum-induced reinitiation of DNA synthesis in G_0-arrested cells was inhibited by PGE_1 and 8-Br cAMP to an extent very similar to that in thrombin-stimulated cells (figs 1,4, inset). Moreover, the restimulation by serum of G_0-arrested cells was similarly inhibited by cAMP in confluent and subconfluent cultures (not shown), which indicates that high cellular density is not required for cAMP-induced inhibition, in contrast with findings in human fibroblasts [23]. Thus, taken together, our data support the contention that in CCL39 cells increased cAMP primarily affects a mitogenic signal which is essential for the exit of resting cells from G_0 but less important for logarithmically growing cells.

However, the most important finding of this study is that cAMP inhibits the mitogenic response to all of the growth-promoting agents for CCL39 cells, irrespective of the signalling pathway activated. Whereas phosphoinositide hydrolysis is believed to play an important role in the mitogenic activity of thrombin [17], it has been shown that this pathway is not involved in FGF-induced mitogenesis [15] or in the potentiating effect of serotonin on FGF-induced DNA synthesis [18]. Since cAMP inhibits the mitogenic response of CCL39 cells to FGF alone or to FGF + serotonin as well as the response to thrombin, it is clear that

cAMP-mediated inhibition of G_1 progression is not solely caused by inhibition of the inositol lipid pathway that cAMP-elevating agents elicit in CCL39 cells [6,16]. Therefore, our results suggest that increased cAMP inhibits, presumably through activation of protein kinase A [24], other early steps of the mitogenic response, in addition to phosphoinositide hydrolysis. These putative sites of inhibition may be located either on the tyrosine kinase-activated signalling pathway, or more downstream of phospholipase C and tyrosine kinases, on some step common to both pathways.

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