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Inhibition of the Proliferation of Nb2 Cells by Femtomolar Concentrations of Cholera Toxin and Partial Reversal of the Effect by 12-O-Tetradecanoyl-Phorbol-13-Acetate

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One hour of exposure to cholera toxin is sufficient to elicit a significant delay in the initiation of DNA synthesis and cell division in lactogenic hormone-dependent Nb2-11C lymphoma cells. The inhibitory effect occurs already at very low concentrations of cholera toxin (5–50 fM), at which it is not accompanied by a detectable increase in intracellular cAMP, or ADP-ribosylation of the alpha subunit of G_s, the stimulatory guanine nucleotide binding protein of adenylate cyclase; IBMX, the phosphodiesterase inhibitor, acts synergistically to cholera toxin, indicating that a minute increase in cAMP may be sufficient for the inhibition. This indication is substantiated by the finding that dibutyryl cAMP also inhibits cell proliferation. Phorbol diester reverses partially the inhibitory activity of cholera toxin. It is most likely that this effect does not result from blocking the increase in cAMP, but rather from some subsequent, yet unidentified, events. The inhibitory effect of cholera toxin is not dependent on the concentration of the proliferation-stimulating lactogenic hormone and cannot be abolished or reduced by excess of the hormone. Cholera toxin also inhibits the autonomous proliferation of a lactogenic hormone-independent cell line (Nb2-SP); however, in this case the inhibition is not affected by TPA.

Key words: Nb2 lymphoma cells, phorbol ester, cAMP

Rat node lymphoma cells (Nb2-11C cells) are completely dependent on lactogenic hormones such as PRL, human GH (hGH), or placental lactogens when cultured in 10% horse-serum-supplemented medium [1,2]. We have found that the tumor promoter 12-O-tetradecanoylphorbol ester (TPA) and other 4-phorbol diesters enhance the hGH- and oPRL-stimulated mitogenesis of Nb2 cells. TPA is devoid of mitogenic activity and its enhancing effect is expressed only in the presence of hGH or oPRL. Interestingly, a lactogenic-hormone-independent, spontaneously proliferating variant of Nb2 cells (Nb2-SP) does not respond to TPA [3]. In addition, the lactogenic-hormone-stimulated mitogenesis of Nb2-11C cells, which is enhanced by TPA, is not mediated by an immediate

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increase in phosphoinositide metabolism [4]. Moreover, the initial events occurring after exposure of the cells to lactogenic hormones include rapid expression of *myc* oncogene [5], activation of ornithine decarboxylase [6,7], and probably also phosphorylation of S6 ribosomal protein [7]. We have shown previously that in order to initiate the mitogenic process, Nb2 cells have to be exposed to lactogenic hormones for at least 4 h [8].

It is evident that these mitogenic effects are mediated through specific lactogenic hormone receptors and that the receptors can be activated not only by lactogenic hormones but also by antireceptor antibodies [9]. The latest report indicates that a recombinant analogue of hGH lacking 13 N-terminal amino acids [10] inhibits the lactogenic-hormone-stimulated mitogenesis of Nb2-11C cells. In spite of the information accumulated through the last few years, the initial questions of how lactogenic hormones signal the Nb2 cell to proliferate and what the signal transduction pathway is remain to be answered.

The role of cAMP and protein phosphorylation by cAMP-dependent protein kinase in relation to Nb2 cell proliferation has not yet been studied. Numerous studies in which cAMP analogues and cAMP-elevating agents were employed in order to investigate the involvement of cAMP in mitogenic processes reveal that the effects of cAMP vary with the cell type. For example, in Schwann cells [11], cultured human epidermal cells, keratinocytes [12], human mammary epithelial cells [13], and Swiss 3T3 cells [14] cAMP acts as a mitogen. On the other hand, in human medullary thyroid carcinoma cells [15] and in cultured human fibroblasts [16] cAMP or its derivatives induce growth inhibition. In this study we have investigated the effect of cholera toxin on Nb2 cell proliferation. In addition, the effect of TPA, which activates protein kinase C via cAMP-independent mechanisms, was evaluated in relation to the cholera toxin effects. Phosphorylation induced by these two agents can either result in the same effect—namely, stimulation of DNA synthesis [14] or induction of growth inhibition [15]—or in opposite effects such as serotinin release in platelets [17]. Interactions between cAMP- and TPA-dependent phosphorylation have been reported [18].

MATERIALS AND METHODS

Materials

hGH (hGH 83-8-29 H; 2.2 IU/mg) was a gift of Dr. H.G. Friesen (University of Manitoba, Winnipeg, Manitoba, Canada). Adenosine 3′, 5′ cyclic phosphoric acid 2′-O-succinyl 3-[^{125}I]-iodo-tyrosyl methyl ester (2,000 Ci/mmol), tritiated thymidine ([^{3}H]-TdR, 90 Ci/mmol), and nicotin amide dinucleotide ([^{32}P]-NAD, 25 Ci/mmol) were purchased from Amersham (Buckinghamshire, England). Pertussin toxin was purchased from List Biological Laboratories, Inc., Campbell, CA, USA. Cholera toxin, cholera toxin subunits A and B, dibutyryl-cAMP, 12-O-tetradecanoyl phorbol ester (TPA), 3-isobutyl-1-methyl-xanthine (IBMX), forskolin, and cAMP were from Sigma (St. Louis, MO, USA). TPA was dissolved in dimethyl sulfoxide (DMSO; 0.1 mg/ml) and stored in aliquots in the dark at −20°C. Fischer's medium for leukemic cells and RPMI-1640 medium were obtained from Gibco (Grand Island, NY, USA). Fetal calf serum (FCS) and lactogen-free horse serum (HS) were purchased from Sera Lab Ltd. (Sussex, England). Anti-cAMP antibody and lyophylized crude protein A were from Bio-Yeda (Rehovot, Israel). Toluene-based scintillation liquid was prepared as described previously [19]. Purified transducin from bovine rod outer segment was a gift from Dr. A. Spiegel, NIH, Bethesda, MD, USA.

Nb2 Lymphoma Cells Culture

A cloned Nb2 cell line (Nb2-11C), developed in Dr. H.G. Friesen's lab (University of Manitoba) by H. Cosby, was used in the current investigation. Growth characteristics were comparable to those of the original PRL-independent cell line [1]. Suspension cultures of Nb2-11C lymphoma cells were maintained in 75-cm^2 tissue culture flasks (Nunc, Kampstrup, Denmark). Culture conditions were similar to those described [1], but Fischer's medium was replaced by RPMI 1640. In order to stimulate maximal cell growth, cells were cultured in RPMI 1640 medium, supplemented with 25 mM HEPES buffer (pH 7.4) containing FCS (10%), penicillin (50U/ml), and streptomycin (50 μg/ml). Under these conditions, the doubling time was approximately 20 hr. As RPMI 1640 medium contains reduced glutathione, no 2-mercaptoethanol was added.

To synchronize cells in the G_0/G_1 phase, cells grown to confluency were spun (5 min, 500g), resuspended (1.5×10^6 cells/ml) in Fischer's medium containing HS (10%), 2-mercaptoethanol (0.1 mM), penicillin (50 U/ml), and streptomycin (50 μg/ml), and left for 20–24 hr. As shown previously [3], this procedure synchronizes over 80% of the cells in the G_0/G_1 phase.

A spontaneously proliferating Nb2 variant (Nb_2-SP) arose after frequent passaging of the original cell line [3]. The growth rate of these cells in the absence of PRL was similar to that of Nb2-11C cells, maximally stimulated by lactogenic hormones (doubling time, 20 hr). Nb2-SP cells had PRL-binding sites but did not respond to lactogen concentrations tenfold greater (ie, 10 ng/ml) than those used in stimulation of PRL-dependent Nb2 cells [3].

Growth of Nb2 cells was quantified as described previously [3] but the growth experiments were performed in 24-well plates (Nunc), 1 ml/well. Alternatively, the cells were cultured in 25-cm^2 flasks (14 ml/flask), and at various times 1-ml aliquots were withdrawn for counting.

cAMP Assay

Aliquots of 0.8 ml of cell suspension were withdrawn, pelleted in Eppendorf tubes (3 min, 500g), resuspended in 0.1 ml of 0.1 M KCl, and boiled for 3 min. Then 0.5 ml of double-distilled H_2O was added, and after centrifugation, 0.5-ml samples were acetylated. For cAMP assay, 50-μ aliquots were removed and the RIA was performed according to Harper and Brooker [20]. The bound ligand was separated from the free ligand by binding the antibody to protein A [19]. The sensitivity of the assay ranged from 3 to 4 fmol/10^6 cells.

Determination of [^{3}H]-TdR Incorporation

Synchronized Nb2-11C cells were diluted to 3×10^5 cells/ml in Fischer's medium supplemented with 10% horse serum. The suspension was distributed to 24-well plates (1 ml/well) and left for 1 hr of preincubation. Then, hGH (2 ng/ml) and cholera toxin (0, 4, 400, 40,000 pg/ml) were added. The hormone and toxin were diluted previously in phosphate-buffered saline (pH 7.4) containing 0.2% BSA. Cells without hGH but with respective concentrations of cholera toxin served as controls. At 2-hr intervals, a [^{3}H]-TdR pulse (1 μCi/ml) was added to the cell suspension. The pulse was terminated by transferring the cells to Eppendorf tubes and centrifugation (5 min, 500g). The pellet was washed twice with 1 ml 4% $HClO_4$; 1 ml of 80% EtOH was added; and the pellets were kept at 4°C. At the end of the experiment the pellets were washed consecutively

with 1 ml of 95% EtOH and 4% $HClO_4$, dissolved in 1 ml 5% $HClO_4$, and heated for 60 min at 80°C; 0.5 ml of each sample was mixed with 5 ml of scintillation liquid and counted.

Membrane Preparation and ADP Ribosylation

Cells were pelleted (5 min, 500g), resuspended in homogenizing buffer (10 mM Tris, pH 7.5, 2 mM magnesium chloride, 2 mM dithiothreitol [DTT] and 0.1 mM EDTA), and homogenized with a motor-driven Teflon pestle. The homogenate was centrifuged at low speed (2,000 rpm in a Sorvall SS-34 rotor) for 5 min, and the pellet was discarded. The supernatant was recentrifuged at 15,000 rpm for 20 min, and the pellet was collected, resuspended in buffer, and frozen at −20°C until use.

Cholera toxin and pertussis toxin were preactivated in 250 mM Tris buffer (pH 8.0) containing 50 mM DTT for 1 hr at 37°C. Membranes (200 μg) were incubated with 10 μg of each of the toxins, with 30 μCi of [^{32}P]NAD for 1 hr and with either 2.5 mM ATP for pertussis toxin labeling [21] or 5 mM GTP and 2 mM thymidine for cholera toxin [22]. Purified bovine transducin was ADP ribosylated as previously described [21]. The reaction was terminated by centrifugation. The supernatant was discharged and the pellet was dissolved in SDS-containing sample buffer, boiled, and subjected to $NaDodSO_4$/PAGE (12.5%) followed by autoradiography.

RESULTS

Results shown in Table I indicate that the hGH-stimulated proliferation of Nb2-11C cells and the spontaneous proliferation of Nb2-SP cells are remarkably inhibited by cholera toxin, at extremely low concentrations, in a dose-dependent manner. However, as shown later (see Table II, Fig. 3), this inhibition resulted most likely from a delay in the first division rather than inhibition of the exponential proliferation. In the case of Nb2-11C cells, in which TPA enhances the hGH-stimulated mitogenesis [3], addition of the latter drastically reduced (except at 40,000 pg cholera toxin/ml) the extent of

TABLE I. Effect of Cholera Toxin on the Proliferation of Nb2-11C and Nb2-SP Lymphoma Cells in the Presence or Absence of TPA (20 nM)

Cholera toxin (pg/ml)	No. of doublings (mean ± SEM) in the presence or absence of TPA (20 nM)[a]			
	Nb2-11C		Nb2-SP	
	−TPA	+TPA	−TPA	+TPA
0	2.30 ± 0.01	2.46 ± 0.02	2.37 ± 0.02	2.34 ± 0.04
0.4	2.08 ± 0.04*	2.42 ± 0.03	ND[b]	ND
4	1.75 ± 0.03*	2.30 ± 0.02*	2.01 ± 0.05*	1.88 ± 0.06*
40	1.54 ± 0.02*	2.22 ± 0.02*	1.40 ± 0.04*	1.53 ± 0.05*
400	1.37 ± 0.03*	2.20 ± 0.05*	1.23 ± 0.02*	1.29 ± 0.05*
4,000	1.26 ± 0.04*	2.10 ± 0.03*	ND	ND
40,000	1.19 ± 0.03*	1.60 ± 0.02*	ND	ND

[a]Cells that were previously synchronized in the C_0/G_1 phase were cultured in 24-well plates (1 ml/well, two wells per treatment, initial concentration 1.6 × 10^5 cells/ml) in the presence of 2 ng/ml hGH (Nb2−11C only), for 66 hr.

[b]ND = not determined.

*$P < .01$ vs the respective treatment without cholera toxin.

inhibition. On the other hand, in Nb2-SP cells that do not respond to TPA [3], addition of the latter had no effect in any of the assays. Pertussis toxin could also inhibit proliferation of both Nb2 cell lines but the minimal concentrations required were not less than 50 ng/ml (not shown). In Nb2-11C cells, both toxins inhibited the proliferation, not only at the optimal concentration of hGH (2 ng/ml) but also at suboptimal (0.05–0.2 ng/ml) concentrations. A higher concentration of hGH (20 ng/ml) did not abolish or affect the inhibition.

Kinetic analysis of proliferation of Nb2-11C cells in the presence of cholera toxin is summarized in Table II. The results show that at least in the range of 4–400 pg/ml, the inhibition did not result from reducing the rate of proliferation, but rather from prolonging the lag period until the first cell division occurred. TPA did not affect the lag period at 400 pg cholera toxin/ml but shortened it at 40 and especially at 4 pg/ml. At higher cholera toxin concentrations, both a prolongation of the lag period and a reduction in the rate of proliferation occurred (not shown). It should be noted that cholera toxin inhibited not only the initiation of mitogenesis in synchronized quiescent cells but also the proliferation of dividing Nb2-11C and Nb2-SP cells (not shown).

Results shown in Figure 1 indicate that at 4 pg cholera toxin/ml, a delay in the initiation and the rate of DNA synthesis occurred. At 400 pg/ml (not shown) and at 40,000 pg/ml, the extent of inhibition was even greater. At these two latter concentrations of cholera toxin, the rate of [^{3}H]-TdR incorporation was almost identical. The inhibition of cell proliferation was verified by counting the cells in the appropriate treatments after 36 and 60 hr. The results were comparable to those presented in Tables I and II.

In order to determine whether the inhibitory effect exhibited by cholera toxin is dependent upon its continuous presence, synchronized cells were exposed to 4 and 400 pg cholera toxin for 1 hr. Subsequently, the cells were washed extensively, resuspended, and cultured in the presence of hGH with or without cholera toxin. As shown in Figure 2, 1-hr exposure to either 4 or 400 pg/ml of cholera toxin was sufficient to delay the initiation of mitogenesis from 13 to approximately 20 hr. This is evident by extrapolating the lines to the initial cell number (2×10^5 cell/ml). Exposure of cells to either 4 or 400 pg/ml followed by culture in the absence or presence of 4 pg cholera toxin/ml

TABLE II. Effect of Cholera Toxin on the Proliferation Rate and the Time Required for the First Division of Nb2-11C Lymphoma Cells in the Presence or Absence of TPA (20nM)

Cholera toxin[a] (pg/ml)	No. of doublings per 24 hr[b]		TPA[c] effect (%)	Time required for the first division (hr)[d]		TPA[e] effect (%)
	−TPA	+TPA		−TPA	+TPA	
0	1.21	1.57	29.8	14.5	13.7	− 5.5
4	1.20	1.51	25.8	23.2	19.8	−15.3
40	1.24	1.55	25.0	27.2	25.1	− 7.8
400	1.18	1.55	35.4	27.3	27.8	None

[a]Cells that were previously synchronized in the G_0/G_1 phase were cultured in 25-cm^2 flasks (14 ml/flask; initial concentration 1.6×10^5 cells/ml; two flasks per treatment) in the presence of 2 ng/ml hGH.
[b]Calculated from the slope obtained by plotting the number of doublings vs time (five or six time points for each hormone concentration); in all cases, the correlation coefficients were higher than 0.98 ($P < .01$).
[c]Expressed as percent increase in the doubling rate.
[d]Extrapolated from the regression equation of the number of doublings, to the point where number of doublings = 0.
[e]Expressed as percent decrease in the time required for the first division.

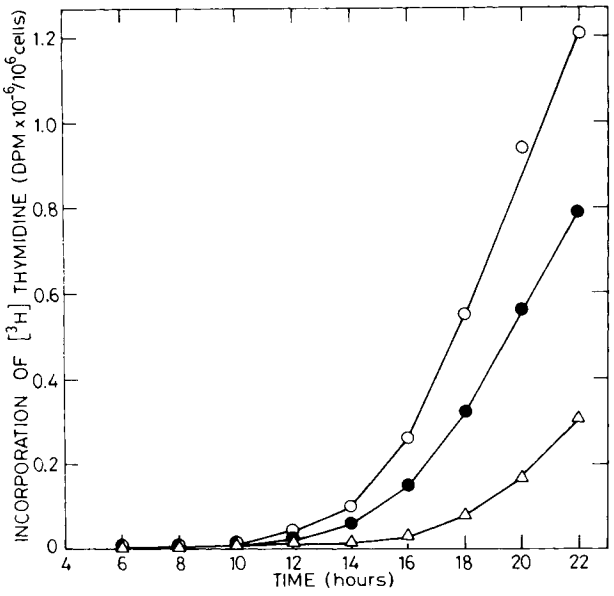

Fig. 1. Effect of cholera toxin on [^{3}H]-TdR incorporation in Nb2-11C lymphoma cells. Synchronized cells were diluted to 3 × 10^5 cells/ml with 10% HS-supplemented medium and distributed into 24-well plates (1 ml/well). After 1 hr, hGH (2 ng/ml) and cholera toxin (none—○ 4 pg/ml—●; 40,000 pg/ml—Δ) were added. A pulse of ^{3}[H]-TdR (1 μCi/ml) was given every 2 hr. Two hours later, the cells (in triplicate) were harvested to determine the extent of incorporation. The values in the figure (means of three wells) are the cumulative sum of the results obtained during the time course of each treatment. Standard errors ranged between 1.7 and 8.2% of the cumulative sum. In the absence of hGH, the incorporation was less than 800 dpm/well. After 14 hr or more, all three treatments differed significantly ($P < .05$) from each other.

yielded almost identical growth curves (Fig. 2A,B) These growth curves were similar to those obtained with cells not pretreated with cholera toxin but grown in the presence of 4 pg cholera toxin/ml (Fig. 2C). However, in cells pretreated with either 4 or 400 pg/ml and then cultured in the presence of 400 pg cholera toxin/ml, a further delay in the initiation of proliferation was observed (Fig. 2A,B). This delay was considerably greater than in cells cultured in the presence of 400 pg/ml but not pretreated with cholera toxin (Fig. 2C). Once cell division had started, the proliferation rate was almost the same, thus confirming the results presented in Table II.

Since it was found that 1 hr of exposure to cholera toxin was sufficient to inhibit the initiation of mitogenesis, the nature of events occurring in the cell during this period was studied by using the following experimental approaches: (1) We attempted to inhibit the proliferation by cholera toxin subunits A or B. No inhibition was observed even at 40 ng/ml, thereby indicating the dependence of inhibition on penetration of the toxin into the cell (data not shown). (2) Membranes from cells, pretreated with different cholera toxin concentrations, were ADP ribosylated in vitro either with cholera toxin or pertussis toxin (Fig. 3). In the control cells, cholera toxin caused ADP ribosylation of two proteins (45 and 52 kDa, lane 3) both corresponding to G_s, the stimulatory guanine nucleotide binding protein, whereas pertussis toxin caused ADP ribosylation of G_i, the inhibitory guanine nucleotide binding protein (40 kDa, lane 9). Transducin, the GTP

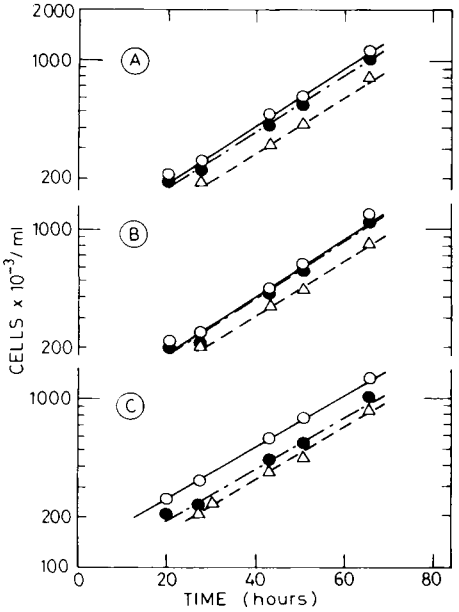

Fig. 2. Effect of preincubation with cholera toxin on the initiation and the rate of proliferation of Nb2-11C lymphoma cells. Synchronized cells were diluted with 10% HS supplemented medium to 9×10^5 cells/ml and incubated for 1 hr with 400 pg/ml (**A**), 4 pg/ml (**B**), or no (**C**) cholera toxin. Subsequently the cells were washed ($\times 3$) with medium to remove cholera toxin, diluted to 2×10^5 cells/ml, and cultured for 68 hr in the presence of 2 ng/ml hGH with 400 pg/ml (Δ), 4 pg/ml (●); or no (○) cholera toxin. Aliquots (1 ml) were withdrawn for counting.

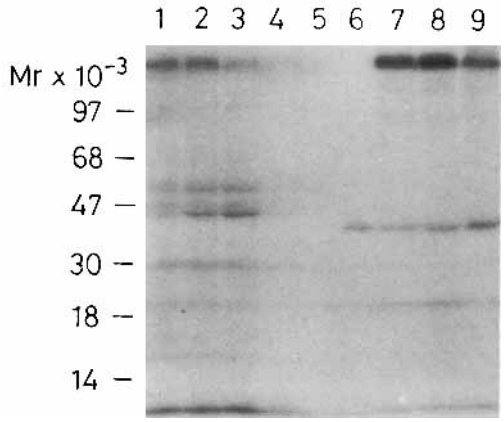

Fig. 3. ADP ribosylation of membranes from cholera-toxin-treated cells by bacterial toxins. Membranes were prepared from cells pretreated for 1 hr without (lanes 3, 9), or with 4 pg/ml cholera toxin (lanes 2, 8) and 400 pg/ml cholera toxin (lanes 1, 7). The membranes were ADP-ribosylated with either cholera toxin (lanes 1–3) or pertussis toxin (lanes 7–9). Lanes 4 and 5 represent toxin-independent ADP-ribosylation. Lane 6 shows the ADP ribosylation of purified transducin (GTP binding protein purified from bovine rod outer segments) by pertussis toxin. At the end of the incubation, the membranes were subjected to $NaDodSO_4$/PAGE on 12.5% gels and autoradiographed.

binding protein purified from bovine rod outer segments, is a 40-kDa protein which undergoes ADP ribosylation by pertussis toxin (lane 6). No ADP ribosylation of the GTP binding proteins was found without the bacterial toxins (lanes 4, 5). No significant changes in the cholera toxin-dependent ADP ribosylation pattern were observed in membranes prepared from cells pretreated with 4 pg/ml cholera toxin as compared with the control cells (lane 2). However, a clear reduction in the labeling of both proteins was found in the cells pretreated with 400 pg/ml (lane 1). A minor reduction of ADP ribosylation of G_i by pertussis toxin was found in membranes from cells pretreated in both cholera toxin concentrations (lanes 7, 8). (3) We have determined the accumulation of cAMP in Nb2-11C cells exposed to various concentrations of cholera toxin, through 0–150 min, in the presence or absence of TPA. This experiment was performed in the absence of any phosphodiesterase inhibitor such as IBMX, since the proliferation of Nb2 cells was inhibited by this material itself, at 10^{-4} M (Table III). As shown in Figure 4B, at 4 pg cholera toxin/ml, there was no significant increase in cAMP content. Some increases were observed at 400 pg/ml after 60 and 90 min (Fig. 4C), and rapid increases (after 30 min) occurred at 40,000 pg/ml (Fig. 4D). Other experiments (data not shown) revealed that at this cholera toxin concentration, a similar increase occurs after as soon as 15 min. After 180 min, the cAMP level dropped to its initial low level (~0.1 pmol/10^6 cells) at least for the next 36 hr. In all the cases where cAMP elevation occurred, TPA significantly potentiated this effect.

Inhibition of cell proliferation was also observed in the presence of dibutyryl cAMP and forskolin. The number of doublings after 72 hr was 2.24 ± 0.01 without the cAMP analog, 2.17 ± 0.01 with 10^{-6} M, 2.04 ± 0.03 with 10^{-5} M, and 0.99 ± 0.03 with 10^{-4} M dibutyryl cAMP, and 1.98 ± 0.03 with 10^{-4} M forskolin. However, no detectable increase in cAMP concentration (0–2 hr after its addition) was observed in the forskolin-treated cells.

Interestingly, when Nb2-11C cells were cultured in the presence of both IBMX and cholera toxin, a strong synergism in growth inhibition was observed (Table III). It should be noted that in the absence of IBMX the relative inhibition caused by cholera toxin was lower than in the experiment described in Table I. This is due to the fact that those cells were counted after 74 hr (as compared to 66 hr in Table I) while the control

TABLE III. Effect of Cholera Toxin and IBMX on the Proliferation of Nb2-11C Lymphoma Cells†

Cholera toxin pg/ml	No. of doublings (mean ± SEM) in the presence or absence of IBMX		
	−IBMX	+IBMX (10^{-5} M)	+IBMX (10^{-4} M)
0	2.51 ± 0.01	2.46 ± 0.02	2.08 ± 0.01*
4	2.46 ± 0.01***	2.12 ± 0.01*	1.81 ± 0.01**
40	2.15 ± 0.01****	1.47 ± 0.02*	0.35 ± 0.006**
400	1.97 ± 0.04****	1.20 ± 0.02*	0.02 ± 0.005**

†Cells were cultured in 24-well plates (1 ml/well, initial concentration 1.6×10^5 cells/ml) in the presence of 2 ng/ml hGH, with or without IBMX and cholera toxin for 74 hr.

*$P < .05$ vs control without IBMX.

**$P < .01$ vs control without IBMX.

***$P < .05$ vs control without cholera toxin.

****$P < .01$ vs control without cholera toxin.

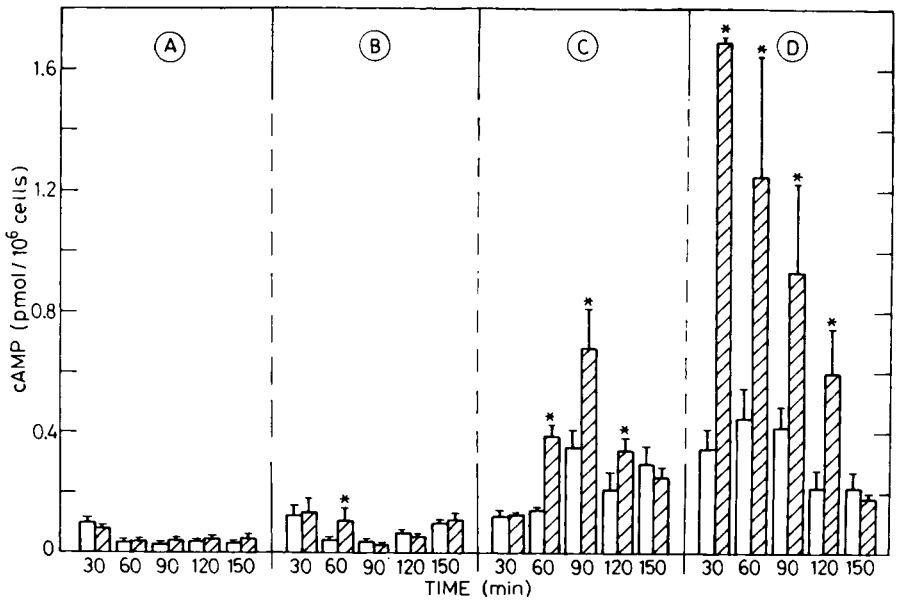

Fig. 4. Effect of cholera and TPA on cAMP accumulation in Nb2 cells. Synchronized cells were diluted to 8 × 10^5 cells/ml (14 ml/flask, three flasks per treatment) with 10% HS-supplemented medium, after 3 hr 0.01% DMSO (empty bars) or TPA (20 nM, full bars) and cholera toxin (**A**: none, **B**: 4 pg/ml, **C**: 400 pg/ml, **D**: 40,000 pg/ml) were added. Aliquots (0.8 ml) were withdrawn for cellular cAMP determination. The results are given as mean ± SEM: all bars marked with an asterisk differ significantly ($P < .05$) from the respective treatments without TPA.

cells reached confluence and ceased to proliferate. At 400 pg cholera toxin/ml, the increase in cAMP after 60 min was threefold higher in the presence of IBMX than in its absence.

DISCUSSION

The results of the present work indicate clearly that cholera toxin inhibits the proliferation of Nb2-11C and Nb2-SP cells. The effect occurs already at extremely low concentrations of the toxin (5–50 fM). A clear dose response exists at 5–5,000 fM, but the inhibition is only partial, even at 500 pM. It is evident that the inhibitory effect results mainly from prolonging the lag period for the first mitosis. Although full cytometric analysis of all divisions was not performed, [^{3}H]-TdR incorporation studies suggest that this phenomenon is mainly related to the prolongation of the G_0/G_1 and S phases.

It is known that to stimulate adenylate cyclase in intact cells, cholera toxin requires the interaction of its A and B subunits. In the case of Nb2 cells, the effect of the toxin on proliferation was dependent on its penetration into the cells, since the A or B subunits alone were unable to show any effect on proliferation, even at high doses.

One-hour exposure to cholera toxin was sufficient to cause the inhibition. This finding indicates that some rapid event occurs shortly after exposure to cholera toxin, leading to almost doubling of the lag period needed for the initiation of DNA synthesis and cell division. At low concentrations of cholera toxin (4 pg/ml), which were sufficient

to induce significant growth inhibition, no increase in the intracellular level of cAMP was detected, and no detectable ribosylation of G_s occurred. These findings raise the possibility that in Nb2 cells, cholera toxin, at least at low concentrations, may not act through activation of adenylate cyclase [23] but rather through a different pathway such as chemotaxis inhibition in mouse macrophage cell line [24]. However, it is more likely that a minute elevation in cAMP levels, below the sensitivity of our assay and confined to a specific cellular compartment, is sufficient to start the chain of events leading to inhibition in cell proliferation. This possibility is supported (1) by the results obtained with dibutyryl cAMP and forskolin and (2) by the finding that IBMX, the phosphodiesterase inhibitor, is highly synergistic to cholera toxin. If this possibility is correct, some cholera-toxin-dependent phosphorylation event(s) that regulates cell proliferation may take place after exposure of the cells to the toxin. This possibility is currently under investigation in our laboratory. The role of cAMP as a mediator of the mitogenic effect of cholera toxin, as reported by others, is somewhat unclear. Pruss and Hershman [25] showed that the concentration dependence for cholera-toxin-induced mitogenesis and elevation of cAMP levels in Swiss 3T3 cells differs by about half an order of magnitude, and it was impossible to mimic the cholera toxin mitogenic effect by agents which elevate cAMP levels in the same cells.

Another factor studied in this work was TPA, which enhances the lactogenic-hormone-stimulated mitogenesis of Nb2-11C cells [3]. Initial results (Table I) gave the impression that the inhibitory activity of cholera toxin and TPA's ability to partially abolish this effect are mediated through related mechanisms. Other results—such as the finding that at 40 pg/ml and particularly at 4 pg/ml cholera toxin TPA shortened the delay in mitosis—support this assumption.

TPA, on the other hand, potentiated the cholera-toxin-stimulated elevation of cAMP, raising the possibility (1) that TPA-enhancing activity does not result from blocking the increase in cAMP but from subsequent events, and (2) that a small increase in cAMP level is sufficient to elicit the full inhibitory effect, and a larger increase does not potentiate it any further. This conclusion can explain the paradoxical finding that TPA augments the cholera-toxin-stimulated rise in cAMP levels but reduces the extent of cholera-toxin-stimulated inhibition of proliferation. Thus it seems that although TPA, like cAMP, exerts its effects via protein phosphorylation, the effects of the two agents on the proliferation of Nb2 lymphoma cells are opposite. One can postulate different substrates for the two protein kinases that trigger opposite pathways.

The time course of the effects of cholera toxin and TPA are also different. Even a brief incubation of the cells with cholera toxin starts the chain of events needed for its effect (Fig. 2), in contrast to TPA, which has to be included all the time in the incubation medium [3]. It is also interesting to note that although cholera toxin inhibits proliferation in both lymphoma cell lines—the one which is completely dependent on lactogenic hormones and the one which proliferates spontaneously—the effect of TPA on proliferation after cholera toxin treatment is observed only in the former. This can be used as a model to understand the differences between these two cell lines.

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