Brief Definitive Report

INCREASED INTRACELLULAR CYCLIC ADENOSINE MONOPHOSPHATE INHIBITS T LYMPHOCYTE-MEDIATED CYTOLYSIS BY TWO DISTINCT MECHANISMS

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The possibility that intracellular cyclic adenosine monophosphate concentration ($[cAMP]_i$) may play a role in the action of CTL was suggested by the observation that target cell lysis was inhibited when CTL were treated with either cholera toxin (CT) or a cAMP analogue (1, 2). Recent data indicate that elevation of $[cAMP]_i$ also blocks receptor-mediated T cell activation and the associated phosphorylation of components of the TCR complex (3). In Jurkat cells, Imboden et al. (4) have shown that cholera toxin inhibits TCR-mediated increases in intracellular calcium concentration ($[Ca^{2+}]_i$), but that this inhibitory effect was not mimicked by other treatments that raised $[cAMP]_i$. More recently, Ledbetter et al. (5) showed that increases in $[Ca^{2+}]_i$ could also be inhibited by pertussis toxin (PT) treatment of Jurkat cells. In the present study, the effects on murine CTL function of CT, PT, and agents that directly elevate $[cAMP]_i$ have been investigated in detail. In this system, both CT and cAMP analogues inhibited conjugate formation and the receptor-stimulated increase in $[Ca^{2+}]_i$, but had no effect on the expression of the TCR or other proteins thought to be involved in target cell binding. PT treatment of these cells was without effect on any measured parameter. These results suggest that cAMP may be an important modulator of CTL function.

Materials and Methods

Reagents. The binding (B) subunit of CT and the cAMP analogues, 8-bromoadenosine 3′:5′-cyclic monophosphate (8Br-cAMP) and 6,2′-*O*-dibutyryladenosine 3′:5′-cyclic monophosphate (dibutyryl-cAMP) were obtained from Sigma Chemical Co. (St. Louis, MO).

Cells. The characteristics and maintenance of the CTL clones and cell lines used here have been previously described (6). A hamster hybridoma (145-2C11) that produces an mAb that is specific for the ε chain of T3 was generously provided by J. A. Bluestone (National Cancer Institute, Bethesda, MD).

Measurement of $[cAMP]_i$. CTL were incubated with the indicated concentrations CT (List Biological Laboratories, Campbell, CA) for 1 h or PT (prepared as previously described, reference 7) for 18 h. Cell pellets were extracted with 0.1 N HCl, and cAMP was determined

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using an automated radioimmunoassay that was performed by the University of Virginia Diabetes Center RIA Core Laboratory, Charlottesville, VA.

Flow Cytofluorimetric Determination of Conjugates and $[Ca^{2+}]_i$. CTL were treated with CT or 8Br-cAMP for 1 h as described above and simultaneously with 5 μM indo-1/AM (8). Both CTL and target cells were washed in a balanced salt solution (BSS) at pH 7.4, containing 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes, 0.1% glucose, and 0.1% BSA, and were resuspended at 1×10^7 cells/ml. 100 μl of the CTL suspension was mixed with an equal volume of the target cell suspension, centrifuged for 2 min, and incubated at 37°C for 10 min. The 400 nm/480 nm fluorescence-emission ratio from indo-1-labeled CTL as determined on a flow cytofluorimeter (Epics V, Coulter Electronics Inc., Hialeah, Fl) was set to ~0.5 on a scale of 0–1 by adjusting instrument settings. Under these conditions, target cells examined alone were outside of the range of the histogram. Conjugates were defined as events falling >2 SD from the peak forward angle light scatter obtained from the examination of CTL alone and were further restricted to those events falling above a line drawn 2 SD below the peak fluorescence ratio of CTL alone. (9) Activated CTL were defined as events falling >2 SD above the peak ratio fluorescence of CTLs measured without added targets. (9)

Determination of antibody-stimulated increases in $[Ca^{2+}]_i$ was performed on a flow cytofluorimeter using indo-1-labeled CTL essentially as previously described (5). The baseline fluorescence emission ratio was recorded for 30 s before protein-A-purified anti-T3 mAb at a final concentration of 50 μg/ml was added to the sample. Data were acquired for 16 s for each point with a total of 64 time points and $3–4 \times 10^5$ events for each determination.

Results

Three independent CTL clones exposed to cholera holotoxin, but not the noncatalytic B subunit, showed a dose-dependent inhibition of specific target cell lysis (Fig. 1 *A*). In contrast, exposure to PT had no effect on cytotoxicity (Fig. 1 *B*). Prior treatment of intact CTL with CT resulted in the modification of 92% of its 44-kD substrate, while pertussis-toxin treatment resulted in the modification of 97% of its 38-kD substrate (data not shown). Thus, the inability of PT to inhibit CTL-mediated cytotoxicity cannot be explained by lack of ADP-ribosylation of the toxin substrate.

CT treatment of CTL clones resulted in an increase in $[cAMP]_i$, whereas PT treatment did not (Table I). Therefore, CT, but not PT, was able to modify a G

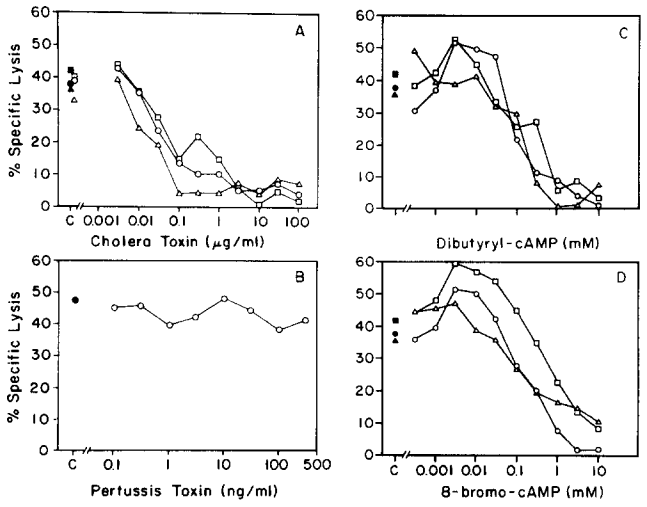

FIGURE 1. The CTL clones III12-2 (○), 1D5 (△), and 1H11 (□) were incubated with the indicated concentrations of CT (*A*) dibutryl cAMP (*C*), or 8Br-cAMP (*D*) for 1 h or PT for 18 h (*B*). They were washed and incubated in duplicate with ^{51}Cr-labeled JY cells at an E/T ratio of 5:1. The cAMP analogues were present throughout the assay. (*C*) The values shown are for CTL treated for 1 h with CT B subunit at a concentration of 10 μg/ml (*A*, *open symbols*) or left untreated (*A* and *B*, *closed symbols*). The spontaneous release of ^{51}Cr from target cells was not altered by the presence of any of the drugs.

TABLE I

Effects of Bacterial Toxins on Intracellular Concentrations of cAMP in Cytotoxic T Cells

Treatment	Picomoles cAMP/milligram protein
Control	8 (1)
CT (μg/ml)	
10	86 (12)
1	93 (13)
0.1	84 (11)
0.01	81 (11)
0.001	34 (5)
0	8 (1)
PT	
10 μg/ml	9 (1)

$[cAMP]_i$ was determined by RIA using CTL clone III12-2 as described in Materials and Methods. Values represent the mean of triplicate determinations, and the numbers in parentheses represent pmol $[cAMP]_i$/106 cells.

protein with consequent activation of endogenous adenylate cyclase. Although CT caused an increase in $[cAMP]_i$, it was still possible that inhibition of cytotoxicity was due to the action of the toxin on another regulatory G protein as has been previously hypothesized (4). However, treatment of CTL with cAMP analogues also inhibited of cytotoxicity (Fig. 1, *C* and *D*). These data suggest that CT may inhibit CTL-mediated lysis through its ability to elevate $[cAMP]_i$.

Conjugate formation was determined by flow cytofluorimetery as an increase in forward angle light scatter of CTL after binding to target cells (9). The reduction in the proportion of CTL bound to JY cells ranged from 27–69% after 8Br-cAMP treatment and from 70–85% after CT treatment. Both CT and 8Br-cAMP also reduced nonspecific conjugate formation with the irrelevant target cell Daudi by >72%. Thus, at least one mechanism by which CT and $[cAMP]_i$ inhibit CTL-mediated lysis is through a reduction in stable conjugate formation with target cells. The CTL in these experiments were loaded with indo-1 in order to determine the proportion of CTL that demonstrated an increase in $[Ca^{2+}]_i$ after interaction with relevant target cells (9). As shown in Table II, 8Br-cAMP reduced the proportion of activated CTL by 68%, which was a significantly greater decrease than that produced in conjugate formation ($p < 0.01$). CT inhibited activation by >75% as compared with untreated CTL (Table II). The proportion of CTL that was counted as activated by incubation with Daudi cells was low in all samples and confirms earlier findings (9).

Although conjugate formation was inhibited by CT and 8Br-cAMP, treatment of CTL with either CT or 8Br-cAMP did not affect the level of expression of TCR, T3, LFA-1, or Lyt-2 as determined by mAb binding (data not shown). Therefore, the effect of CT and 8Br-cAMP on increases in $[Ca^{2+}]_i$ induced by anti-TCR mAbs was investigated. Addition of anti-T3 mAb resulted in an increase in $[Ca^{2+}]_i$, evident as an increased fluorescence ratio (Fig. 2 *A*). This increase was eliminated by prior treatment of CTL with either CT (Fig. 2 *B*) or 8Br-cAMP (Fig. 2 *C*). Since the target cell binding defect was bypassed using mAbs, these data indicate that another separate mechanism by which elevated $[cAMP]_i$ inhibits CTL-mediated cytotoxicity is likely to involve inhibition of the receptor-stimulated increase in $[Ca^{2+}]_i$.

TABLE II

Inhibition of Conjugate Formation and CTL Activation by Elevated Intracellular cAMP Concentration

Target cell	Exp. 1		Exp. 2		Exp. 3	
	Conjugation	Activation	Conjugation	Activation	Conjugation	Activation
	%		%		%	
JY						
CTR	43.0	48.0	47.0	39.7	40.9	46.2
8Br	13.3	6.5	34.2	10.3	18.8	26.7
CT	6.5	9.7	14.2	11.2	NT*	NT
Daudi						
CTR	20.9	11.1	14.6	2.6	14.2	9.3
8Br	8.5	10.1	5.3	1.8	0.9	2.0
CT	8.1	6.3	5.7	3.2	NT	NT

CTL were examined by flow cytofluorimetry as described in Materials and Methods. When tested alone, the percentage of both treated and untreated CTL within the window defined for conjugation had a mean value for the three experiments of 6.1% and was 2.5% for activation. The value obtained for a particular experiment was subtracted from the data presented in the table. A total of 30,000 events were obtained for each determination.

* Not tested.

Discussion

CT or 8Br-cAMP treatment abolished the increase in $[Ca^{2+}]_i$ that normally results from binding of anti-T3 mAb and significantly reduced the increase stimulated by relevant cellular antigen. These results stand in contrast to those reported by Imboden et al. (4), who showed that CT, but not forskolin or cAMP analogs,

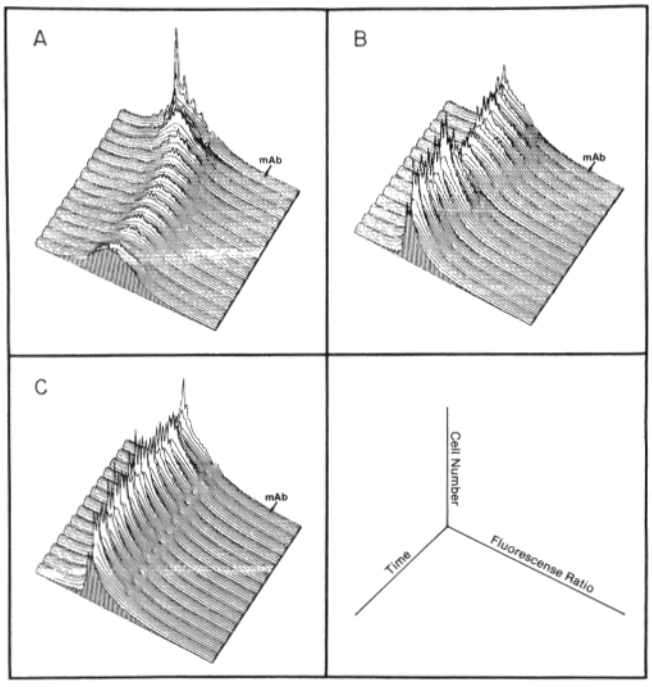

FIGURE 2. CTL clone III12-2 was labeled with indo-1 and concurrently incubated with medium (*A*), 1 μg/ml CT (*B*), or 10 mM 8Br-cAMP (*C*) for 1 h. Anti-T3 mAb (50 μg/ml) was added at times indicated by the arrows. The scalloping, which is evident along the *x*-axis, is an artifact of the computer program used to generate the graphs.

abolished the increase in $[Ca^{2+}]_i$ induced in Jurkat cells by anti-T3 antibodies. The disparity in the effects of cAMP analogs in these two studies may reflect differences between Jurkat and cloned CTLs. It is also possible that CT does modify a G protein, distinct from Gs, that is a participant in signal transduction in CTL. However, if this is the case, then the effect of CT on this putative G protein must be masked in CTL by the actions of elevated $[cAMP]_i$ alone. In this regard, CT appeared more effective in inhibiting the interaction of CTL with target cells than was 8Br-cAMP.

There are similarities between the actions of cAMP reported here and those reported to occur in platelets and T cells by other groups. In platelets, elevated $[cAMP]_i$ eliminates receptor-stimulated increases in $[Ca^{2+}]_i$ by inhibiting Ca^{2+} influx (10), mobilization (10), and, possibly, sequestration (11). Furthermore, it has been shown that treatment of Jurkat cells with a cAMP analogue inhibits the antigen-induced production of inositol polyphosphates that have been implicated in control of Ca^{2+} influx and mobilization (3). Taken together, these results are in accord with the idea that CT acts at least partially through elevation of $[cAMP]_i$ to inhibit antigen-induced increases in $[Ca^{2+}]_i$ by its effects on inositol polyphosphate production.

Elevated $[cAMP]_i$ also results in a marked decrease in the ability of CTL to bind to either relevant or irrelevant target cells. This is not due to a decrease in the expression of the membrane proteins currently thought to be responsible for initiation or maintenance of the CTL-target cell interaction. However, cAMP-dependent protein phosphorylation has been shown to reduce receptor affinity in some systems (12), and it is possible that a similar phenomenon is occurring in T lymphocytes. LFA-1 has been shown to play a key role in conjugate formation (13). Because elevated $[cAMP]_i$ inhibited conjugate formation with both relevant and irrelevant targets, it is tempting to speculate that one mechanism of action of cAMP may involve this protein.

The relationship between cAMP-mediated inhibition of the antigen-stimulated increase in $[Ca^{2+}]_i$ and the inhibition mediated by membrane hyperpolarization (8) is not clear and will require further investigation. However, the present study has provided evidence for at least two independent sites of inhibitory action by elevated $[cAMP]_i$ on cytotoxic T lymphocyte function. Because of its effects, an attractive hypothesis is that an increase in $[cAMP]_i$ serves to terminate the lytic interaction by ending CTL-target cell adhesion as well as the increase in $[Ca^{2+}]_i$, which are both required for lytic function. Regardless of the actual mechanism, these results provide suggestive evidence concerning the mechanisms by which CTL activity may be regulated.

Summary

Cholera toxin (CT), but not pertussis toxin (PT), treatment of cloned murine CTL inhibited target cell lysis in a dose-dependent fashion. The effects of CT were mimicked by forskolin and cyclic adenosine monophosphate (cAMP) analogues. Inhibition of cytotoxicity by CT and cAMP analogs was mediated in part by attenuation of conjugate formation. Additionally, both CT and cAMP analogs blocked the increase in intracellular Ca^{2+} induced by stimulation of the TCR complex by mAbs. These findings indicate that cAMP inhibits the activity of CTL by two distinct mechanisms and suggests a role for this second messenger in CTL-mediated cytolysis.

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