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Cholera Toxin Inhibits the Increase in Cytoplasmic Free Calcium Induced Via the CD2 Pathway of Human T-Lymphocyte Activation

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We investigated the action of cholera toxin on the intracellular ionized calcium $[Ca^{2+}]i$ increase induced by anti-CD2 and anti-CD3 monoclonal antibodies in the leukemic human T-cell line Jurkat. Cholera toxin inhibits in a dose-dependent manner these two pathways of human T-lymphocyte activation but with different half maximal inhibition doses (75 ng/ml for CD3, 30 ng/ml for CD2). This effect cannot be accounted for only by the increase in cAMP induced by cholera toxin because forskolin, which raises cellular cyclic adenosine monophosphate (cAMP) to the same levels, induced only a small inhibition of the $[Ca^{2+}]i$ increase in similar conditions. Cholera toxin induced a decrease in the surface expression of the CD3 molecule, suggesting a down-regulation of the CD3 molecules. On the other hand, the expression of CD2 remained unchanged. Cell surface disappearance of the CD3 molecule cannot account for all the inhibitory effects of cholera toxin because CD2 molecule expression was not affected (no modifications in the half maximal binding of anti-CD2 monoclonal antibodies). All together, these results suggest that cholera toxin acts on substrates, possibly G proteins, that could regulate the $[Ca^{2+}]i$ increase induced by anti-CD2 and anti-CD3 mAbs in Jurkat cells. In addition, the present study demonstrated that the rise in cellular cAMP partially inhibits the $[Ca^{2+}]i$ increase induced by anti-CD2 and anti-CD3 mAbs.

Key words: cAMP, cholera toxin, CD3 molecules, CD2 molecules

Abbreviations used: $[CA^{2+}]i$, Intracellular ionized calcium concentration; CD, Cluster of differentiation; CT, Cholera toxin; mAb, Monoclonal antibody; G proteins, Guanine nucleotide binding protein; IP3, Inositol trisphosphates; PIP2, Phosphatidyl inositol biphosphates; 1-2 DAG, 1-2 diacylglycerol; Indo-1, [1-[2-amino-5-[carboxylindol-2-yl]-2-[2′-amino-5′-methylphenoxy]-ethane-N,N,N′,N′-tetra-acetic acid]; FITC, Fluorescein isothiocyanate; EGTA, Ethyleneglycol-bis-(β-aminoethylether)-N,N′-tetra-acetic acid.

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The development of monoclonal antibodies (mAb) has allowed characterization of human T-lymphocyte activation pathways, which are different from the T-cell receptor-CD3 complex (TCR-CD3). One of the most intriguing molecules involved in T-cell activation is CD2 (T11/E-rosette receptor). T-cell activation via the CD2 pathway is metabolically regulated by the TCR-CD3 molecule on mature T cells [1], but it could be functionally independent of the TCR-CD3 complex on particular cell types [2–4]. T-cell activation via the CD2 molecule requires pairs of antibodies directed against different epitopes on the molecule [1,5,6]. T-cell activation can also occur in presence of one anti-CD2 (anti-T11.2) mAb in combination with sheep red blood cells (SRBC) or the purified CD2 ligand LFA-3 (Dustin ML, Olive D, Springer TA:J Exp Med, in press).

Activating pairs of anti-CD2 mAbs induce an increase in the concentration of cytoplasmic free calcium ($[Ca^{2+}]i$) in T-lymphocytes [7,8]. The initial increase in $[Ca^{2+}]i$ is due to the release of Ca^{2+} from internal stores [8,9], which seems to be mediated by inositol trisphosphate (IP3) because transmembrane signaling by the CD2 molecule involves a phospholipase C that hydrolyzes phosphatidylinositol biphosphates (PIP2) to IP3 and 1-2 diacylglycerol (1-2 DAG) [9]. Guanine nucleotide-binding G proteins have been implicated as signal transducers in a wide variety of cell types [11]. Bacterial toxins have greatly helped the characterization of these G proteins. Cholera toxin (CT), for example, activates the stimulatory G protein of adenylate cyclase (Gs) by catalyzing adenosine diphosphate (ADP)-ribosylation of its α chain [12,13]. These bacterial toxins act upon G proteins that regulate processes other than adenylate cyclase. Imboden et al. [14] found that CT acts on a G protein other than Gsα that could regulate transmembrane signaling via the TCR-CD3 complex in the leukemic T-cell line Jurkat. These results have not been confirmed by others [15,16]. Rosoff et al. [15] found no modifications of the anti-CD3-induced $[Ca^{2+}]i$ rise in CT-treated Jurkat cells. Lerner et al. [16] showed an inhibitory effect of CT on the IP3 generation and the increase in $[Ca^{2+}]i$ after stimulation of murine T-cell clones by concanavalin A, but their time-response as well as dose-response curves suggest that this inhibition is due essentially to an increase in cyclic adenosine monophosphate (cAMP).

We have tested the action of cholera toxin on the $[Ca^{2+}]i$ rise induced on Jurkat cells by stimulation via the CD2 molecule, which is another pathway of human T-cell activation linked to a phospholipase C. CD2, like CD3, was inhibited by cholera toxin. The CD2 pathway appears to be more sensitive than the CD3 pathway to this inhibitory effect. This effect cannot be ascribed to the cAMP rise induced by cholera toxin because forskolin, which induces similar cAMP levels, inhibited only weakly and to the same extent the calcium increase following CD2 or CD3 activation. In addition, we confirmed that cholera toxin, but not forskolin, induced a partial loss of CD3 from the cell surface, whereas CD2 expression was unaffected. Thus, the CD2 pathway could be linked via G proteins to a phospholipase C activity.

MATERIALS AND METHODS

Cells and mAbs

The leukemic T-cell line Jurkat was maintained in RPMI 1640 supplemented with 10% (vol/vol) fetal bovine serum. Anti-CD3 (×35.7) and anti-CD28 mAbs (CK 248) were generous gifts of D. Bourrel and A. Moretta, respectively. The anti-CD2

mAbs, CD2.1, and CD2.9 are directed against the T11.2 ant T11.1 epitopes of the CD2 molecule and have already been described [6].

Reagents

Ionomycin, cholera toxin, and forskolin were obtained from Calbiochem. 8-Bromo cAMP and EGTA were purchased from Boehringer and Sigma, respectively. Indo-1 AM was obtained from Molecular Probes.

Intracellular Ionized Calcium Assay

Measurement of $[Ca^{2+}]i$ in single cells was as in Rabinovitch et al. [17] with slight modifications. Cells were loaded with the acetoxymethylester of Indo-1 (3 μM) at 37°C in RPMI 1640 containing 2% heat-inactivated fetal bovine serum, 25 mM Hepes, pH 7.4. After 45 min, cells were washed and resuspended in fresh medium at 1×10^7 cells/ml and then stored in the dark at room temperature. Indo-1-loaded cells were transferred to the 37°C thermostatically controlled sample cup and analyzed using an ODAM-BRUCKER ATC 3000 cytofluorograph (Wissembourg, France). Ultraviolet excitation was obtained from an argon ion laser (COHERENT 90/5 UV, Palo Alto, CA). Violet fluorescence emission was measured at 380 to 410 nM (DF 395: 25 nM: Omega Optical Inc, Brattleboro, MN), blue emission measured at 480 to 520 nM (480- to 520-band pass filter: Corion, Holliston, MA), and violet-blue separation performed using a 450-nM dichroic filter (Mellis Giriot, France).

The Indo-1 violet/blue fluorescence ratio was digitally calculated, and data were collected using an "ATC 3000 kinetic" mode (ODAM-BRUCKER, France). The rise in $[Ca^{2+}]i$ after activation of Jurkat cells induces an augmentation of the ratio value due to the increase in the Ca^{2+} bound (violet emission) and a decrease in the Ca^{2+} free (blue emission) Indo-1. The percentage of responding cells was defined by the cells that had a test ratio after stimulation two standard deviations above the control curve. This is digitally calculated by subtraction of the control curve from the test curve. The ratio value remained unchanged during the incubation period, so we used the basal ratio at time 0 before the addition of the stimuli as control curve. After each experiment, the efficiency of cell loading was checked by the addition of Ionomycin, which induces a ratio increase that is seven to eight times superior to the basal ratio; otherwise, the experiment is discarded. Modifications of the results due to leakage of the Indo-1 from the cells is avoided by analyzing exclusively living cells as defined by biparametric analysis using volume and wide angle light scatter.

Measurement of cAMP Concentration

Cells (0.8×10^6 cells of a 4×10^6/ml suspension) were stimulated for various periods. Samples were quickly frozen and stored at −80°C until analysis. After thawing and sonication in 1N $HClO_4$, cAMP was assayed in the 10,000 g supernatants with a radioimmunoassay kit according to the manufacturer's instructions (Immunotech SA, Marseille-Luminy, France).

Analysis of Cell Surface Antigens

For indirect immunofluorescence, 2×10^5 cells (2 to 4×10^6/ml) were incubated 45 min at 4°C with saturating concentrations of antibodies. Cells then were washed and incubated with a saturating concentration of fluorescein-labeled goat anti-mouse immunoglobulin antibodies (Jackson Laboratories, West Grove, PA, USA). In

some experiments, fluorescein isiothiocyanate (FITC)-conjugated CD2.9 mAb (Immunotech SA, Marseille-Luminy, France) was used.

Samples were analyzed by flow cytometry using an ATC 3000 cytofluorograph (ODAM-BRUCKER, France), as already described [6].

RESULTS

Using the calcium-sensitive fluorescent indicator Fura-2 and spectrofluorimetry, the present authors have previously reported that selected pairs of anti-CD2 mAbs (CD2.1 + CD2.9) induced an increase in $[Ca^{2+}]i$ in Jurkat cells [9]. This increase is due in part to the release of $[Ca^{2+}]i$ from internal stores, as there is still an initial increase in $[Ca^{2+}]i$ in presence of EGTA. Also, it was confirmed that CD2, like TCR-CD3, complex was linked to a phosphodiesterase that hydrolyzes phosphatidyl inositol bisphosphate (PIP2) to inositol tris phosphates (IP3) and 1-2 diacylglycerol [9,10].

These results were confirmed and extended using Indo-1 and flow cytometry (Fig. 1). Anti-CD3 mAbs induced a rapid increase in $[Ca^{2+}]i$, which peaked at 30–45 s followed by a plateau. The $[Ca^{2+}]i$ increase induced by anti-CD2 mAbs was a slower process (half-maximal response 30 to 45 s for anti-CD2 and <30 s for anti-CD3). In both cases, most cells responded. The kinetic differences between the CD3 and CD2 pathways could be due to differences in the association kinetics of anti-CD2 and anti-CD3 mAbs for their ligands or to a conformational change induced by one of the anti-CD2 mAbs that would be required for the binding of the second. Because preincubation of Jurkat cells with either anti-CD2 mAb (CD2.1 or CD2.9) for 30 min at 37°C, followed by the addition of the other anti-CD2 mAb, did not modify the half-maximal response (not shown), the last hypothesis can be ruled out.

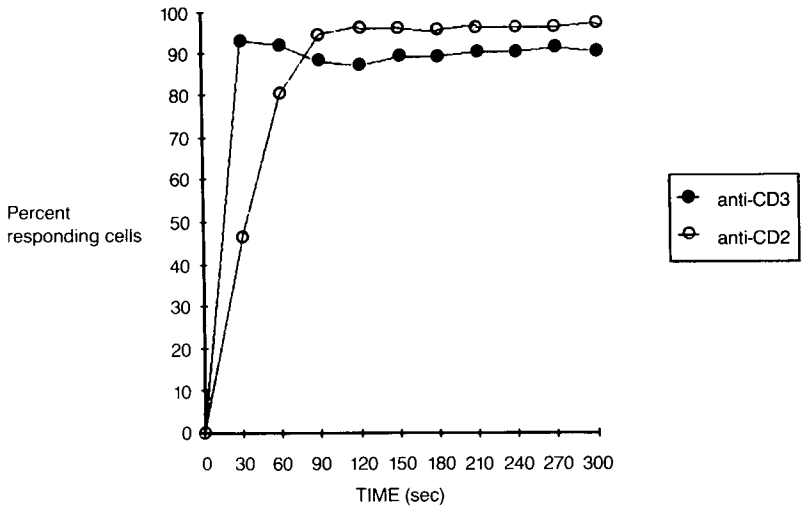

Fig. 1. $[Ca^{2+}]i$ increase in Jurkat cells after stimulation by anti-CD3, anti-CD2 mAbs. Jurkat cells were loaded with 3 μM Indo-1 AM for 45 min at 37°C, washed, and then stimulated with anti-CD3 (×35.7) and anti-CD2 (CD2.1 + CD2.9) ascites (1/200). The percentage of cells having a calcium response above threshold is plotted as a function of time.

Preincubation of Jurkat cells with CT inhibited the number of responding cells (as defined in Materials and Methods) upon anti-CD3 and anti-CD2 mAbs induced stimulation (Fig. 2). However, cells that are still responsive have a $[Ca^{2+}]i$ increment per cell that is equal to the cells not treated with CT. This suggests an all or none effect of CT on the $[Ca^{2+}]i$ rise induced by anti-CD3 or anti-CD2 mAbs. This effect was not due to alterations in cell viability, as cytofluorimetric analysis was performed on viable cells as defined by biparametric analysis of electric cell volume and wide-angle light scattering; in addition, these cells continued to grow in usual culture conditions. Half-maximal inhibition occurred around 75 ng/ml for the CD3 pathway and 30 ng for the CD2 pathway, in the absence or presence of 10 mM EGTA (not shown).

Cholera toxin induced an increase in cAMP concentrations (Table I) via adenylate cyclase activation due to the ADP ribosylation of Gs. This increase in cAMP was maximal after 1 h and reached a stable plateau for at least 3 h. Both forskolin, a known adenylate cyclase activator, and cholera toxin generated the same levels of cAMP in Jurkat cells (100 ng cholera toxin and 10 uM forskolin generated 10 pM cAMP/10^6 cells). Because cholera toxin generated high levels of cAMP, the action of forskolin on the $[Ca^{2+}]i$ induced by anti-CD2 and anti-CD3 mAbs was tested. Forskolin only partially inhibited the $[Ca^{2+}]i$ induced by anti-CD3 (Fig. 3A) and

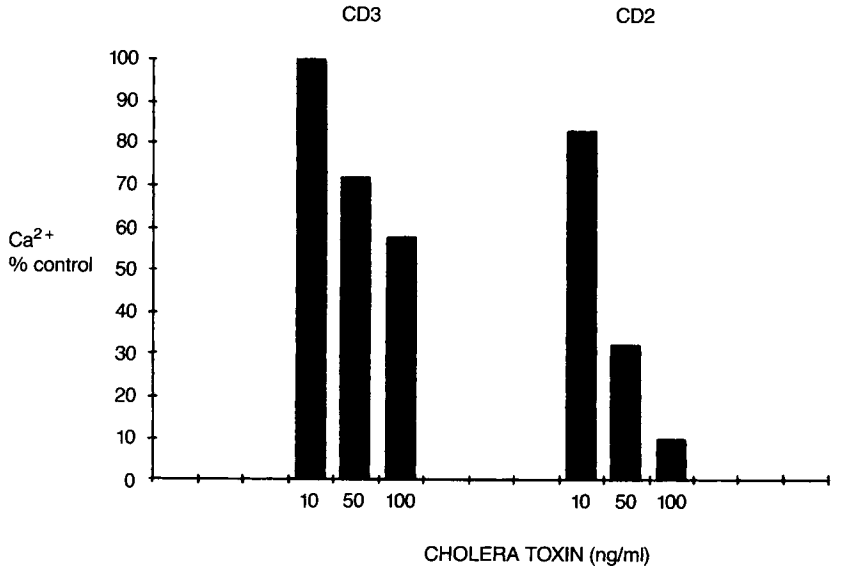

Fig. 2. Inhibition of the anti-CD3 and anti-CD2 induced $[Ca^{2+}]i$ increase by cholera toxin. Cells (5×10^6/ml) were incubated with different doses of cholera toxin for 3 h at 37°C, with 3 μM Indo-1 AM added during the last 45 min. Cells were then washed and resuspended at 10^7/ml at room temperature in the dark until used. Cells were stimulated by an optimal dose of mAb (ascites 1/200). On the y-axis, the $[Ca^{2+}]i$ response of Jurkat cells is presented as a percentage of the responses of control cells not treated with cholera toxin. This percentage of responsive cells was measured at the plateau of the $[Ca^{2+}]i$ increase (anti-CD3: 2 min; anti-CD2: 3 min). Treatment with cholera toxin does not influence the ability to detect changes in $[Ca^{2+}]i$, as the response to ionomycin is not affected by cholera toxin (5 μM Ionomycin induce a $[Ca^{2+}]i$ rise in >90% cells, with a mean ratio fluorescence intensity of 169 for untreated cells and 175 for cells treated with cholera toxin).

TABLE I. Increase in a AMP Induced by Cholera Toxin and Forskolin in Jurkat Cells*

	Time (min)			
Stimuli	0	30	60	180
Medium	1.12[a]	1.12	1.25	1.25
Cholera toxin 100 ng/ml		6	10	10
Forskolin 10 μM		9	NT[b]	10

*Jurkat cells were incubated for various periods at 37°C; then samples were frozen and stored at −80°C until analysis, at which time they were sonicated in 1N $HClO_4$. cAMP was assayed in the 10,000 g supernatant with a Immunotech RIA kit according to the manufacturer's instructions.
[a]Results are expressed at pM/10^6 cells.
[b]Not tested.

anti-CD2 mAbs (Fig. 3B). The inhibition affected the kinetics of the $[Ca^{2+}]i$ increase and the number of responsive cells.

Cholera toxin decreased the binding of anti-CD3 mAbs by a factor of 2 to 4 as assessed by immunofluorescence using flow cytometry (Table II). This effect was dose dependent and restricted to the CD3 molecule. Because CD2 expression was unmodified in the same condition, this effect cannot be due solely to the increase in cAMP, as forskolin (10 uM) did not affect CD3 expression (Table III).

Inhibition of the $[Ca^{2+}]i$ increase induced by anti-CD3 mAbs could be due to the CD3 modulation. This appears unlikely because variants isolated by cell sorting, which expressed fewer CD3 molecules than the wild-type Jurkat cell line, responded equally well to anti-CD3 mAbs (not shown). Furthermore, the $[Ca^{2+}]i$ increase induced by anti-CD2 was dramatically affected despite the fact that cholera toxin does not induce any decrease in CD2 expression. Nevertheless, the mere modulation of the CD3 molecule could still account in part for the high sensitivity in inhibition by CT of the CD2 pathway of activation [1,25].

Inhibition of the Ca^{2+} response to anti-CD2 mAbs could be due to a change in affinity of the mAb for its ligand. To elucidate this, the capacities of untreated and treated cells to bind FITC-labeled CD2.9 at equilibrium were measured. Indeed, the half-saturation value was very sensitive to variations in affinity of mAbs for their ligands. There was no modification in the half-saturation of binding between treated and untreated cells (Table IV). Forskolin did not affect FITC CD2.9 binding.

DISCUSSION

Recently, the involvement of G proteins has been suspected in the activation of human T-lymphocytes via the CD3-TCR complex [14].

The CD2 molecule is a pathway of human T-lymphocyte activation identified by mAbs that, like CD3, is linked to a phospholipase C activity [9,14]. The present study tested the action of cholera toxin on the $[Ca^{2+}]i$ rise induced by anti-CD2 mAbs in Jurkat cells. Indeed, cholera toxin is a bacterial toxin that activates adenylate cyclase by ADP ribosylation of $Gs\alpha$ [12,13], but it could have pleiotropic effects, including an action on other G proteins [23,24,14].

Cholera toxin inhibited the $[Ca^{2+}]i$ increase induced in Jurkat cells by anti-CD2 and, as already reported, by anti-CD3 mAbs [14]. This effect was linked only in part to the increase in cAMP induced by cholera toxin. In addition, cholera toxin, but not other agents (such as forskolin) that increase cAMP, was confirmed to induce a partial loss in the expression of the CD3, but not of the CD2 molecule.

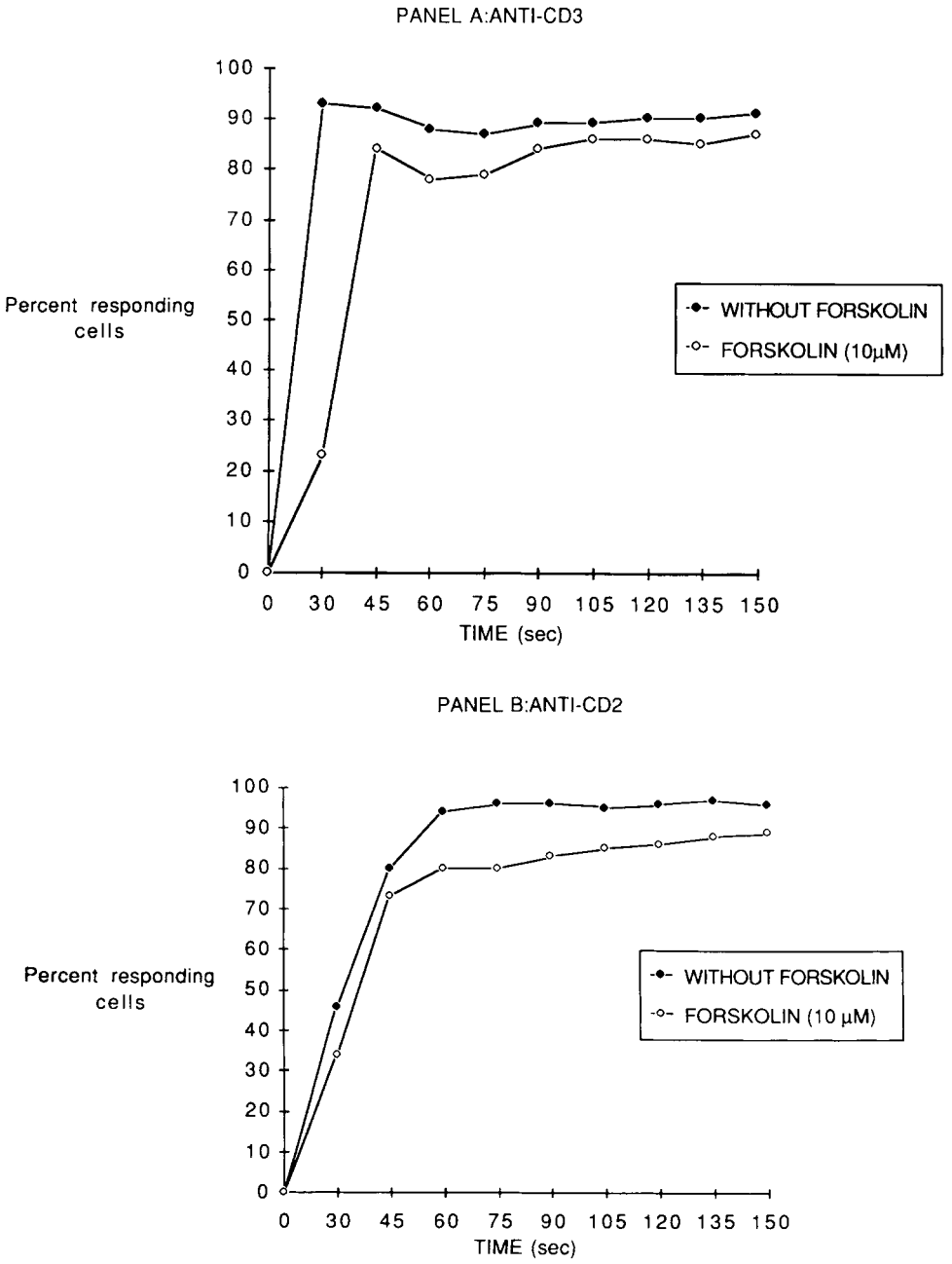

Fig. 3. Action of forskolin on the anti-CD2 and anti-CD3 induced $[Ca^{2+}]i$ rise in Jurkat cells. Cells (5 $\times$ 10^6/ml) were incubated with 10 μM forskolin for 3 h at 37°C; 3 μM Indo-1 AM added during the last 45 min and then processed as in Figure 2. Cells were stimulated with anti-CD3 mAb (Panel **A**) or anti-CD2 mAbs (Panel **B**). The percentage of cells having a calcium response above threshold is plotted as a function of time. Treatment with forskolin does not influence the ability to detect the changes in $[Ca^{2+}]i$, as the response to ionomycin is not affected by forskolin, 5 μM ionomycin induce a $[Ca^{2+}]i$ rise in >90% cells with a mean ratio fluorescence intensity of 169 for untreated cells and 167 for cells incubated with forskolin.

TABLE II. Cholera Toxin Modulates the Expression of the CD3 Molecule From the Cell Surface*

Reagents	Concentration ng/ml	Mean fluorescence intensity	
		CD3	CD2
Medium		47	74
Cholera toxin	100	12	68
	30	23	74
	10	33	70

*Jurkat cells (5×10^6/ml) were incubated at 37°C with different doses of cholera toxin. After 3 h, cells were incubated 45 min at 4°C with a saturating amount of antibody, washed, and stained with fluorescein-labeled goat antibodies against mouse immunoglobulin. Samples were analyzed by flow cytometry.

TABLE III. Modulation of the CD3 Molecule Is Induced by Cholera Toxin but Not by Forskolin*

Reagents		Mean fluorescence intensity	
		CD3	CD2
Medium		90	105
Cholera toxin	100 ng/ml	45	118
Forskolin	10 μM	86	124
TPA	10^{-8}M	48	158

*Jurkat cells (5×10^6/ml) were incubated at 37°C with either cholera toxin (100 ng/ml), forskolin (10 μM), or TPA (10^{-8}M). After 3 h, the cells were incubated (45 min at 4°C) with a saturating amount of antibody, washed, and stained with fluorescein-labeled goat antibodies against mouse immunoglobulin. Samples were analyzed by flow cytometry.

TABLE IV. Half-Saturation Binding of FITC-Labeled CD2.9 on Jurkat Cells Treated With Medium, Cholera Toxin or Forskolin*

	Reagents		
	Medium	Cholera toxin (100 ng/ml)	Forskolin (10 μM)
FITC-labeled CD2.9	0.94[a]	1.25	1.06

*Jurkat cells were treated with cholera toxin (100 ng/ml), forskolin (10 μM), or left untreated 3 h at 37°C. Then cells were washed and incubated with different concentrations of FITC-labeled CD2.9 at 4°C for 45 min. CD2.9 binding was analyzed by flow cytometry and expressed as mean fluorescence intensity.

[a]Results are expressed in nM and correspond to the half-maximal binding of CD2.9.

Cholera toxin is known to activate adenylate cyclase and so increases the intracellular cAMP levels, but this is not sufficient to explain the totality of its inhibitory effect on the $[Ca^{2+}]i$ rise induced in Jurkat cells by anti-CD2 and anti-CD3 mAbs. Forskolin increased intracellular cAMP to the same levels as cholera toxin after 3 h, but its inhibitory effect on the anti-CD2 and anti-CD3 induced $[Ca^{2+}]i$ rise was marginal in comparison to the profound inhibition due to cholera toxin.

Nevertheless, weak inhibition of forskolin on the CD2- and CD3-induced calcium increment was reproducible. These results differ from the work of Imboden et al. [14], but this could be due to differences in the cell lines used (we used a subclone from the parental Jurkat cell line) or in the assay for $[Ca^{2+}]i$ measurement (Imboden used Fura-2 and spectrofluorimetry, whereas we used Indo-1 and cytofluorimetry).

Our results are partly in line with recent data on murine T cells that demonstrated a role for cAMP in the regulation of the activity of the phospholipase C linked to the TCR-CD3 complex [16,18]. The present work has demonstrated that the same or the different phospholipases C [19] linked to CD2 and CD3 are regulated by cholera toxin that could act on G proteins other than $Gs\alpha$.

Whether the inhibitory effect of cholera toxin on the CD2 pathway is direct or linked to down-regulation of the CD3 molecule remains unanswered. The complete modulation of the CD3-TCR complex induced by anti-CD3 mAbs is known to inhibit a further stimulation via the CD2 pathway [1, 25].

Nevertheless, after treatment with cholera toxin, the loss of CD3 expression was only partial, whereas the $[Ca^{2+}]i$ rise induced by anti-CD2 mAbs was completely inhibited.

This partial loss of CD3 expression, which was not induced by forskolin treatment, confirms that cholera toxin has other effects in addition to activation of adenylate cyclase.

The basis for the down-regulation of the CD3 molecule is known in two systems. The stimulation of a T-cell clone by its antigen or the treatment of T cells by activators of protein kinase C induce both the phosphorylation of the CD3 and, to a lesser extent, chains, which correlates with the disappearance of the CD3 molecule from the cell surface [21,22]. On the contrary, activators of protein kinase C neither down-regulate nor phosphorylate the CD2 molecule [22]. An attractive hypothesis could be an action of cholera toxin on protein kinase C activity that would induce the phosphorylation of CD3, resulting in its disappearance from the cell surface. This could correlate with the inhibitory effect of phorbol esters on the $[Ca^{2+}]i$ increase induced in Jurkat cells by anti-CD2; anti-CD3 stimulation was affected only to a lesser extent (D. Olive, unpublished observations).

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