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Elevated Intracellular Concentrations of Cyclic AMP Inhibited Serum-Stimulated, Density-Arrested BALB/c-3T3 Cells in Mid G_1

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The stimulation of DNA synthesis in quiescent, density-arrested BALB/c-3T3 cells by platelet-derived growth factor in plasma-supplemented medium was inhibited by the presence of isobutylmethylxanthine (IBMX) and cholera toxin, although neither IBMX or cholera toxin when used alone inhibited the stimulation of DNA synthesis. The cells were reversibly inhibited in mid G_1 at a point 6 hr prior to the initiation of DNA synthesis. The inhibition of cell cycle traverse was associated with a 10–15 fold increase in cellular cyclic AMP concentration over basal levels. The reversal of this inhibition by removal of IBMX was correlated with a dramatic decrease in cyclic AMP levels. The traverse of late G_1 and the initiation of DNA synthesis after release from the cholera toxin and IBMX inhibition was dependent on the presence of plasma in the medium. Either somatomedin C (10–20 ng/ml) or insulin (10^{-6}–10^{-5} M) completely replaced the plasma requirement for late G_1 progression and entry into S phase. Once the inhibited cells were released from the IBMX and cholera toxin block a subsequent increase in cyclic AMP did not prevent entry into S phase. The presence of cholera toxin alone inhibited the stimulation of human dermal fibroblasts. The elevation of intracellular cyclic AMP levels in the human dermal fibroblasts by cholera toxin was two to three fold greater than that found in the BALB/c-3T3 cells in the presence of cholera toxin and IBMX.

Key words: cyclic AMP, BALB/c-3T3 cells, mid G_1, DNA synthesis

Platelet-derived growth factor (PDGF) initiates cell growth by rendering quiescent BALB/c-3T3 cells competent; the progression of competent cells through G_0/G_1 and entry into S phase proceeds by an ordered sequence of events dependent on the continuous presence of plasma-derived factors [1,2]. Two plasma specific G_1

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arrest points have been previously described in stimulated quiescent BALB/c-3T3 cells: V and W. The V point was operationally defined as being 6 hr prior to S phase and the W point was determined to be located at the G_1/S boundary and it apparently was the point of commitment to DNA synthesis [2,3].

In a previous paper we have shown that a transient increase in intracellular cyclic AMP level potentiated the action of competence factors to render quiescent BALB/c-3T3 cells competent to initiate cellular proliferation [4]. However, a dichotomous effect of cyclic AMP could be expected from previously reported data. In Swiss 3T3 cells cholera toxin was mitogenic when added alone [5], while in epithelial cells the toxin potentiated the action of serum factors [6]. However, cholera toxin has also been reported to be a potent inhibitor of proliferation. Hollenberg and Cuatrecasas [7] showed that cholera toxin inhibited serum-stimulated human fibroblasts in G_1 by a mechanism that was postulated to involve increases in cyclic nucleotide concentrations. In addition, high levels of cyclic AMP analogs inhibited protease-stimulated cell cycle traverse in Swiss 3T3 cells [8], as well as serum-stimulated human fibroblasts [9] and bovine adrenocortical cells [10]. In the experiments reported in this paper we have investigated the inhibitory effects of cyclic AMP as compared to the cyclic AMP growth potentiating actions that we have reported for the same culture system [4]. Although the cholera toxin induced increase in cyclic AMP was sufficient to potentiate the induction of cellular proliferation of BALB/c-3T3 cells, it did not inhibit the initiation of DNA synthesis. We have now demonstrated, however, that the addition of isobutylmethylxanthine (IBMX) with cholera toxin to density-arrested BALB/c-3T3 cells enhanced, potentiated, and sustained the elevation of intracellular cyclic AMP concentration and produced inhibition in cell cycle traverse at mid-G_1.

MATERIALS AND METHODS

Cell Culture and DNA Synthesis

BALB/c-3T3 cells (clone A31) were grown to confluence in Nunc (0.3 cm^2) 96-well microtiter plates in 0.2 ml Dulbecco-Vogt Modified Eagles medium (DME) containing 10% calf serum, 4 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2. Two to three days after the cells reached confluence the spent medium was removed and medium containing 5 μCi/ml ^{3}H-thymidine (6.7 Ci/mmole) plus the appropriate supplements was added. DNA synthesis was stopped by the addition of 1 M ascorbic acid and the plates were washed with phosphate buffered saline and fixed with two 10-min applications of methanol. After extensive washing with water the cultures were processed for autoradiography by the method described by Antoniades et al [11]. The data illustrated show results from typical experiments.

Human dermal fibroblasts were derived from newborn foreskin and were maintained in minimal essential medium (MEM) supplemented with 10% calf serum and 2 mM glutamine. To measure DNA synthesis, cells were grown to confluence in Limbro Multiwell plates. When the cells had been density-arrested for 2–3 days, epidermal growth factor (EGF) was added directly to the spent medium so that the final concentration was 10 ng/ml. Twenty-four hrs later the cells were pulsed for 60 min with ^{3}H-thymidine (5 μCi/ml, 6 Ci/mM). Cultures were then fixed with 5% trichloroacetic acid (TCA), washed extensively with water, solubilized in 0.1 M NaOH 1% SDS and measured by liquid scintillation counting.

Cyclic Nucleotide Assay

Cells were grown to confluence in 35-mm culture dishes. At the appropriate times after the addition of fresh medium the cultures were washed with cold, serum-free DME and extracted with cold 5% TCA. Cyclic AMP was then purified by ion exchange chromatography [12] and measured by radioimmunoassay [13].

Preparation of Serum Components and Growth Factors

Platelet-poor plasma (PPP) was prepared from freshly drawn human venous blood. The tubes were centrifuged at 4°C (2500 g for 30 min) to pellet the formed elements. The supernatant was carefully aspirated and recentrifuged, after which it was incubated at 37°C for 3–5 hr and the fibrin clot was removed. The platelet-poor plasma was heat inactivated at 56°C for 30 min and stored at −20°C.

Partially purified PDGF was prepared from boiled extracts of outdated human platelets that were passed through CM Sephadex as described by Antoniades et al [14]. Active fractions were collected, pooled, and stored at −20°C.

Epidermal growth factor was prepared from extracts of male submaxillary glands according to the method of Savage and Cohen [15]. Somatomedin C was a gracious gift from Dr. Judson J. Van Wyk and was prepared by the method of Svoboda et al [16]. Multiple criteria showed it to be not less than 95% pure. Fibroblast growth factor (FGF) was a gift from Dr. Denis Gospodarowicz.

Chemicals and Reagents

DME was obtained from Flow Laboratories (McLean, Virginia) and calf serum from Colorado Serum Company (Denver, Colorado). Isobutylmethylxanthine was purchased from Aldrich Chemical Company, Inc. (Milwaukee, Wisconsin), cholera toxin from Schwartz-Mann (Orangeburg, New York) and ^{3}H-thymidine from ICN (Irvine, California). Antibody to cyclic AMP was purchased from Biotek Inc. (Shawnee, Kansas). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, Missouri).

RESULTS

IBMX Inhibition of Serum-Stimulated BALB/c-3T3 Cells in the Presence of Cholera Toxin

Cholera toxin potentiated serum stimulation of DNA synthesis in density-arrested BALB/c-3T3 cells (Fig. 1A). This potentiated stimulation was apparently due to an effect of an increased cyclic AMP level of PDGF-induced competence formation [4]. Although cholera toxin brought about a significant increase in cyclic AMP levels (5–6-fold) it did not inhibit cellular proliferation. It was noticed however, that by 2 hr after cholera toxin addition the increased cyclic AMP levels had decreased to half their maximum stimulated level [4].

In order to determine whether a sustained increase in cyclic AMP would inhibit the initiation of DNA synthesis quiescent cells were stimulated with serum-supplemented medium containing cholera toxin and IBMX for the length of the experiment (36 hr). IBMX produced a concentration dependent decrease in the number of cells capable of initiating DNA synthesis (Fig. 1B) with 90% inhibition of serum-stimulated DNA synthesis at a concentration of 0.01 mM IBMX. It must be emphasized that this inhibition was observed when IBMX and cholera toxin were present

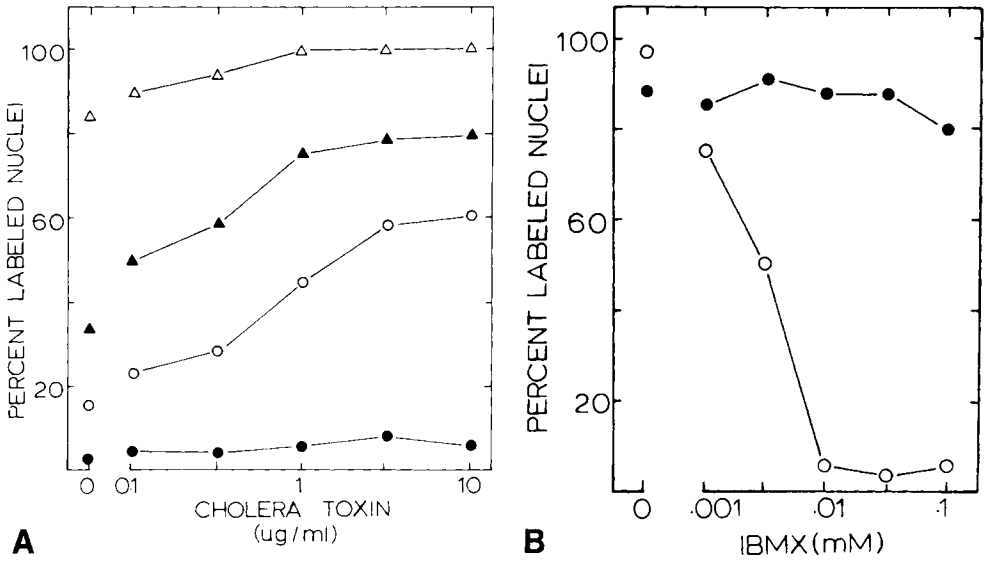

Fig. 1. (A) Effects of cholera toxin on serum stimulated 3T3 cells. Quiescent density-arrested cells were stimulated at 0 hrs with fresh medium containing 5 μ Ci/ml ^{3}H-thymidine and either 0.25% (●), 2.5% (○), 5% (▲), or 10% (△) calf serum plus the indicated concentrations of cholera toxin. After an incubation of 36 hr the cells were fixed, processed for autoradiography, and the percentage of cells incorporating ^{3}H-thymidine was determined. (B) Inhibition by IBMX to serum-stimulated 3T3 cells. Quiescent density-arrested cells were stimulated with DME supplemented with 10% calf serum (●) or 10% calf serum plus 1 μg/ml cholera toxin (○) and ^{3}H-thymidine. IBMX was added to both cultures at the indicated concentration and the percentage labeled nuclei was determined 36 hr later.

continuously during the incubation of quiescent cells with the fresh serum-supplemented medium. These experimental results are contrasted by the potentiation of serum stimulation of quiescent cells when IBMX was present for only the first 5 hr during an exposure to PDGF and then removed for the remainder of the experiment [4].

IBMX and Cholera Toxin Inhibited Serum-Stimulated Cells at Mid-G_0/G_1

The inhibition of the initiation of DNA synthesis after the addition of fresh serum-supplemented medium containing cholera toxin and IBMX to density-arrested cells could occur by several different mechanisms. Since it was previously reported that methylxanthines were capable of inhibiting the intracellular transport of nucleic acid precursors, as well as a variety of other compounds [17], one possible mechanism was that the cells had entered S phase and we had simply observed an effect on uptake of ^{3}H-thymidine thereby resulting in no ^{3}H-thymidine incorporation into DNA. This explanation was unlikely since IBMX did not inhibit DNA synthesis unless cholera toxin was also present (Fig. 1B) and the addition of IBMX to cells in S phase did not inhibit ^{3}H-thymidine incorporation (data not shown). Alternative mechanisms could be that IBMX inhibited the culture at either random locations in G_1 or at a specific G_1 arrest point. To delineate between these last two mechanistic possibilites the experiments illustrated in Figure 2 were performed. Density-arrested

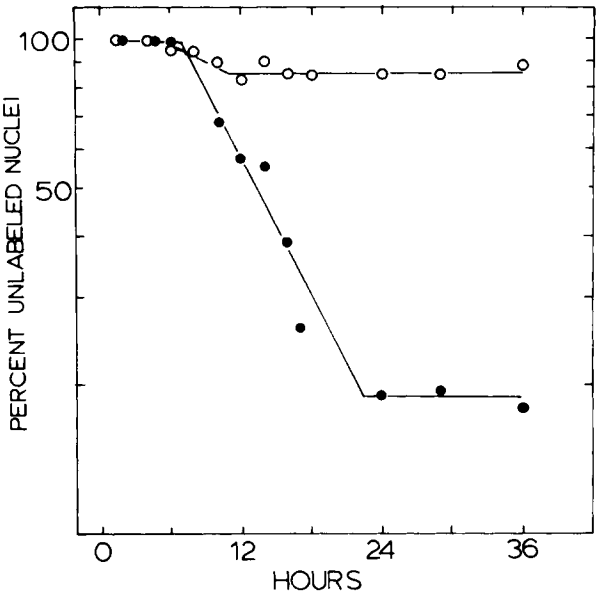

Fig. 2. IBMX and cholera toxin inhibit 3T3 cells at the V point. BALB/c-3T3 cells were incubated with medium supplemented with PDGF (1.06 μg), PPP (5%), cholera toxin (1 μg/ml), and IBMX (0.1 mM) for 11 hr. The cultures were washed and either DME (○) or DME supplemented with 5% PPP (●) was added. Medium contained 5 μ Ci/ml ^{3}H-thymidine. At the indicated times the cells were fixed by the addition of 1 M ascorbic acid and the number of labeled nuclei determined.

BALB/c-3T3 cells were incubated in medium with PDGF, PPP, cholera toxin, and IBMX for 11 hours. The cultures were then washed and placed in DME containing only ^{3}H-thymidine or DME supplemented with 5% PPP plus ^{3}H-thymidine. At the indicated times after the removal of IBMX the cultures were fixed, autoradiography performed and the number of labeled nuclei was determined. The addition of PPP-supplemented medium permitted the cells to enter S phase after a minimum lag of approximately 6 hr from the time of removal of the IBMX, while cells transferred to medium alone did not enter DNA synthesis (Table I and Fig. 2). At present we have operationally termed this point the V point [2]. As seen in Figure 2 and Table I plasma was required for the continued progression of G_1 after the IBMX was removed from the cultures.

Somatomedin C could substitute for the plasma requirement in allowing progression of the last 6 hr of G_1 and entry into S phase (Table I). Insulin concentrations of 10^{-6}–10^{-5} could be used instead of somatomedin C (Table I). At these hyperphysiological concentrations, insulin has been shown to bind to somatomedin C receptors in a wide variety of tissues and is believed to function as a somatomedin-like peptide [18]. Addition of unsupplemented medium of DME supplemented with either EGF, PDGF, or FGF could not substitute for plasma in allowing the culture to enter S phase (data not shown). Incubation of stimulated cell cultures with cholera toxin and IBMX for times as long as 18 hr before removal of IBMX did not alter the 6-hr lag before initiation of DNA synthesis (data not shown). The data in

TABLE I. Somatomedin C Commits Cells to DNA Synthesis After Release From Cholera Toxin V Point

	Percentage labeled nuclei
DME	14
Somatomedin C (20 ng/ml)	75
Insulin (10^{-5}M)	75
PPP (5%)	82

Quiescent BALB/c-3T3 cells were inhibited at the V point as in Fig. 2. The cultures were then washed and either DME or DME supplemented with the indicated growth factors plus ^{3}H-thymidine was added. The percentage labeled nuclei was determined 24 hr later.

TABLE II. Cholera Toxin and IBMX Increase cAMP Concentrations in V Point Arrested Cells

	pmoles cAMP/plate	Fold increase
No addition (0 time)	1.07	1
PDGF (5.3 μg)	1.84	1.72
PPP (10%)	1.30	1.22
PDGF + PPP	1.92	1.79
PDGF + PPP + cholera toxin (1 μg/ml)	4.09	3.82
PDGF + PPP + IBMX (0.1 mm)	2.30	2.15
PDGF + PPP + cholera toxin + IBMX	13.91	13.00

Quiescent 3T3 cells were incubated for 12 hr in DME supplemented with the indicated factors. The plates were then washed and cyclic AMP levels were measured. The cell numbers did not vary during the course of the experiment and were determined to have been 4.5×10^5 cells/P-35 plate.

Figure 2 clearly show that although the initial 11-hr incubation in cholera toxin and IBMX had been sufficient time for progression past mid-G_1, there was a 6-hr lag before DNA synthesis after removal of IBMX. Since the entry into DNA synthesis resulted in a kinetic profile similar to entry into S phase after normal serum stimulation, these results were consistent with the hypothesis that the inhibition observed with cholera toxin and IBMX was due to growth arrest at a unique point six hours prior to DNA synthesis: the V point as operationally defined by Pledger et al [2].

cAMP Concentrations Were Increased in Cells Arrested at Mid-G_1

The addition of IBMX and cholera toxin to PDGF and PPP-supplemented medium was capable of inhibiting BALB/c-3T3 cells at a point operationally defined as the V point: 6-hr prior to S phase (Fig. 2). When IBMX was omitted, or was present in the absence of cholera toxin (with PDGF and PPP), there was no inhibition in the number of cells which initiated DNA synthesis (data not shown). Since both cholera toxin and IBMX are known to bring about increased intracellular concentrations of cAMP, and elevated cAMP levels have been implicated in growth inhibition in a variety of cell types [19,20], we measured cAMP levels in the cholera toxin and IBMX inhibited BALB/c-3T3 cells (Table II). Density-arrested cells were stimulated with PDGF in plasma-supplemented medium in the presence or absence of cholera toxin (1 μg/ml) and IBMX (0.1 M) for 12 hr. The addition of cholera toxin or IBMX to PDGF and PPP resulted in a 3.82- or 2.15-fold increase, respec-

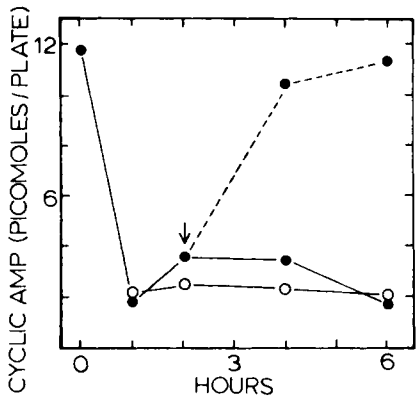

Fig. 3. Cyclic AMP levels fall after IBMX removal. BALB/c-3T3 cells were grown to confluence in 35 mm culture didshes. The spent medium was removed and 1 ml DME supplemented with PDGF (5.3 μg), PPP (10%), cholera toxin (1 μg/ml) and IBMX (0.1 mM) was added. After a 12-hr incubation (0 time) at 37°C the plates were washed and refed with either DME (○) or DME containing 10% PPP (●). Two hours after PPP addition duplicate cultures were remade 0.1 mM in IBMX (– – –). The arrow indicates readdition of IBMX. Cyclic AMP levels were determined at the indicated times for all conditions.

tively, over basal levels of cAMP. However, when cholera toxin and IBMX were simultaneously present in the PDGF and PPP-supplemented medium cAMP was maintained at a level 13.0-fold greater than basal levels. Therefore, if the inhibition seen with cholera toxin and IBMX was mediated by elevated cAMP concentrations these results suggest that a critical level of cAMP is necessary before inhibition occurs. Though we have demonstrated an increase in total intracellular cAMP, the data are also compatible with a compartmentalized increase in cAMP, only occurring in the presence of both cholera toxin and IBMX, mediating the inhibition in progression.

Cyclic AMP Decreased After IBMX Removal and Progression of G_1 Resumed

For cholera toxin and IBMX inhibited cells to continue progression it is necessary, but not sufficient, for cAMP levels to be decreased. This is illustrated in Figure 3. Density-arrested BALB/c-3T3 cells were incubated for 12 hr in medium containing PDGF, PPP, cholera toxin, and IBMX. The cultures were washed with DME and refed with either DME or DME supplemented with 5% PPP. At the indicated times cAMP levels were determined. The removal of IBMX and the addition of either DME or DME supplemented with PPP resulted in a significant decrease in cAMP levels. This decrease in cAMP, however, was in itself not sufficient to allow progression through the last 6 hr of G_1 since the cells did not enter S phase unless plasma or somatomedin C was present (Table I and Fig. 2).

When stimulated cultures that were inhibited by cholera toxin and IBMX were released from this inhibition by the removal of IBMX and refed with medium containing PPP, there was an immediate decrease in cAMP levels followed by the initiation of DNA synthesis 6 hr later (Figs. 2 and 3). The readdition of 0.1 mM IBMX 2

hr after this release into PPP-supplemented medium resulted in a second increased cAMP concentration that was equal to that sufficient to arrest cells at the V point (Fig. 3). This increase in cAMP, however, was not capable in itself of preventing entry into S phase since IBMX addition from 0.25–8 hr after the release did not inhibit the culture from entering DNA synthesis (Table III).

Cholera Toxin Inhibited Serum Stimulation of Human Dermal Fibroblasts

Although IBMX had to be present with cholera toxin to inhibit the stimulation of DNA synthesis in BALB/c-3T3 cells, cholera toxin (5ng/ml) alone completely inhibited quiescent human dermal fibroblasts from entering DNA synthesis in response to serum supplemented medium [data not shown and 7]. Since cholera toxin potentiated the action of competence factors on BALB/c-3T3 cells [4] and progression was only inhibited when cAMP levels were elevated and sustained above a critical threshold (Fig. 1), it is possible that the differing responses to cholera toxin (stimulation versus inhibition) by BALB/c-3T3 cells and human dermal fibroblasts could be due to the relative cAMP levels attained in each cell line after cholera toxin treatment. The results of cAMP measurements in response to 5 ng/ml cholera toxin on human dermal fibroblasts are consistent with that hypothesis. A forty-fold increase in cAMP was seen 6 hr after treatment of dermal fibroblasts with medium supplemented with nanogram concentrations of cholera toxin alone (Fig. 4) and was maintained for the duration of the G_1 period (data not shown). For elevated cAMP concentrations to be maintained in BALB/c-3T3 cells at a level necessary for inhibition a concentration of 1 μg/ml cholera toxin as well as the presence of a phosphodiesterase inhibitor was required (Table II). The requirement for the addition of IBMX to obtain a sustained elevation in cAMP sufficient to inhibit serum stimulation suggests that the phosphodiesterase(s) in BALB/c-3T3 cells may be more active or inducible than that in the dermal fibroblasts. However, a sustained elevation of cyclic AMP accompanied the inhibition of DNA synthesis in both BALB/c-3T3 and human dermal fibroblasts although IBMX was required in BALB/c-3T3 cells. Various cell types may require different conditions to sustain elevated cyclic nucleotide levels.

DISCUSSION

The reported actions of increased concentrations of cyclic AMP on cellular proliferation have varied dramatically. This may be, in part, a result of cyclic AMP having both stimulatory and inhibitory effects in the cell cycle. Previous reports have implicated a role(s) for cyclic nucleotides in growth inhibition of serum-stimulated cultures. Various cell lines show growth inhibition in differing portions of the cell cycle. For example, Dipasquale and McGuire [21] found that dibutyryl cAMP arrested Cloudman melanoma cells in late S and G_2 while Willingham et al [22] reported that BALB/c-3T3 cells were sensitive to dibutryl cAMP in early G_1. Similar results were obtained by Rechler et al [9]. In contrast to these reports of their inhibitor activity, Pruss and Hershman [5] demonstrated a mitogenic activity for cholera toxin in Swiss 3T3 cells while Taylor-Papadimitrious et al [6] reported similar findings for human mammary epithelial cells. In light of this apparent dichotomy regarding increased intracellular cyclic nucleotide concentrations, we have examined the effects of increased cAMP during G_0/G_1 progression of BALB/c-3T3 cells.

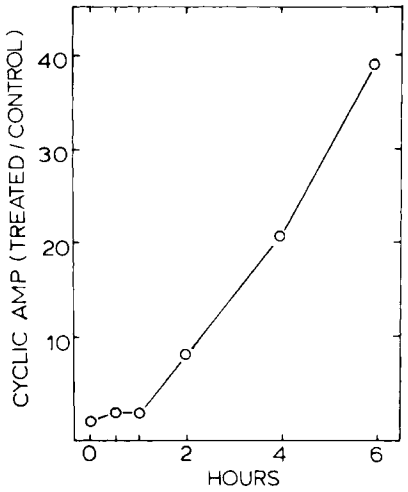

Fig. 4. Cyclic AMP concentrations increase after cholera toxin treatment of human dermal fibroblasts. Human dermal fibroblasts were grown to confluence in 35-mm culture dishes. The medium was removed and MEM supplemented with 5 ng/ml cholera toxin was added. At the indicated times cAMP concentrations were determined.

TABLE III. Readdition of IBMX Does Not Inhibit DNA Synthesis After Release From Cholera Toxin V Point

Final IBMX conc.	% Labeled nuclei					
	Time of IBMX addition (hours)					
	0	0.25	2	4	6	8
0.01 mM	22	94	93	95	96	95
0.03 mM	18	96	91	93	87	92
0.1 mM	17	95	90	89	89	82

Cultures were arrested at the V point as described in Fig. 2. The cultures were then washed and DME supplemented with 5% PPP plus ^{3}H-thymidine was added. At the indicated times IBMX was directly added and the percent labeled nuclei was determined 24 hr later.

Elevations of cAMP levels mediated by cholera toxin and IBMX were capable of reversibly inhibiting serum stimulated cultures in mid-G_1. For continued progression after the removal of IBMX from the culture medium it was necessary but not sufficient for cAMP levels to be decreased to basal concentrations. Recently, Wharton et al [23] reported that somatomedin C could replace PPP for progression of the last 4 hr of G_1. We have extended these findings and have shown that 10–20 ng/ml of somatomedin C will sustain G_1 traverse from a point (possibly V) 6 hr prior to S phase. Additionally, a subsequent increase in cAMP levels in late G_1 between the mid-G_1 cholera toxin and IBMX inhibition point (possibly V) and the G_1/S border was incapable of arresting the culture and preventing subsequent DNA synthesis. These results are in contrast to the findings of Gill et al [10] who demonstrated that 1 mM monobutyryl cAMP inhibited bovine adrenocortical cells at the

G_1/S border. We have also observed that BALB/c-3T3 cells released into the presence of PPP from a nutritional block (possibly V) at mid-G_1, as described by Stiles et al [24], are not inhibited by cholera toxin and IBMX (or additions of cyclic AMP analogs) from entering the S phase (data not shown). When density-arrested BALB/c-3T3 cells were stimulated by PDGF in plasma-supplemented medium in the presence of cholera toxin and IBMX a continuous incubation for longer than 40 hr allowed some cells to start to escape the mid-G_1 inhibition and enter DNA synthesis. The escape from this inhibition may be related to our observation that cholera toxin and IBMX did not inhibit growing sparse cultures of BALB/c-3T3 cells (data not shown).

Our data also demonstrated that different cells vary in their response to agents that stimulate cyclic AMP production. For example, both cholera toxin and IBMX are required to produce elevated sustained cyclic AMP levels capable of inhibiting G_1 traverse in BALB/c-3T3 cells. However, in human dermal fibroblasts only nanogram concentrations of cholera toxin alone are required for sustained high levels of cyclic AMP and inhibition of growth. Such differences may occur because the phosphodiesterase activity in BALB/c-3T3 cells is more active or present in greater amounts.

The results reported here, and in a recent paper by Wharton et al [4], demonstrate opposing cell cycle dependent regulatory effects of elevated cyclic AMP concentrations on stimulated density-arrested BALB/c-3T3 cells. An initial increase in cAMP level was capable of potentiating the action of competence factors to initiate the cell cycle while higher and sustained levels inhibited the progression of the competent cells at the V point. Though the role(s) of cyclic nucleotides in cellular proliferation still remains controversial, our results suggest that model systems that are capable of examining discrete portions of the cell cycle, rather than one overall parameter (ie, growth), might provide a means of integrating seemingly conflicting data.

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