Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 7260–7264, October 1988
Cell Biology

Cholera toxin potentiates IgE-coupled inositol phospholipid hydrolysis and mediator secretion by RBL-2H3 cells

(G proteins/pertussis toxin/cyclic AMP/isobutylmethylxanthine/Ro20-1724)

MICHAEL A. MCCLOSKEY

Department of Physiology and Biophysics, University of California, Irvine, CA 92717

Communicated by Harden M. McConnell, June 17, 1988 (received for review August 27, 1987)

ABSTRACT **Binding of polyvalent antigens to IgE present in Fcε receptors on the surface of mast cells and the RBL-2H3 cell line triggers the exocytotic release of allergic mediators. Preincubation of RBL-2H3 cells with cholera toxin (CT) was found to potentiate ≥2- to 3-fold the rate and final amount of antigen-induced secretion of [^{3}H]serotonin and *N*-acetyl β-D-glucosaminidase. This was accompanied by a more variable increase in the initial rate of antigen-triggered formation of inositol phosphates. The holotoxin was required for potentiation, as neither the A nor the B subunit was effective when added separately. Four observations indicate that cAMP was not the primary effector of the augmentation of secretion caused by CT: (*i*) culture conditions were found in which CT caused large increases in secretion but very modest (or no) increases in cAMP; (*ii*) under other conditions, progressive increase in [CT] caused a maximum 2.5- to 3-fold increase in cAMP followed by a return to basal levels, whereas the secretory response saturated and remained stable; (*iii*) permeant cAMP analogs consistently enhanced secretion at low doses and inhibited at higher doses, but the peak enhancement was always much less than that achieved by an optimal dose of CT; (*iv*) the selective phosphodiesterase inhibitor Ro 20-1724 exhibited similar biphasic dose–response curves, the maximum enhancement again being small compared to that caused by CT itself. Both *in vitro* and *in vivo*, CT catalyzed transfer of ADP-ribose from NAD to two membrane proteins that comigrated on NaDodSO$_4$/polyacrylamide gel electrophoresis with two CT substrates in other cell types, and these were identified by immunoblotting as $G_s\alpha$. These results suggest that ADP-ribosylation of a cholera toxin substrate potentiates IgE-mediated secretion from RBL-2H3 cells by a largely cAMP-independent route.**

The initial stimulus in antigen-evoked secretion of allergic mediators from mast cells and basophils is cross-linkage of cell-surface receptors for the Fc domain of IgE (1). In rat mast cells and basophilic leukemia (RBL-2H3) cells, clustering of Fcε receptors triggers inositol phospholipid hydrolysis (2) as well as an increase in the concentration of ionized cytosolic calcium (3). The manner in which these events are coupled to Fcε receptor aggregation is unknown, although by analogy with other systems (see, e.g., ref. 4) it is speculated that a GTP-binding protein (G protein) may be involved. Available evidence has not implicated any particular member of this class of proteins.

Rat peritoneal mast cells can be triggered to degranulate by loading them with nonhydrolyzable GTP analogs, either by permeabilization (5, 6) or through patch pipettes (7). But whether the critical target proteins are actually components of the IgE receptor-linked route for inositol phospholipid hydrolysis or mediator secretion is unknown. Pertussis toxin (PT), a bacterial toxin that catalyzes ADP-ribosylation of $G_i\alpha$ and $G_o\alpha$, inhibits compound 48/80-induced inositol phospholipid breakdown and histamine secretion by rat peritoneal mast cells (8); PT also partially blocks GTP[γS]-induced exocytosis from permeabilized rat mast cells (9). Yet it does not affect IgE-mediated degranulation of the same cell type (10). Similarly, PT does not affect IgE-mediated secretion from RBL cells (1) or human basophils (11).

In the present work, I examined the effects of cholera toxin (CT), a bacterial toxin that ADP-ribosylates $G_s\alpha$, on IgE-mediated events in RBL cells. Knoop and Thomas (12) reported that CT increases $^{45}Ca^{2+}$ uptake by resting RBL cells, although they did not explore the effects of CT on antigen-evoked events. I found that intact CT markedly increased antigen-triggered inositol phospholipid hydrolysis and mediator secretion. Although the enhanced secretion was accompanied by ADP-ribosylation of $G_s\alpha$ and required the enzymatic A subunit of CT, it appeared to be largely independent of cAMP accumulation. This suggests that in RBL cells, $G_s\alpha$ or another CT substrate directly or indirectly modulates transmembrane signaling via the IgE receptor.

MATERIALS AND METHODS

Materials. Toxins were obtained from either List Biological Laboratories (Campbell, CA) or Calbiochem. Of the radiochemicals, 5-[1,2-^{3}H]hydroxytryptamine (5-HT), cAMP-2′-*O*-succinyl-([^{125}I]iodotyrosine methyl ester), and ^{125}I-labeled goat anti-rabbit IgG F(ab′)$_2$ were purchased from New England Nuclear, [^{32}P]NAD was from ICN, and *myo*-[^{3}H]inositol was from Amersham. Cyclic nucleotides and their permeant analogs were supplied by Sigma, as were isobutylmethylxanthine (IBMX) and *p*-nitrophenyl β-D-*N*-acetylglucosamine. Peter Sorter (Hoffmann-La Roche, Nutley, NJ) generously supplied Ro 20-1724. The anion exchange resin AG 1-X8 was purchased from Bio-Rad. Goat anti-cAMP antibody, goat carrier IgG, and donkey anti-goat IgG were from Bio-Tek (Lenexa, KS).

Cell Culture. The 2H3 subline of RBL-4 cells was provided by Reuben Siraganian (National Institutes of Health) and maintained as described (13). The RAW 264.7 macrophage cell line was obtained in 1981 from the Cell Distribution Center of the Salk Institute. Diploid human fibroblasts (HF cells) and Swiss mouse 3T3 fibroblasts were provided by H. Haigler and D. Cunningham, respectively, of this institution. Wild-type S49 cells were a gift from L. Brunton (University of California, San Diego).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: CT, cholera toxin: G protein, GTP-binding regulatory protein; G_i and G_s, G proteins that mediate inhibition (G_i) and stimulation (G_s) of adenylate cyclase; α, GTP-binding subunit of G proteins; 5-HT, 5-hydroxytryptamine; IBMX, 3-isobutyl-1-methylxanthine; PDE, phosphodiesterase; TNP, trinitrophenyl; TNP-BSA, trinitrophenylated bovine serum albumin; PT, pertussis toxin.

Secretion Assays. Cells were grown as described for the cAMP assay. Ascites fluid containing anti-dinitrophenyl IgE (14) was diluted 1:1500 to 1:500 into the growth medium at the time of seeding. For measurement of 5-HT release, [^{3}H]5-HT was added at 1 μCi/ml (1 Ci = 37 GBq) to the culture at the time of seeding. Twelve to 24 hr later the cultures were treated with Hepes-buffered Eagle's minimal essential medium (plus 0.5 mg of bovine serum albumin per ml) containing toxin or other additive and incubated for various times at 37°C. Monolayers were then rinsed three times with Hepes-buffered saline (HBS) (in mM: 135 NaCl/5 KCl/0.5 $MgCl_2$/1.0 $CaCl_2$/5.6 glucose/10 Hepes, pH 7.4/0.05% bovine serum albumin), and 0.8 ml of HBS containing trinitrophenylated-bovine serum albumin (TNP-BSA) was added. The cultures were placed in a 37°C H_2O bath for 60 min. Supernates were then removed and the cells were lysed with 0.8 ml of 1% $NaDodSO_4$ (for 5-HT release) or with 0.15% Brij 35 in HBS (for β-hexosaminidase release). Radioactivity in supernates and cell lysates from each vial was determined by liquid scintillation counting and β-hexosaminidase was assayed with the chromogenic substrate *p*-nitrophenyl β-D-*N*-acetylglucosamine (15). None of the additives present in supernates (e.g., IBMX) affected the enzyme assay.

cAMP Assay. Cells were grown as monolayers either in 24-well plates or in silicone-stoppered glass shell vials. Normally, 4×10^5 cells in 1 ml of medium were seeded in each well or 3-dram vial ($1–2 \times 10^5$ per 1-dram vial) and then cultured for 12–24 hr at 37°C. The medium was aspirated from triplicate vials or wells and CT or other additives were then added in Hepes-buffered Eagle's minimal essential medium (plus 0.5 mg of bovine serum albumin per ml). Vials were restoppered and incubated in a 37°C water bath, usually for 2 hr. cAMP was extracted either with 0.1 M HCl and freeze–thawing or with 11% trichloroacetic acid followed by removal of the acid with ether. Recovery of [^{3}H]cAMP was 80–100%. Radioimmunoassay for cAMP was performed according to standard procedures using the antibodies listed above. Protein was determined as described (16).

Assay of Inositol Phosphates. Assay of total inositol phosphates using anion-exchange chromatography was performed as described (2), with the following modifications: (*i*) dialyzed serum (or 2 mg of bovine serum albumin per ml) was used in place of normal serum to obtain efficient labeling of the inositol phospholipid fraction; (*ii*) cells were grown in stoppered shell vials rather than 24-well plates, and were seeded 12–24 hr before use in 0.5 ml of medium (2×10^5 cells).

ADP-Ribosylation. CT was activated as described (17) and PT (135 μg/ml) was incubated 30 min at 37°C in 90 mM dithiothreitol/90 mM KP_i, pH 7.4. Membrane suspensions (50–200 μg of protein at 5 μg/μl) were mixed with 100 μl of the ADP-ribosylation reaction mixture (in mM: 100 KPO_4, pH 7.4/5 MgATP/5 *m*-aminobenzamide/2-dithiothreitol/5 $MgCl_2$/58 μg of activated CT per ml or 17 μg of activated PT per ml) and the reaction was started by adding 12 μM [^{32}P]NAD at 2 Ci/mmol. The mixture was incubated for 30 min at 32°C and the reaction was stopped by dilution with 1.2 ml of ice-cold 15% trichloroacetic acid. The precipitate was collected by centrifugation, rinsed with cold acetone, and subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis on 12% gels (18). Gels were dried and autoradiographed at −80°C with Kodak X-Omat AR film and an intensifying screen. Immunoblotting with rabbit antiserum (A-572) raised against a peptide determinant unique to $G_s\alpha$ was performed as described by Mumby *et al.* (19).

RESULTS

Potentiation of Secretion by CT. Pretreatment of RBL cells with CT caused a large potentiation of subsequent antigen-induced release of the secretory granule enzyme, β-*N*-acetylhexosaminidase, and of [^{3}H]5-HT that had been incorporated into the secretory granules (Fig. 1*A*). The enhancement was dependent on the time of preincubation with CT as well as the [CT] (Fig. 1*B*). Expression of maximal effects required 2–3 μg of CT per ml and an incubation time of ≈2 hr (CT at 3 μg/ml). Spontaneous release was unaffected except at supraoptimal concentrations of CT. Note from Fig. 1*A* (*Inset*) that addition of TNP-BSA (0.1 ng/ml) to control cells caused no release of 5-HT above the basal level, whereas the same dose of antigen released 23% of the 5-HT from CT-treated cells. Similar enhancement of secretion was observed in 20 β-hexosaminidase and 25 [^{3}H]5-HT release assays using cells from four primary frozen stocks and CT from two suppliers. The effect was therefore consistent and not restricted to a particular RBL variant arising during prolonged cell culture. PT alone (0.4 μg/ml for 3–15 hr) did not affect 5-HT secretion, but in two of three trials it did partially block the enhancement due to CT.

Potentiation of Inositol Phospholipid Hydrolysis by CT. Fig. 2 demonstrates that pretreatment of RBL cells with CT was capable of increasing markedly the antigen-elicited formation of inositol phosphates. This enhancement varied more than that of secretion, however, with the average increase at 5–20 min post-challenge being 90% ± 67% (range, 22–207%; *n* =

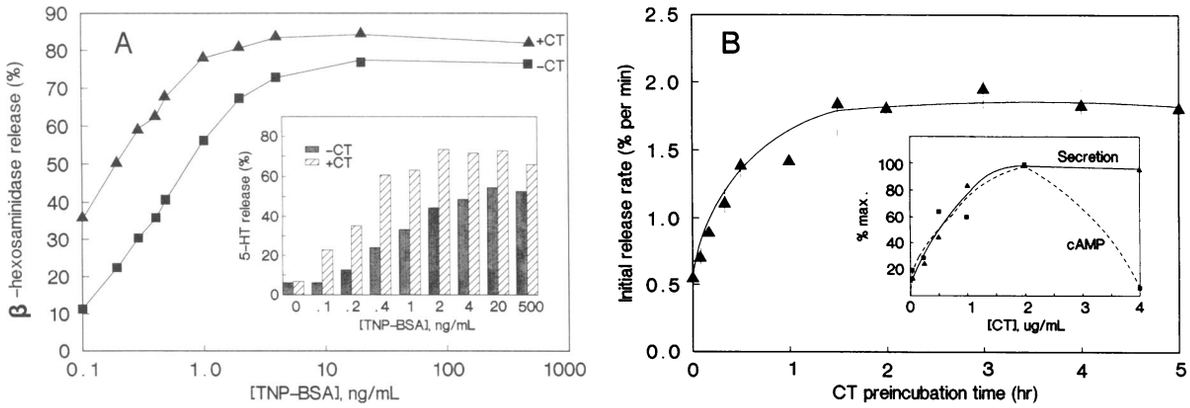

FIG. 1. The effect of CT on antigen (TNP-BSA)-induced secretion from RBL cells. (*A*) Antigen dose–response curve showing total release (60-min assay) of β-hexosaminidase before and after treatment for 6 hr with 2 μg of CT per ml. (*Inset*) [^{3}H]5-HT release from cells incubated 2.5 hr with 2 μg of CT per ml. Duplicate measurements of cells cultured in 24-well plates. (*B*) Dependence of initial rate of β-hexosaminidase release over first 8 min (0.4 ng of TNP-BSA) on time of preincubation with 3 μg of CT per ml. Triplicate samples. (*Inset*) Dependence of cAMP formation and antigen-triggered hexosaminidase release on [CT] after 2-hr preincubation in 24-well plates.

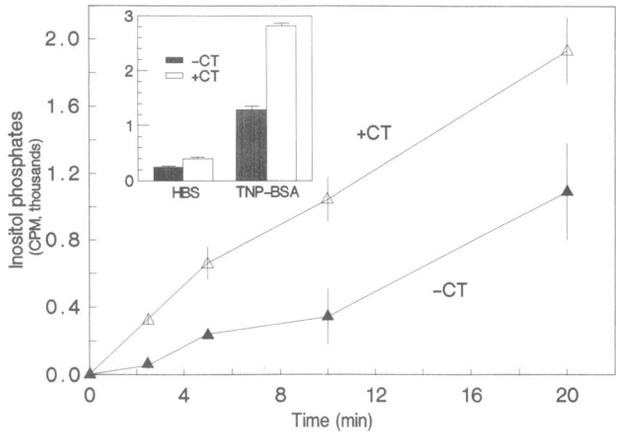

FIG. 2. The effect of CT on rate of antigen-triggered formation of [^{3}H]inositol phosphates at an optimal dose of TNP-BSA (20 ng/ml). Note that at 20 ng/ml the rates have nearly equalized within 10 min. Preincubation was 5 hr with CT at 2 μg/ml. Time zero values have been subtracted from each time point; absolute cpm at time zero was 247 (−CT) and 464 (+CT). (*Inset*) Separate experiment in which cells were exposed for 20 min at 37°C to buffer with or without TNP-BSA (20 ng/ml) (absolute cpm, in thousands). Preincubation was for 3 hr with CT at 2 μg/ml.

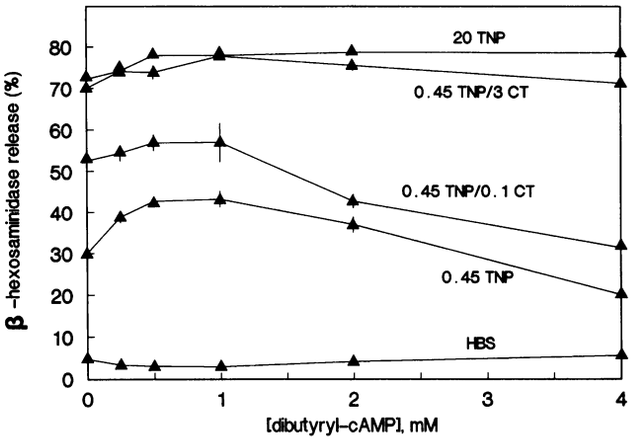

FIG. 3. Permeant cAMP analogs have biphasic effects on secretion. Dose–response curve for dibutyryl-cAMP under five different triggering protocols. Label numbers refer to TNP-BSA (ng/ml) or CT (μg/ml) (2-hr preincubation); HBS, spontaneous release. Note that peak enhancement by dibutyryl-cAMP is much less than that achieved with CT alone. Three such experiments were conducted (using stoppered shell vials), and three more with 8-bromo-cAMP; the latter gave very similar results, although the peaks were displaced to the right by ≈1 mM. Error bars give SD of triplicate samples.

7). It depended on the dose and time of exposure to both CT and antigen. In the kinetic experiment shown, the average rate within the first 5 min of antigen addition was 40 cpm·min^{-1} in control and 125 cpm·min^{-1} in CT-pretreated cells, a 3-fold difference. Note that at this optimal antigen dose (20 ng of TNP-BSA per ml) the rates were equal within ≈10 min, as was true for 5-HT secretion at the same concentration of TNP-BSA (data not shown). CT also caused some increase in the "spontaneous" formation of inositol phosphates.

Lack of Correlation Between CT Effects on cAMP and Secretion. In contrast to the effects of CT on secretion, the effects of CT on cAMP levels depended markedly on the method of cell culture. If the cells were grown in stoppered shell vials they responded very poorly, with an average increase in cAMP in 10 RIAs of 22% ± 24% (2-hr incubation with 2 μg of CT per ml). Using this system, parallel cAMP and secretion assays were run four times, in which I found an average 2.3-fold enhancement of secretion (at 0.3–0.5 ng of TNP-BSA per ml) and no difference in cAMP between CT-treated and control cell cultures (22.2 ± 5.3 vs. 22.1 ± 3.4 pmol per mg of protein, respectively).

When cells were cultured in 24-well plates or the vial stoppers were replaced with gas-permeable caps, a 2-hr treatment with CT caused substantial increases in cAMP of 2.5- to 3-fold. Not only was antigen-triggered secretion excellent in both the 24-well plate and stoppered-vial culture systems, but CT caused the same large enhancement of secretion in both systems. Thus, the effects of CT on cAMP and secretion can be manipulated independently of one another. Further evidence that the effects of CT on cAMP and secretion are experimentally dissociable stems from the dose–response curve shown in Fig. 1*B*. Here one sees that the cAMP response to increasing [CT] was biphasic, whereas the secretory response saturated and remained stable. Measurements of endogenous cAMP thus provide two separate lines of evidence that prior elevation of cAMP by CT does not account for the effects of CT on secretion.

Effects of Permeant Cyclic Nucleotides on Secretion. Preincubation of RBL cells for 2 hr with "low" concentrations of either 8-bromo-cAMP or N^6,$O^{2'}$-dibutyryl-cAMP consistently enhanced antigen-triggered secretion of both 5-HT and β-hexosaminidase, the effect reversing at higher concentrations (Fig. 3). In contrast, the cGMP analogs 8-bromo-cGMP and dibutyryl-cGMP (0.5–3.0 mM) did not affect spontaneous or antigen-evoked secretion. Note that the peak enhancement caused by dibutyryl-cAMP reflects a balance between inhibitory and stimulatory effects, and that the peak enhancement was much less than that caused by CT alone (Fig. 3). This suggests that CT acts by some mechanism in addition to or entirely apart from cAMP elevation.

Effects of Phosphodiesterase Inhibitors on Secretion. In four of five experiments, the selective cAMP phosphodiesterase (PDE) inhibitor Ro 20-1724 yielded a biphasic dose–response curve similar to that of permeant cAMP analogs (Fig. 4*A*). Within the range 25–60 μM it increased by ≈9% (absolute) the enhancement by CT (2 μg/ml) of release due to suboptimal antigen, and at higher Ro 20-1724 concentrations there was a more gradual return to control values or below. cAMP levels at 2 μg of CT per ml were ≈2- to 3-fold higher in the presence of 0.1 mM Ro 20-1724 than in its absence. In each of four trials, the nonselective PDE inhibitor IBMX had triphasic effects on secretion (Fig. 4*B*), a marked inhibition saturating at ≈20 μM IBMX (50% inhibition at 2–4 μM) and at higher [IBMX] a biphasic effect of similar magnitude to that occurring with Ro 20-1724. The latter may be due to an increase in cAMP, as a similar increase in cAMP was found at 0.1 mM IBMX and 0.1 mM Ro 20-1724, and preincubation with IBMX was required for this effect on secretion.* Both in the presence and absence of CT, the magnitude of the enhancement was much smaller than that caused by CT alone. These findings again suggest that progressive elevation of cAMP has opposing effects on secretion, but that the maximum gain possible by the cAMP-dependent process is less than that caused by CT.

Holotoxin Requirement for Potentiation. The A subunit of CT did not affect antigen-induced 5-HT secretion. The "B subunit" had a slight effect equal to 2.5% that of the holotoxin at saturating doses. This may have been due to contaminating holotoxin as indicated by the fact that at a saturating dose the B subunit was 2.6% as effective as holotoxin in causing an

*The initial inhibitory phase may not have been due to an increase in cAMP because (*i*) no increase in cAMP was found at 10 μM IBMX, (*ii*) no preincubation of the cells with IBMX was required to attenuate the initial rate of secretion, and (*iii*) after a 2-hr preincubation with IBMX, two quick rinses of the cell monolayer prior to antigen addition reversed the initial phase of inhibition.

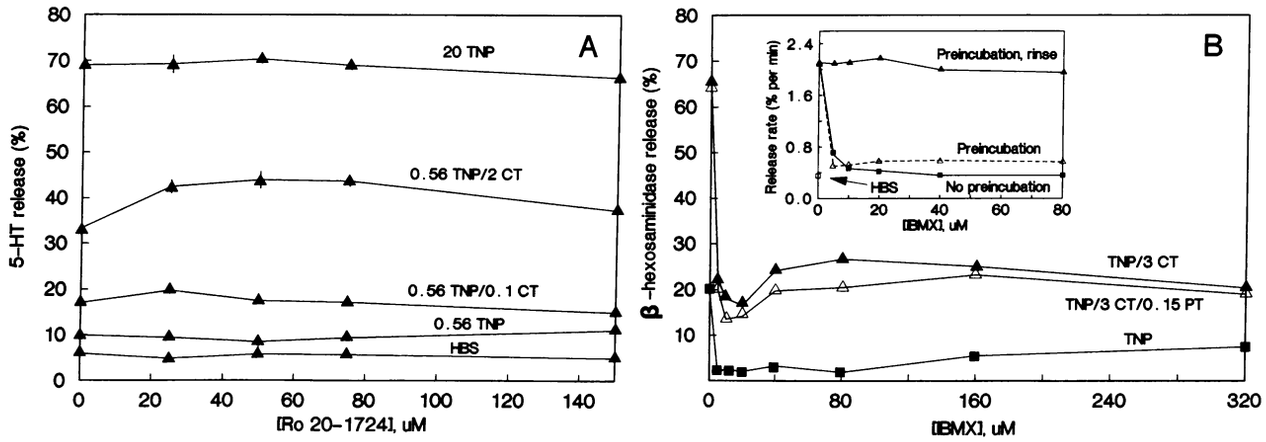

FIG. 4. Effect of the PDE inhibitors on antigen-triggered secretion. (*A*) Dose–response curve for Ro 20-1724 showing biphasic effect on 5-HT secretion at 2–3 μg of CT per ml (60-min assay); substantial inhibition was observed at 200–500 μM Ro 20-1724. (*B*) IBMX has triphasic effects on enzyme secretion. (*Inset*) Average release rate over first 14 min. Note that strong initial inhibition does not require preincubation with IBMX but secondary potentiation does, and that quick rinse of pretreated monolayer reverses initial inhibitory phase. CT (in μg/ml) and PDE inhibitors at the indicated concentrations were present for 2 hr prior to addition of antigen (TNP-BSA in ng/ml).

increase in cAMP in HF cells. [The cAMP content (pmol of cAMP per mg of protein) was 17.4 ± 1.8 (SEM) in control cells, 156 ± 6.2 in cells treated for 2 hr with 2 μg of holotoxin per ml, and 21 ± 0.9 in cells treated for 2 hr with 2 μg of B subunit per ml).] $NaDodSO_4$ electrophoresis also showed in the commercial B subunit a faint band that comigrated with the A subunit. The holotoxin was thus required for enhancement of secretion, and the commercial B subunit may have contained a trace of A subunit, evidently as holotoxin.

Substrates for ADP-Ribosylation. Fig. 5 shows that CT catalyzed the labeling of two membrane proteins from RBL cells. These comigrated with two membranous CT substrates from three other cell types (HF cells not shown), and they had apparent masses of ≈43 and 47 kDa. Variable molecular masses have been reported for the two forms of $G_s\alpha$, although from the cDNA one predicts values of 44.5 and 46 kDa (4). Immunoblotting with the A-572 antiserum against $G_s\alpha$ (19) revealed two major immunoreactive proteins that nearly comigrated with a $G_s\alpha$-reactive doublet in wild-type S49 lymphoma cells (Fig. 5*B*). The CT substrates detected by *in vitro* labeling were also physiological substrates, as prior treatment of the cells with CT prevented their subsequent labeling *in vitro* (Fig. 5*C*). I assume here that CT catalyzed transfer of nonradioactive ADP-ribose from endogenous NAD to the acceptor proteins, thereby blocking subsequent transfer of [^{32}P]ADP-ribose in the cell-free system.

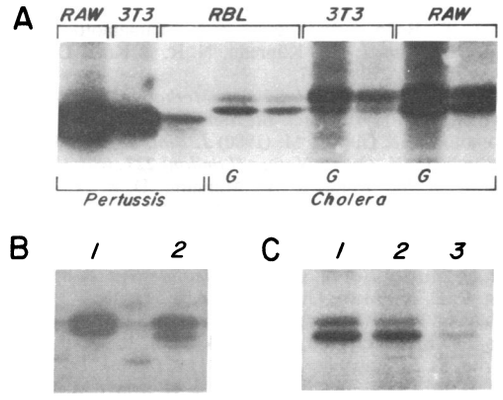

FIG. 5. Autoradiograms of 12% gels containing [^{32}P]ADP-ribosylated proteins. (*A*) PT and CT substrates in membrane fractions from three different cell types. Note that the two membranous CT substrates from RBL cells comigrate with those from RAW 264.7 macrophages and Swiss 3T3 fibroblasts, and that the membranous PT substrate(s) from RBL cells comigrates with membranous PT substrates in the other cell types (shorter exposure of the negative resolved two bands in the first two lanes). G indicates the presence of 1 mM GTP[γS]. (*B*) Immunoblot probed with A-572 antiserum against $G_s\alpha$; lane 1, membrane proteins from wild-type S49 cells; lane 2, membrane proteins from RBL cells. (*C*) Preincubation of RBL cells for 6 hr with 8 μg of CT per ml prevents subsequent [^{32}P]ADP-ribosylation of two CT substrates. Additions: lane 1, control membranes; lane 2, same plus RBL cytosol; lane 3, membranes from CT-pretreated cells plus cytosol.

PT mediated the ADP-ribosylation of a membrane-associated protein of ≈41 kDa (Fig. 5*A*). PT substrates of similar mass were present in RAW 264.7 macrophages, Swiss 3T3 fibroblasts, and HF cells (the latter not shown). The reduced labeling of the CT substrates in RBL cells (≈200 μg of protein per lane) was possibly due to a greater proportion of nuclei in the crude RBL membrane fraction.

DISCUSSION

CT catalyzes ADP-ribosylation of $G_s\alpha$ (20), a process that enhances both basal and agonist-stimulated adenylate cyclase activities in many cell types (21). In considering possible means by which CT might potentiate secretion from RBL cells, the first question is whether cAMP is involved. Data on the effects of cAMP on secretion from primary serosal mast cells (see, e.g., refs. 22 and 23) indicate that, although a transient increase in cAMP occurs within 5–15 sec of antigen binding, chronic elevation of cAMP by a variety of other means usually inhibits IgE-mediated secretion. I found only two published accounts of cAMP measurements on RBL cells (12, 24), and neither dealt with the effects of cAMP on secretion.

If cAMP does mediate the potentiation of secretion by CT, one might expect permeant cAMP analogs to mimic this effect. From the data for 8-bromo-cAMP and dibutyryl-cAMP (Fig. 3) one surmises that progressive elevation of endogenous cAMP may in fact have dual actions on antigen-triggered secretion, an initial stimulation followed by inhibition. The balance of these opposing effects will determine the maximum augmentation possible via the cAMP-dependent route. That the peak enhancement produced by either cAMP analog was well below that achieved with CT is a good indication that cAMP is not the primary effector of CT action in this system. That permeant cAMP analogs enhanced secretion even at a maximally effective dose of CT suggests that in these experiments CT did not raise cAMP levels enough to saturate the cAMP-dependent process.

Direct measurements of endogenous cAMP gave two indications that effects of CT on cAMP and secretion are

dissociable. First, the cAMP response was markedly dependent on the method of cell culture, whereas the enhancement of secretion was not. Second, the CT dose–response curve shows that antigen-induced secretion stabilized beyond ≈2 μg of CT per ml, even though cAMP levels dropped off in this region. Although this decline is unusual, CT is known to activate a PDE in rat liver cells (25), and it also increases ^{45}Ca uptake by RBL cells (12), a process that could stimulate calmodulin-regulated PDE. One might speculate that this stimulated Ca uptake also affects another PDE—namely, phospholipase C, and that this contributes to the enhancement of antigen-triggered inositol phospholipid hydrolysis. Whatever the cause of the decline in cAMP, elevation of cAMP prior to antigen addition does not appear to explain the subsequent enhancement of secretion.

This leaves open the possibility that CT acts by potentiating an increase in cAMP caused by antigen binding. Morita and Siraganian reported that Fcε receptor cross-linkage does not elevate cAMP in RBL cells (24), and in three of four experiments (data not shown) I found no difference in cAMP between control and antigen-treated cultures (within 4 min of antigen addition). In one trial a brief spike in cAMP was observed at 8–20 sec, but CT at 3 μg/ml did not amplify this spike. It is possible that the quench method I used was not rapid enough to consistently resolve a cAMP transient. However, CT has no effect on antigen-induced secretion from rat peritoneal mast cells (10), in which antigen-binding is known to cause a brief increase in cAMP (26). Perhaps this ephemeral peak in cAMP is not mediated by G_s, or, if so, it may not be crucial for mediator secretion.

As a further test of the cAMP hypothesis, I asked whether PDE inhibitors could boost the enhancement of secretion caused by CT. The nonselective PDE inhibitor IBMX had complex effects on secretion, part of which may not involve cAMP elevation.* Ro 20-1724 is a fairly potent inhibitor of the low K_m cAMP-selective PDE (27). In four of five trials Ro 20-1724 (plus 2–3 μg of CT per ml) had a biphasic effect on secretion similar to that of the permeant cAMP analogs (Fig. 4*A*). This is consistent with the notion that increases of cAMP within a certain range cause a modest enhancement of secretion, with the increases normally induced by 2–3 μg of CT per ml (using the stoppered vial system) being too small to saturate this cAMP-dependent process.

CT is known to have cAMP-independent effects on other cell types (25, 28, 29). A simple hypothesis to explain the present results as well as those of Knoop and Thomas (12) is that $G_s\alpha$ directly links the IgE receptor to phospholipase C or to a Ca channel. By inhibiting the intrinsic GTPase activity of $G_s\alpha$ (30, 31), ADP-ribosylation could potentiate its ability to stimulate esteratic or channel activity, just as it amplifies the response of adenylate cyclase to catecholamines (21). Previously, $G_s\alpha$ has been implicated in other functions distinct from its role in cAMP formation—namely, inhibition of Mg uptake (32) and regulation of voltage-gated Ca channels (33). Two problems with this hypothesis are (*i*) β-adrenergic agonists, which act via G_s, do not mediate secretion from RBL cells, and (*ii*) cross-linkage of IgE receptors does not appear to consistently activate adenylate cyclase. Perhaps the relevant CT substrate escaped detection in the present work.

A more serious concern with the direct coupling hypothesis is that systematic increase in the proportion of cross-linked IgE receptors causes the secretory response to saturate long before the Ca or total inositol phosphate signals do (34). Thus, one should not observe enhancement of secretion at supraoptimal antigen levels if potentiation of the total inositol phosphate or Ca signals is the sole mechanism of enhancement. Perhaps the increased mediator release occurring in this region reflects in part the cAMP-regulated process described above. Obviously, CT could also act by a variety of indirect means, although there is not room here to speculate as to their nature.

In summary, I found that intact CT dramatically potentiated IgE-coupled transmembrane signaling and mediator secretion by RBL cells. This potentiation was accompanied by ADP-ribosylation of $G_s\alpha$, although the bulk of it did not appear to depend on cAMP accumulation. It is intriguing to speculate that some fraction of $G_s\alpha$ (or another CT substrate) directly links the IgE receptor to an intracellular effector other than adenylate cyclase, although the possibility that CT acts indirectly also must be considered. In either case, given the robust nature of the phenomenon its further characterization is likely to offer new clues as to the mechanism of transmembrane signaling via the high-affinity Fcε receptor.

I am very grateful to Gloria Carandang for her skilled technical assistance with many of the experiments. Thanks are also due to Dr. Fu-tong Liu of the Medical Biology Institute (La Jolla, CA) for his gift of ascites fluid containing anti-dinitrophenyl IgE, and to Drs. Susanne Mumby and Alfred Gilman of the University of Texas Health Science Center (Dallas) for their gift of A-572 antiserum. This work was supported by a grant from the National Institute of General Medical Sciences (GM-35901) and a Medical Research and Education Society award.

1. Metzger, H., Alcaraz, G., Hohmam, R., Kinet, J.-P., Pribluda, V. & Quarto, R. (1986) *Annu. Rev. Immunol.* **4**, 419–470.
2. Beaven, M. A., Moore, J. P., Smith, G. A., Hesketh, T. R. & Metcalfe, J. C. (1984) *J. Biol. Chem.* **259**, 7137–7142.
3. Beaven, M. A., Rogers, J., Moore, J. P., Hesketh, T. R., Smith, G. A. & Metcalfe, J. C. (1984) *J. Biol. Chem.* **259**, 7129–7136.
4. Gilman, A. G. (1987) *Annu. Rev. Biochem.* **56**, 615–649.
5. Gomperts, B. D. (1983) *Nature (London)* **306**, 64–66.
6. Howell, T. W., Cockcroft, S. & Gomperts, B. D. (1987) *J. Cell Biol.* **105**, 191–197.
7. Fernandez, J. M., Neher, E. & Gomperts, B. D. (1984) *Nature (London)* **312**, 453–455.
8. Nakamura, T. & Ui, M. (1985) *J. Biol. Chem.* **260**, 3584–3593.
9. Nakamura, T. & Ui, M. (1984) *FEBS Lett.* **173**, 414–418.
10. Saito, H., Okajima, F., Ui, M. & Ishizaka, T. (1987) *Fed. Am. Soc. Exp. Biol.* **46**, 1028 (abstr.).
11. Warner, J. A., Yancey, K. B. & MacGlashan, D. W., Jr. (1987) *J. Immunol.* **139**, 161–165.
12. Knoop, F. C. & Thomas, D. D. (1984) *Int. J. Biochem.* **16**, 275–280.
13. Barsumian, E. L., Isersky, C., Petrino, M. B. & Siraganian, R. P. (1981) *Eur. J. Immunol.* **11**, 317–323.
14. Liu, F.-T., Bohn, J. W., Ferry, E. L., Yamamoto, H., Molinaro, C. A., Sherman, L. A., Klinman, N. R. & Katz, D. H. (1980) *J. Immunol.* **124**, 2728–2737.
15. Landegren, U. (1984) *J. Immunol. Methods* **67**, 379–388.
16. Hartree, E. F. (1972) *Anal. Biochem.* **48**, 422–427.
17. Enomoto, K. & Gill, D. M. (1980) *J. Biol. Chem.* **255**, 1252–1258.
18. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
19. Mumby, S. M., Kahn, R. A., Manning, D. R. & Gilman, A. G. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 265–269.
20. Cassel, D. & Pfeuffer, T. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2669–2673.
21. Gill, D. M. (1977) *Adv. Cyclic Nucleotide Res.* **8**, 85–118.
22. Alm, P. E. & Bloom, G. D. (1982) *Life Sci.* **30**, 213–218.
23. Healicon, R. M. & Foreman, J. C. (1984) in *The Receptors*, ed. Conn, P. M. (Academic, New York), Vol. 1, pp. 83–140.
24. Morita, Y. & Siraganian, R. P. (1981) *J. Immunol.* **127**, 1339–1344.
25. Heyworth, C. M., Wallace, A. V. & Houslay, M. D. (1983) *Biochem. J.* **214**, 99–110.
26. Holgate, S. T., Lewis, R. A. & Austen, K. F. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 6800–6804.
27. Weishaar, R. E., Cain, M. H. & Bristol, J. A. (1985) *J. Med. Chem.* **28**, 537–545.
28. Aksamit, R. R., Backlund, P. S., Jr., & Cantoni, G. L. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 7475–7479.
29. Biffen, M. & Martin, B. R. (1987) *J. Biol. Chem.* **262**, 7744–7750.
30. Cassel, D. & Selinger, A. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 3307–3311.
31. Levinson, S. L. & Blume, A. J. (1977) *J. Biol. Chem.* **252**, 3766–3774.
32. Maguire, M. E. & Erdos, J. J. (1980) *J. Biol. Chem.* **255**, 1030–1035.
33. Yatani, A., Codina, J., Imoto, Y., Reeves, J. P., Birnbaumer, L. & Brown, A. M. (1987) *Science* **238**, 1288–1292.
34. Maeyama, K., Hohman, R. J., Metzger, H. & Beaven, M. A. (1986) *J. Biol. Chem.* **261**, 2583–2592.