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Immunology

Cholera toxin inhibits the T-cell antigen receptor-mediated increases in inositol trisphosphate and cytoplasmic free calcium

(GTP binding proteins/inositol phospholipid turnover/T-cell activation)

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ABSTRACT **The addition of monoclonal antibodies to the antigen receptor complex on the malignant human T-cell line Jurkat generates increases in inositol trisphosphate and in the concentration of cytoplasmic free calcium. Exposure of Jurkat cells to cholera toxin for 3 hr inhibited these receptor-mediated events and led to a selective, partial loss of the antigen receptor complex from the cellular surface. None of the effects of cholera toxin on the antigen receptor complex were mimicked by the B subunit of cholera toxin or by increasing intracellular cAMP levels with either forskolin or 8-bromo cAMP. These results suggest that a cholera toxin substrate can regulate signal transduction by the T-cell antigen receptor.**

Perturbation of the antigen receptor complex by monoclonal antibodies (mAbs) results in an increase in the concentration of cytoplasmic free calcium ($[Ca^{2+}]_i$) in T lymphocytes (1–6). The initial antigen receptor-induced increase in $[Ca^{2+}]_i$ is due to the release of Ca^{2+} from intracellular stores and appears to be mediated by inositol trisphosphate ($InsP_3$) (1), the putative Ca^{2+}-mobilizing signal generated by a number of receptors (reviewed in ref. 7). A critical step in transmembrane signaling by the T-cell antigen receptor, then, is the activation of a phosphodiesterase that hydrolyzes phosphatidylinositol bisphosphate ($PtdInsP_2$) to $InsP_3$ and diacylglycerol.

Receptor-mediated hydrolysis of $PtdInsP_2$ can be enhanced by GTP (8–10). Ca^{2+}-mobilizing receptors, therefore, may be linked to the $PtdInsP_2$ phosphodiesterase by regulatory guanine nucleotide binding proteins (G proteins) in a fashion analogous to the coupling of receptors to adenylate cyclase (11). Critical to the characterization of the G proteins that regulate adenylate cyclase was the observation that these G proteins serve as substrates for certain bacterial toxins. Cholera toxin, for example, activates the stimulatory G protein of adenylate cyclase (G_s) by catalyzing the transfer of ADP-ribose to its 43-kDa α subunit (12, 13). Pertussis toxin, on the other hand, ADP-ribosylates the 41-kDa α subunit of the inhibitory G protein G_i (14, 15). As a consequence of the action of pertussis toxin, G_i is inactivated, and hormonal inhibition of adenylate cyclase is blocked (14, 15). Cholera toxin and pertussis toxin can act upon G proteins that regulate processes other than adenylate cyclase. Both toxins ADP-ribosylate transducin, the G protein that mediates rhodopsin-induced activation of cyclic GMP phosphodiesterase in the retina (16, 17). In addition, pertussis toxin ADP-ribosylates G_o, a G protein purified from the brain (18) that can functionally interact with muscarinic cholinergic receptors (19).

A substrate for pertussis toxin appears to regulate signal transduction by Ca^{2+}-mobilizing receptors in the neutrophil. Exposure of neutrophils to pertussis toxin blocks the ability of the chemotactic peptide fMet-Leu-Phe to activate a membrane-associated GTPase, to decrease $PtdInsP_2$, to increase $[Ca^{2+}]_i$, and to induce secretion (20–24). This inhibition of the action of fMet-Leu-Phe by pertussis toxin is temporally associated with the ADP-ribosylation of a 41-kDa protein (23, 24). Although identity of this substrate with the α subunit of G_i has not been established, purified G_i can reconstitute the fMet-Leu-Phe-stimulated GTPase activity in membranes prepared from pertussis toxin-treated neutrophils (20). In other cellular systems, however, signal transduction by Ca^{2+}-mobilizing receptors is not sensitive to pertussis toxin and does not appear to involve G_i (25, 26). For example, exposure of chicken heart cells to pertussis toxin inhibits G_i activity but does not inhibit muscarinic receptor-mediated phosphatidylinositol hydrolysis and Ca^{2+} mobilization (25).

In this communication, we examine the effects of cholera toxin on signal transduction by the T-cell antigen receptor. Exposing the malignant human T-cell line Jurkat to cholera toxin blocks the ability of the antigen receptor to generate inositol phosphates and to increase $[Ca^{2+}]_i$. In addition, cholera toxin causes a selective, partial loss of the antigen receptor heterodimer and its associated structures, T3, from the cellular surface. The effects of cholera toxin on the antigen receptor require the addition of the intact toxin, are temporally dissociated from the ADP-ribosylation of putative $G_s\alpha$, and are not mimicked by simply increasing the intracellular level of cAMP with either forskolin or 8-bromo cAMP. These findings suggest that cholera toxin acts directly on a substrate, possibly a G protein distinct from $G_s\alpha$, that can regulate the antigen receptor-mediated generation of $InsP_3$.

MATERIALS AND METHODS

Cells and mAbs. Jurkat and J.T30.7 were maintained in RPMI 1640 supplemented with penicillin, streptomycin, L-glutamine (2 mM), and 10% (vol/vol) fetal bovine serum (medium) as described (27). C305 mAb, an IgM with κ light chains, recognizes the antigen receptor heterodimer displayed by Jurkat and J.T30.7; C373, another IgM κ mAb, reacts with the E-rosette receptor on human T cells (27). mAbs, OKT3 and Leu 4, were obtained from Ortho Diagnostics and Becton Dickinson, respectively.

Reagents. Ionomycin was a gift from Squibb. Cholera toxin, cholera toxin A subunit, and cholera toxin B subunit were purchased from List Biologicals (Campbell, CA). Forskolin and quin-2 AM [the tetrakis(acetoxymethyl) ester

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Abbreviations: mAb, monoclonal antibody; $[Ca^{2+}]_i$, concentration of cytoplasmic free calcium; $InsP_3$, inositol trisphosphate; $PtdInsP_2$, phosphatidylinositol bisphosphate; G protein, guanine nucleotide binding protein; $InsP_2$, inositol bisphosphate; $InsP$, inositol phosphate; s, stimulatory; i, inhibitory.

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of quin-2] were obtained from Calbiochem. [α-^{32}P]NAD (29.5 Ci/mmol; 1 Ci = 37 GBq) was from New England Nuclear. Electrophoresis supplies were from Bio-Rad. All other chemicals were from Sigma.

Determination of $[Ca^{2+}]_i$. Cells were loaded with 10 μM of the acetoxymethyl ester of quin-2, and the fluorescence of the cellular suspension (5×10^6 cells per ml) was monitored with a Perkin–Elmer 650-40 spectrofluorometer as described (3). $[Ca^{2+}]_i$ was calculated by the method of Tsien *et al.* (28).

[^{32}P]ADP-Ribosylation of Membrane Proteins. Jurkat cells (5×10^6 cells per ml) were incubated at 37°C for 1 hr in medium alone or in medium with cholera toxin (0.1 μg/ml). After incubation, cells were washed twice in ice-cold Dulbecco's phosphate-buffered saline and then homogenized in 25 mM Tris·HCl (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol. Following removal of debris by centrifugation at $500 \times g$ for 10 min, a crude membrane fraction was sedimented by centrifugation at $12,000 \times g$ for 10 min. [^{32}P]ADP-ribosylation of membrane proteins was carried out as described (29, 30) in a final volume of 0.1 ml containing 100 mM potassium phosphate (pH 7.5), 10 mM thymidine, 1 mM ATP, 0.1 mM GTP, 2.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 2.5 μg of cholera toxin A subunit, and 100 μg of membrane protein. After 30 min at 32°C, 1 ml of ice-cold buffer containing 25 mM Tris·HCl (pH 7.5), 1 mM EDTA was added, and the membranes were sedimented by a $12,000 \times g$ centrifugation for 10 min. The pellet was resuspended in 0.1 ml of Laemmli sample buffer (31) with 5% (vol/vol) 2-mercaptoethanol, and samples were electrophoresed in 8% $NaDodSO_4$/polyacrylamide gels. Autoradiography was performed by exposing the dried gel to Kodak X-Omat AR film in the presence of an intensifier screen for 72 hr at −70°C.

Determination of Cellular cAMP Content. cAMP was extracted from cells with three 1-ml washes of absolute ethanol. Following evaporation of the ethanol in a Brinkman sample concentrator, cAMP was determined by the competitive protein binding assay of Gilman (32).

Measurement of [^{3}H]Inositol Phosphates. Incorporation of [^{3}H]inositol into phospholipid was achieved by incubating Jurkat cells (10^7 cells per ml) in a Hepes-buffered saline solution with [^{3}H]inositol (40 μCi/ml; 37 MBq/ml; Amersham) for 3 hr followed by dilution in medium to 10^6 cells per ml. After an overnight incubation at 37°C in 5% CO_2/95% air, cells were washed and then resuspended in medium alone, medium with forskolin (10^{-4} M), or medium with cholera toxin (0.1 μg/ml). After 3 hr, aliquots (5×10^6 cells) were removed and either processed directly or treated with C305 (final dilution 1:100). The [^{3}H]inositol phosphates were extracted, separated by anion exchange chromatography, and quantified as described (1).

Flow Cytometric Analysis. After a 3-hr incubation in medium alone or medium with either cholera toxin (0.1 μg/ml), 8-bromo cAMP (10^{-3} M), forskolin (10^{-4} M), or cholera toxin B subunit (1 μg/ml), indirect immunofluorescence was performed by staining, on ice, with the indicated mAb followed by fluoresceinated F(ab')$_2$ goat anti-mouse immunoglobulin (Cappel Laboratories, Cochranville, PA). Cells were analyzed on a FACS IV cell sorter (Becton Dickinson) as described (3).

RESULTS

Monoclonal antibodies with specificity for the antigen receptor complex cause prompt, sustained increases in $[Ca^{2+}]_i$ when added to Jurkat cells that are loaded with the Ca^{2+}-sensitive fluor, quin-2 (2, 3). A representative experiment using C305, a mAb to the antigen receptor heterodimer displayed by Jurkat cells (27), is shown in Fig. 1*A*. C305 causes an increase in $[Ca^{2+}]_i$ that reaches a peak value within 60 sec and then falls to a plateau, remaining elevated above basal levels for at least 30 min. The peak value attained with C305 ranges from 350 nM to 1 μM Ca^{2+}, and the plateau level varies from 200 to 280 nM Ca^{2+} (2). Comparable increases in $[Ca^{2+}]_i$ are seen with mAbs to T3 components of the antigen receptor complex (Fig. 1*C* and ref. 3).

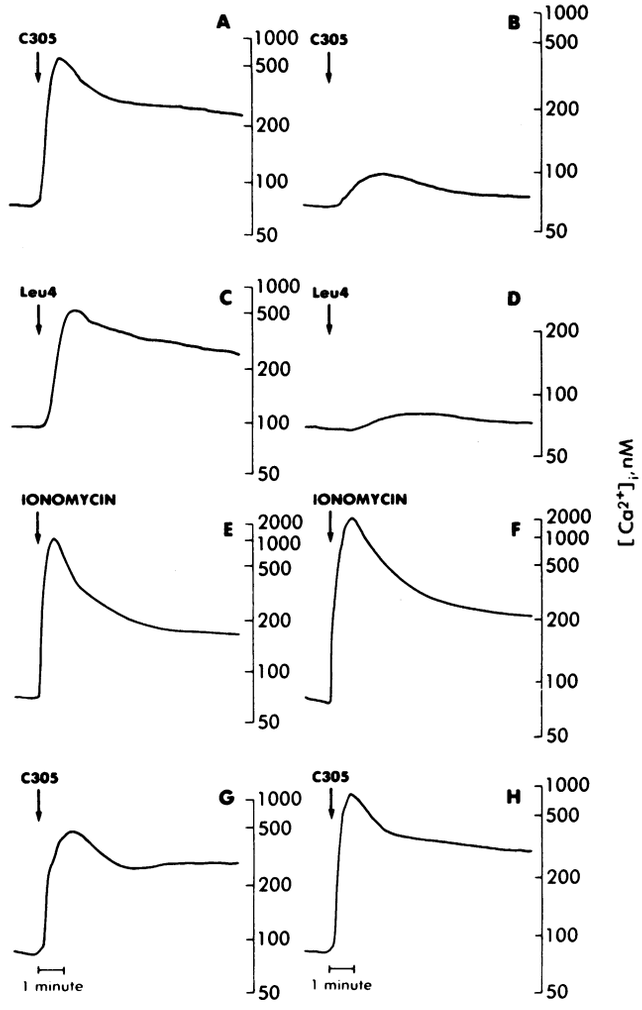

FIG. 1. The antigen receptor-mediated increase in $[Ca^{2+}]_i$ is inhibited by exposure to cholera toxin. Shown are representative tracings of the fluorescence of quin-2-containing Jurkat cells monitored over time. $[Ca^{2+}]_i$, calculated by the method of Tsien *et al.* (28), is displayed on the vertical axis. C305 (mAb to the antigen receptor heterodimer), Leu 4 (mAb to T3), and the Ca^{2+} ionophore, ionomycin, were added at the indicated time points to Jurkat cells that had been incubated for 3 hr in medium alone (*A*, *C*, and *E*) or medium with either cholera toxin at 0.1 μg/ml (*B*, *D*, and *F*), 10^{-4} M forskolin (*G*), or 10^{-3} M 8-bromo cAMP (*H*). During the final hour of this incubation, the cells were loaded with quin-2 by the addition of 10 μM quin-2 AM. C305 was added from culture supernatants in a final dilution of 1:100 (equivalent in activity to HPLC-purified C305 at 1 μg/ml) to the cholera toxin-treated cells and in a final dilution of 1:1000 to the cells that were untreated or exposed to either forskolin or 8-bromo cAMP. Leu 4 was added from ascites in final dilutions of 1:1000 to the cholera toxin-treated cells and 1:10,000 to the untreated cells. The final concentration of ionomycin was 1 μM. Each tracing is representative of at least three separate experiments.

Exposure of Jurkat cells to cholera toxin (0.1 μg/ml) for 3 hr markedly reduces both the peak and the sustained increases in $[Ca^{2+}]_i$ that follow the addition of C305 (Fig. 1*B*). In nine experiments, $[Ca^{2+}]_i$ rose to a peak value of only 107 ± 7 nM (mean ± SEM) when C305 was added in a final dilution of 1:100 to cholera toxin-treated Jurkat cells. Similar results are obtained with Leu 4, a mAb to T3 determinants (Fig. 1*D*) as well as with two additional T3 mAbs (OKT3 and

64.1.1; data not shown). In the presence of this concentration of cholera toxin, Jurkat cells remain viable and continue to grow with the same doubling time as untreated cells (data not shown). Treatment with cholera toxin does not influence the ability to detect changes in $[Ca^{2+}]_i$ as the response to the Ca^{2+} ionophore, ionomycin, is not affected by cholera toxin (Fig. 1 *E* and *F*). In contrast to the effects of the intact toxin, the purified binding B subunit of cholera toxin, even in concentrations of 1 μg/ml, has no effect on the antigen receptor-mediated increase in $[Ca^{2+}]_i$ (data not shown). Effective binding of the B subunit was confirmed by the ability of the B subunit to completely protect Jurkat cells from the inhibitory effects of intact cholera toxin (0.1 μg/ml; data not shown).

Through its ability to ADP-ribosylate and to activate $G_s\alpha$, cholera toxin increases cellular cAMP content. Acute elevations in cellular cAMP content can inhibit the ability of certain receptors to increase $[Ca^{2+}]_i$, but this is not a universal finding (33). The predominant substrate for cholera toxin in Jurkat cells has a M_r of 43,000, which is identical to that of $G_s\alpha$ in most cells (12, 13) (Fig. 2*A*). Within 1 hr of the addition of cholera toxin to intact cells, this 43-kDa substrate appears to be completely ADP-ribosylated (Fig. 2*B*). Consistent with this observation, the maximal increase in cellular cAMP content occurs 1 hr after the addition of cholera toxin to Jurkat cells (Fig. 3). At that time point, however, there is no inhibition of the antigen receptor-mediated increase in $[Ca^{2+}]_i$ (Fig. 3). Inhibition of the antigen receptor-mediated increase in $[Ca^{2+}]_i$ is detected 90 min after the addition of cholera toxin and is maximal only after 3 hr of exposure to the toxin. The maximal effects of cholera toxin on the antigen receptor, therefore, occur 2 hr after ADP-ribosylation of the putative $G_s\alpha$ in Jurkat cells and 2 hr after the peak cAMP response.

To examine the possibility that prolonged elevation of the intracellular cAMP level inhibits the ability of the antigen receptor to increase $[Ca^{2+}]_i$, we exposed Jurkat cells to either 8-bromo cAMP (10^{-3} M) or to forskolin (10^{-4} M) for 3 hr. In this concentration, forskolin, an activator of adenylate cyclase (34), generates higher cellular cAMP contents at all time points throughout a 3-hr incubation than does cholera toxin

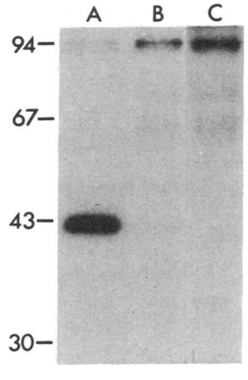

FIG. 2. Effect on Jurkat cells of prior exposure to cholera toxin on subsequent ADP-ribosylation of the 43-kDa cholera toxin substrate. Intact Jurkat cells were incubated for 1 hr at 37°C in medium alone (lanes A and C) or in medium with cholera toxin at 0.1 μg/ml (lane B). Cells were washed, and a membrane fraction was prepared and incubated with [^{32}P]NAD in the presence (lanes A and B) or absence (lane C) of cholera toxin A subunit. Labeled proteins were separated by electrophoresis in 8% $NaDodSO_4$/polyacrylamide gels. The molecular weights ($\times 10^{-3}$) of protein standards are shown on the left. These data are representative of six separate experiments. In several experiments, a faint 50-kDa cholera toxin substrate was also observed; in all cases this band was not detected in membranes obtained from cells that had been previously exposed to cholera toxin for 1 hr.

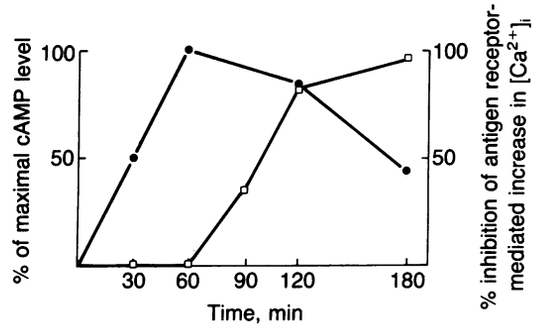

FIG. 3. Time courses for the increases in cellular cAMP content (●) and for the inhibition of the antigen receptor-mediated increase in $[Ca^{2+}]_i$ (□) following the addition of cholera toxin to Jurkat cells. The cAMP response is expressed as the percentage of the maximal intracellular cAMP level after the addition of cholera toxin (0.1 μg/ml). The cellular cAMP content, measured as the means of cAMP content of triplicate cell samples and determined by the method of Gilman (32), was 0.5 pmol per 10^6 cells in the untreated cells and rose to 35.4 pmol per 10^6 cells after cholera toxin. To follow the time course of the inhibition of the antigen receptor-mediated increase in $[Ca^{2+}]_i$, Jurkat cells were loaded with quin-2 and then incubated with either medium alone or medium with cholera toxin (0.1 μg/ml). At the indicated time points, single determinations of the peak C305-induced increase in $[Ca^{2+}]_i$ in the treated and untreated cells were made. The data are presented as the percentage inhibition of the peak increase by cholera toxin and are representative of three separate experiments.

at a concentration of 0.1 μg/ml (data not shown). Simply increasing the intracellular cAMP level for prolonged periods does not mimic the effects of cholera toxin on the antigen receptor-mediated increase in $[Ca^{2+}]_i$. C305, in a final dilution of 1:1000, induces peak increases in $[Ca^{2+}]_i$ that exceed 450 nM, and plateau increases that are greater than 200 nM, when added to Jurkat cells that have been exposed to either forskolin (Fig. 1*G*) or 8-bromo cAMP (Fig. 1*H*) for 3 hr. No inhibitory effect is detectable with either agent acutely, after 15 min, or at regular 30-min intervals up to 4 hr (data not shown). In marked contrast, C305 causes no detectable change in $[Ca^{2+}]_i$ when added to cholera toxin-treated Jurkat cells in a final dilution of 1:1000 (see Fig. 7).

As $InsP_3$ is the putative Ca^{2+}-mobilizing signal generated by perturbation of the antigen receptor (1), we studied the ability of C305 to generate $InsP_3$ in cholera toxin-treated cells (Fig. 4). The addition of C305 to untreated Jurkat cells generates substantial increases in $InsP_3$ as well as in its breakdown products, inositol bisphosphate ($InsP_2$) and inositol phosphate (*InsP*) (Fig. 4*A*). Identical antigen receptor-mediated increases in all three inositol phosphates are seen in Jurkat cells exposed to forskolin (Fig. 4*B*). In contrast, perturbation of the antigen receptor results in no detectable change in either *InsP*, $InsP_2$, or $InsP_3$ in Jurkat cells that have been treated with cholera toxin for 3 hr (Fig. 4*C*).

Exposure to cholera toxin results in the reduction in the binding of both OKT3 and C305 by approximately a factor of 2 as assessed by indirect immunofluorescence using flow cytometry (Fig. 5 *A* and *B*). This effect is specific for mAbs to the antigen receptor complex in that cholera toxin has no effect on the expression of the E-rosette receptor (Fig. 5*C*) or HLA class I antigens (data not shown). The reduction in binding sites for OKT3 and C305 is detectable at 2 hr and is maximal at 3 hr (data not shown). Jurkat cells that have been exposed to cholera toxin for 3 hr, washed, and then resuspended in fresh medium express a normal density of the antigen receptor complex within 18 hr (data not shown). Neither 8-bromo cAMP nor forskolin affect the expression of either T3 determinants or the antigen receptor heterodimer

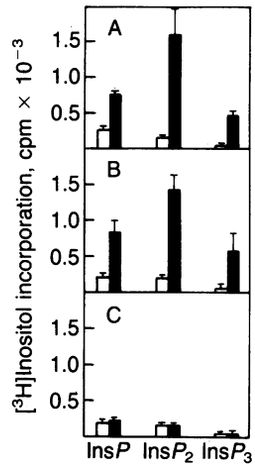

FIG. 4. Cholera toxin inhibits the antigen receptor-mediated increase in [^{3}H]inositol phosphates. Jurkat cells, labeled with [^{3}H]inositol, were incubated for an additional 3 hr in medium alone (*A*), medium with 10^{-4} M forskolin (*B*), or medium with cholera toxin at 0.1 μg/ml (*C*). [^{3}H]Ins*P*, [^{3}H]InsP_2, and [^{3}H]InsP_3 were then extracted, separated, and quantified as described (1) from unstimulated cells (open bars) and from cells stimulated for 10 min with C305 (closed bars). Data are presented as the mean ± SEM in counts per minute of three separate experiments. In untreated Jurkat cells, the C305-induced increase in [^{3}H]InsP_3 is maximal at 10 min (1). There are no detectable increases in any of the [^{3}H]inositol phosphates at earlier time points (1, 3, and 5 min) after the addition of C305 to cholera toxin-treated cells (data not shown).

(Fig. 5). This effect of cholera toxin is not simply due to the binding of toxin to the cellular surface as demonstrated by the inability of cholera toxin B subunit to affect expression of determinants for OKT3 and C305 (Fig. 5).

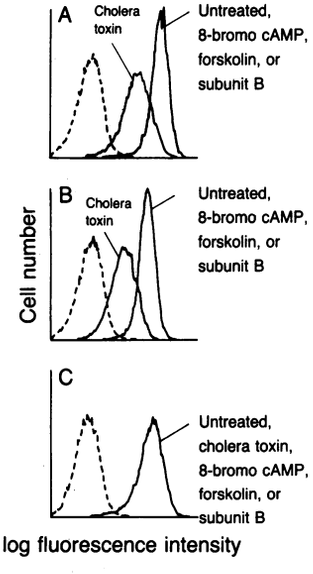

FIG. 5. Cholera toxin induces a reduction by a factor of 2 in the binding of mAbs to the antigen receptor complex on Jurkat. Shown are the fluorescence histograms obtained by flow cytometric analysis of Jurkat cells stained by indirect immunofluorescence with: (*A*) OKT3, a mAb to T3 components of the antigen receptor complex; (*B*) C305, a mAb to the antigen receptor heterodimer; and (*C*) C373, a mAb to the E-rosette receptor. The histogram obtained with a nonreactive control antibody (MOPC 195) is shown as a dotted line. In these experiments, Jurkat cells were incubated as indicated with medium alone (untreated), or medium with cholera toxin (0.1 μg/ml), 8-bromo cAMP (10^{-3} M), forskolin (10^{-4} M), or cholera toxin B subunit (1 μg/ml) as indicated. After 3 hr, cells were washed and then stained for indirect immunofluorescence. These results are representative of at least three separate experiments.

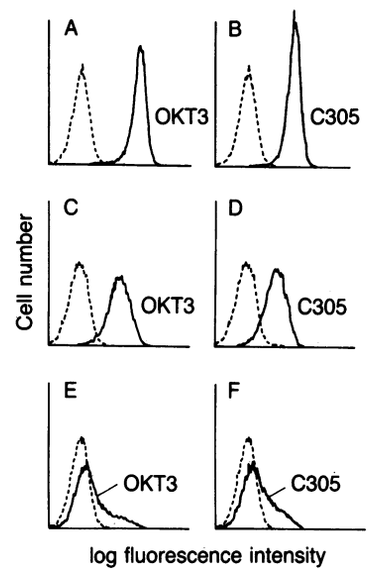

FIG. 6. Comparison of the binding of mAbs to the antigen receptor complex on untreated wild-type Jurkat (*A* and *B*); wild-type Jurkat exposed to cholera toxin (0.1 μg/ml) for 3 hr (*C* and *D*); and an untreated mutant of Jurkat, J.T30.7 (*E* and *F*). Shown are fluorescence histograms obtained by flow cytometric analysis of cells stained with indicated mAbs (solid lines) or a nonreactive control antibody (MOPC 195; dotted lines).

The observation that cholera toxin decreases the cell-surface expression of the antigen receptor complex raises the possibility that receptor loss is the primary mechanism for the cholera toxin-mediated inhibition. To address this issue, we studied J.T30.7, a mutant derived from Jurkat cells (27). J.T30.7 expresses considerably fewer receptors than does the cholera toxin-treated wild-type Jurkat (Fig. 6). We compared the untreated wild-type Jurkat, the cholera toxin-treated wild-type, and untreated J.T30.7 by measuring both the peak and the plateau increases in $[Ca^{2+}]_i$ in response to 1:10 serial dilutions of C305 (Fig. 7). The antigen receptor mAb causes

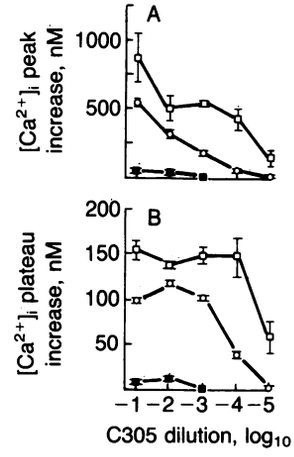

FIG. 7. Comparison of the peak and plateau increases in $[Ca^{2+}]_i$ following the addition of serial 1:10 dilutions of the antigen receptor mAb, C305, to untreated wild-type Jurkat cells (□), untreated J.T30.7 (○), and cholera toxin-treated wild-type Jurkat cells (■). The data represent the mean ± SEM of three separate experiments. The peak increase in $[Ca^{2+}]_i$ is the difference between basal $[Ca^{2+}]_i$ and the highest level of $[Ca^{2+}]_i$ following the addition of C305 to quin-2-loaded cells. The plateau increase in $[Ca^{2+}]_i$ is the difference between the basal level, and the value for $[Ca^{2+}]_i$ measured in 8 min after the addition of C305. Cells were incubated for 3 hr in medium alone or medium with cholera toxin (0.1 μg/ml) and loaded with quin-2 during the final hour of incubation.

substantial peak and plateau increases in $[Ca^{2+}]_i$ when added to J.T30.7 in final dilutions up to 1:1000. In contrast, C305, even in a final dilution of 1:10, induces only trivial changes in $[Ca^{2+}]_i$ in the cholera toxin-treated wild-type cells. These data show that receptor loss alone does not account for the effects of cholera toxin on the antigen receptor complex. The receptors that remain on the cellular surface after treatment with cholera toxin have an impaired ability to generate a signal leading to an increase in $[Ca^{2+}]_i$.

DISCUSSION

Cholera toxin inhibits the ability of mAbs to the antigen receptor complex to increase $[Ca^{2+}]_i$ in Jurkat cells. The toxin almost certainly exerts this effect by blocking the generation of InsP_3 and thereby preventing the antigen receptor-induced release of Ca^{2+} from intracellular stores. Cholera toxin, then, affects events that occur subsequent to the binding of mAbs to the antigen receptor complex but prior to the activation of the PtdInsP_2 phosphodiesterase that generates InsP_3. In addition, cholera toxin causes a selective, partial loss of the antigen receptor complex from the cellular surface.

It is difficult to attribute the cholera toxin effects on the antigen receptor complex to the well-recognized abilities of cholera toxin to ADP-ribosylate $G_s\alpha$ and to increase intracellular cAMP levels. These effects are not mimicked by increasing cAMP with either forskolin or 8-bromo cAMP. Moreover, ADP-ribosylation of the putative $G_s\alpha$ in Jurkat cells appears to be complete within 1 hr after the addition of cholera toxin. The inhibitory effects on the antigen receptor, however, require at least 90 min of exposure to cholera toxin and are not maximal until 3 hr. The effects of cholera toxin on the antigen receptor do appear to require the ADP-ribosyltransferase activity of the A subunit, because purified B subunit does not inhibit the antigen receptor-mediated increase in $[Ca^{2+}]_i$ and does not decrease cell-surface expression of the antigen receptor complex. These observations suggest that a substrate for cholera toxin distinct from $G_s\alpha$ can regulate signal transduction by the antigen receptor.

Cholera toxin ADP-ribosylates two well-characterized G proteins that regulate transmembrane signaling by receptors: G_s and transducin (12, 13, 16). Several observations, however, indicate that cholera toxin can affect additional G proteins. Using membranes isolated from adipocytes, Owens *et al.* (35) found that, under certain conditions, cholera toxin ADP-ribosylates 39- and 41-kDa proteins that also serve as substrates for pertussis toxin. Treatment of membranes by cholera toxin under these conditions inhibits G_i activity (35). Like transducin, therefore, G_i (and perhaps other G proteins) can be ADP-ribosylated by either cholera toxin or pertussis toxin with functional consequences. Consistent with the ability of cholera toxin to ADP-ribosylate G proteins other than G_s, the addition of cholera toxin to intact cells can have effects that are not mediated by cAMP (36, 37). For example, the addition of intact cholera toxin, but not the B subunit and not forskolin, inhibits chemotaxis of the RAW264 murine macrophage cell line (36).

Although there is as yet no direct link between the ability of cholera toxin to ADP-ribosylate certain pertussis toxin substrates and the effects of cholera toxin on the antigen receptor, it is of interest that a pertussis toxin substrate appears to be involved in receptor-mediated activation of the PtdInsP_2 phosphodiesterase in neutrophils (20–24). If the effects of these toxins are indeed a consequence of ADP-ribosylation of G proteins, these findings suggest that either cholera toxin or pertussis toxin can ADP-ribosylate the putative G protein that couples receptors to the PtdInsP_2 phosphodiesterase. Alternatively, more than one distinct G protein may regulate receptor-mediated hydrolysis of inositol phospholipids.

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