A Novel Approach to Detect Toxin-catalyzed ADP-ribosylation in Intact Cells: Its Use to Study the Action of *Pasteurella multocida* Toxin

James M. Staddon, Mark M. Bouzyk, and Enrique Rozengurt

Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, United Kingdom

Abstract. Certain microbial toxins are ADP-ribosyltransferases, acting on specific substrate proteins. Although these toxins have been of great utility in studies of cellular regulatory processes, a simple procedure to directly study toxin-catalyzed ADP-ribosylation in intact cells has not been described. Our approach was to use [2-^{3}H]adenine to metabolically label the cellular NAD^+ pool. Labeled proteins were then denatured with SDS, resolved by PAGE, and detected by fluorography. In this manner, we show that pertussis toxin, after a dose-dependent lag period, [^{3}H]-labeled a 40-kD protein in intact cells. Furthermore, incubation of the gel with trichloroacetic acid at 95°C before fluorography caused the release of label from bands other than the pertussis toxin substrate, thus, allowing its selective visualization. The modification of the 40-kD protein was ascribed to ADP-ribosylation of a cysteine residue on the basis of inhibition of labeling by nicotinamide and the release of [^{3}H]ADP-ribose from the labeled protein by mercuric acetate. Cholera toxin catalyzed the [^{3}H]-labeling of a 46-kD protein in the [2-^{3}H]adenine-labeled cells. Pretreatment of the cells with pertussis toxin before the labeling of NAD^+ with [2-^{3}H]adenine blocked [2-^{3}H]ADP-ribosylation catalyzed by pertussis toxin, but not that by cholera toxin. Thus, labeling with [2-^{3}H]adenine permits the study of toxin-catalyzed ADP-ribosylation in intact cells. *Pasteurella multocida* toxin has recently been described as a novel and potent mitogen for Swiss 3T3 cell and acts to stimulate the phospholipase C-mediated hydrolysis of polyphosphoinositides. The basis of the action of the toxin is not known. Using the methodology described here, *P. multocida* toxin was not found to act by ADP-ribosylation.

THE pathological basis of the action of certain bacteria is accounted for by their ability to produce toxins that enter eukaryotic cells and subvert cellular regulatory processes. Some of these toxins catalyze the transfer of ADP-ribose from NAD^+ to specific target proteins, resulting in a protein that may be either inactive or altered in properties. Diphtheria toxin and *Pseudomonas aeruginosa* exotoxin A block protein synthesis by ADP-ribosylating elongation factor-2 (Collier, 1990; Wick and Iglewski, 1990). *Clostridium botulinum* C2 toxin ADP-ribosylates nonmuscle actin (Aktories et al., 1990) and the C3 toxin ADP-ribosylates *rho*, a *ras*-related GTPase (Aktories and Just, 1990). *P. aeruginosa* exoenzyme S preferentially ADP-ribosylates $p21^{c\text{-}H\text{-}ras}$ in vitro (Coburn et al., 1989*b*) and has also been described to ADP-ribosylate vimentin (Coburn et al., 1989*a*). Pertussis toxin, cholera toxin, and *Escherichia coli* heat-labile enterotoxin ADP-ribosylate the α subunits of heterotrimeric signal transducing G proteins (Moss and Vaughan, 1988; Pfeuffer and Helmreich, 1988; Ui, 1990). The specificity of the action of these toxins and the modification of target protein function can be exploited to make them extremely useful as probes of cellular regulatory processes.

The classic approach to investigate the role of ADP-ribosylation in toxin action has been to identify ADP-ribosylated toxin substrates in cell extracts using radiolabeled NAD^+. This requires activation of the holotoxin in vitro and its use at concentration orders of magnitude greater than those used on intact cells. Furthermore, this approach can be thwarted by the absence of ADP-ribosylation factors and excessive NADase activity in the cell extracts. It can also be sensitive to the composition of the reaction mixture (see, e.g., Ribeiro-Neto et al., 1985, and Gill and Woolakis, 1988 for methodological aspects of cholera and pertussis toxin catalyzed ADP-ribosylation).

The evidence that toxin-catalyzed ADP-ribosylation occurs in intact cells has mainly been inferred from an indirect procedure (see, e.g., Ui, 1990). Since radiolabeled NAD^+ is membrane impermeant, the toxin substrate identified in vitro is prepared from intact cells treated with the same toxin. The subsequent inability of this substrate to accept further ADP-ribose from NAD^+ in vitro is interpreted to mean that the ADP-ribosylation site is already occupied, presumably ADP-ribosylated by the toxin in the intact cells. An alternative approach is to directly measure toxin-catalyzed ADP-ribosylation in intact cells. However, in spite of its importance, a simple method for directly studying the action of toxin ADP-ribosyltransferases in intact cells has not been described.

Employing a new approach, we now demonstrate for the first time pertussis toxin and cholera toxin-catalyzed ADP-

ribosylation of specific target proteins in intact cells. We studied the action of these toxins in Swiss 3T3 cells, a model system to study signal transduction mechanisms involved in cell proliferation (Rozengurt, 1986). The approach that we describe should be applicable to the study of the cellular action of other ADP-ribosylating toxins and also to explore the role of ADP-ribosylation in the action of recently identified bacterial toxins. We describe our findings with *Pasteurella multocida* toxin, a potent mitogen (Rozengurt et al., 1990) that activates phospholipase C to stimulate the hydrolysis of polyphosphoinositides (Staddon et al., 1990, 1991).

Materials and Methods

Materials

[2-^{3}H]adenine (20 Ci/mmol), [^{14}C]methylated molecular weight markers, cyclic AMP [^{125}I] assay kits and [adenylate-^{32}P]NAD$^+$ (1,000 Ci/mmol) were from Amersham International (Amersham Buckinghamshire, UK). Cholera toxin and salicylic acid (American Chemical Society reagent grade) were from Sigma Chemical Co. (St. Louis, MO) and pertussis toxin was from List Biological Laboratories (Campbell, CA). Recombinant *P. multocida* toxin was prepared as described previously (Lax and Chanter, 1990). Gel electrophoresis reagents were from Bio-Rad Laboratories (Richmond, CA). All other reagents were of the highest grade commercially available.

Cell Culture

Stock cultures of Swiss 3T3 cells (Todaro and Green, 1963) were propagated as described (Dicker and Rozengurt, 1980). For experimental purposes, 10^5 cells were subcultured in 33-mm Nunc dishes with 2.5 ml of DME containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. After 6 d at 37°C under a humidified atmosphere of 10% CO_2, 90% air, the cells were confluent and arrested in the G_1/G_0 phase of the cell cycle (Dicker and Rozengurt, 1980).

ADP-Ribosylation of Membranes

Swiss 3T3 cell membranes were prepared (Sinnett-Smith et al., 1990) and suspended at 3 mg of protein/ml in 50 mM Hepes/Na, 1 mM EGTA, 5 mM $MgCl_2$, 1 mg/ml bacitracin, 1 mg/ml soybean trypsin inhibitor, 10 μg/ml aprotinin and 50 μM PMSF, pH 7.2. Cholera toxin (0.5 mg/ml) and pertussis toxin (0.25 mg/ml) were preactivated by incubation at 37°C for 20 min in 12.5 mM DTT. An aliquot (50 μl) of the membrane suspension was added to 30 μl of buffer at pH 7.5, comprising 0.33 M Tris/HCl, 3.3 mM thymidine, 16.7 mM DTT, and 167 μM [^{32}P]NAD$^+$ (10 Ci/mmol). An aliquot (20 μl) of activated toxin was added to the membranes which were then incubated at 37°C for 30 min. The reaction was terminated by the addition of 1 ml of ice-cold 20% TCA. After 30 min on ice, precipitated protein was sedimented by centrifugation. The pellet was dissolved in SDS-sample buffer (Laemmli, 1970) comprising: 62.5 mM Tris/HCl, pH 6.8; 2% SDS (wt/vol); 10% glycerol (vol/vol); 5% β-mercaptoethanol (vol/vol); 0.025% bromophenolblue (wt/vol), heated at 100°C for 5 min, and then analyzed by SDS-PAGE and autoradiography at −70°C using Fuji x-ray film and intensifying screens.

Cell Incubation Procedures and the Analysis of Acid-soluble Material Labeled with [2-^{3}H]adenine

All incubations were at 37°C under a humidified atmosphere of 10% CO_2, 90% air. The cell cultures were washed twice with 2 ml of DME/Waymouth medium (1:1) and then incubated for 16 h with 1 ml of this medium containing 50 μCi of lyophilized [2-^{3}H]adenine. To preserve the pH of the incubation medium, additions were rapidly made to the dishes under an atmosphere of CO_2.

For the analysis of the incorporation of [^{3}H]adenine into acid-soluble material, the labeling medium was rapidly replaced with 1 ml of ice-cold 5% PCA (wt/vol). After 15 min at 4°C, the cells were scraped from the dishes and the extract was centrifuged at 10,000 *g* for 5 min at 4°C. The supernatant was added to 50 μl of 0.1 M EDTA/Na, pH 7.4, and 1 ml of tri-n-octylamine: 1,1,2-trichlorotrifluoroethane (1:1) followed by vigorous vortex mixing and phase separation by centrifugation (Downes et al., 1986). An aliquot (0.75 ml) of the upper phase containing the neutralized extract was diluted to 5 ml with 0.3 M NH_4COOH, pH 9. Dihydroxyboronyl-Bio-Rex 70 resin, which binds NAD$^+$ and ADP-ribose (Alvarez-Gonzalez et al., 1983), was used to separate these metabolites from unincorporated [^{3}H]adenine and adenine nucleotides. The resin was prepared as described by Alvarez-Gonzalez et al. (1983) and 0.6 ml was equilibrated with 0.25 M NH_4COOH, pH 9, in an Econo-column (Bio-Rad Laboratories). The sample was applied to the column and the effluent was collected. The column was then washed with 5 ml of 0.25 M NH_4COOH, pH 9, which was then combined with the initial effluent to give the adenine nucleotide fraction. To remove unincorporated [2-^{3}H]adenine, the column was washed three times with 5 ml of 0.25 M NH_4COOH, pH 9. NAD$^+$ and ADP-ribose were then eluted from the column with 10 ml of H_2O.

The samples were lyophilized and then dissolved in 10 ml of buffer A comprising 10 mM Hepes/Na, 0.1 mM EDTA, pH 7.4, and containing appropriate standards. The samples were applied at a flow rate of 1 ml/min to a Pharmacia 5/5 Mono Q column (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden) equilibrated in buffer A and fitted into an FPLC system. Fractions were collected every minute. NAD$^+$ did not bind to the column and was therefore collected in the flow through. After washing the column for a further 10 min, the concentration of Na_2SO_4 in buffer A was then increased at a rate of 5 mM/min to elute ADP-ribose at ~50 mM.

The samples containing the adenine nucleotides were similarly applied to the Mono Q column. Then, after washing the column for 10 min with buffer A, the concentration of Na_2SO_4 in buffer A was increased at a rate of 5 mM/min to elute AMP, ADP, and ATP, respectively, at ~50 mM, 70 mM, and 100 mM. The samples were mixed with 4.5 ml of Pico-Fluor 15 (Packard, Downers Grove, IL) and counted.

Sample Preparation for SDS-PAGE

Swiss 3T3 cells were labeled with [2-^{3}H]adenine as described above. After the required additions and times, the cells were extracted by rapidly replacing the medium with 0.15 ml of SDS-sample buffer. The cell lysates were scraped from the dishes, transferred to microfuge tubes and then heated at 100°C for 5 min. The samples were stored at −20°C until electrophoresis, prior to which they were reheated at 100°C for 5 min.

Gel Electrophoresis

Discontinuous SDS-PAGE was by the method of Laemmli (1970). Specifically, the slab gels were 0.75 mm thick with 1.5 cm of a 3% acrylamide stacking gel and 12 cm of 12.5% acrylamide resolving gel. Samples (50 μl) were electrophoresed at 20 V for 30 min, then overnight at 50 V and finally at 150 V for 30 min before terminating the run. Gels were fixed in 25% methanol/10% acetic acid (vol/vol).

TCA Treatment and Fluorography

For fluorography, the gels were rinsed in H_2O and then incubated in H_2O for 2 min. The gels were then shaken gently in 1 M sodium salicylate, pH 6.0, for 20 min (Chamberlain, 1979) and then dried for 3 h under vacuum at 80°C. Radioactivity was detected at −70°C using preflashed (Laskey and Mills, 1975) Fuji x-ray film with exposure times of 4–7 d. For the TCA treatment, the gels were equilibrated in 500 ml of 7% TCA (wt/vol) at room temperature and then placed in a 95°C oven for 45 min. The gels were then equilibrated in 25% methanol/10% acetic acid and processed for fluorography as described above.

Analysis of Toxin-catalyzed Labeling

Gel pieces containing [^{3}H]-labeled protein derived from the equivalent of the cultures of five 33-mm dishes were excised from gels treated with TCA at 95°C by reference to the fluorogram. The gel pieces were rehydrated in water and after 5 min were washed for two further 5-min periods in water. The gel was then homogenized in six times the gel volume of 50 mM NH_4HCO_3 containing 0.1% SDS (wt/vol). β-mercaptoethanol was added to a final concentration of 5% (vol/vol) and the mixture was heated at 100°C for 5 min. After shaking overnight at 20°C the mixture was centrifuged at 5,000 *g* for 30 min. The gel fragments were then further extracted with three times the gel volume of 50 mM NH_4HCO_3, 0.1% SDS (wt/vol) for 2 h. The two extracts were combined and protein was precipitated by adding carrier BSA (0.25 mg in 0.1 ml of H_2O) followed by 5 vol of ice-cold acetone. After 16 h at 4°C the precipitated protein was sedimented by centrifugation.

The pellet was washed with acetone, dried, and then resuspended in 0.5 ml of 98% HCOOH. In duplicate, an aliquot (0.1 ml) was then mixed with an equal volume of H_2O or, to hydrolyze thioglycoside linkages (Meyer et al., 1988; Jacobson et al., 1990), freshly made 20 mM mercuric acetate. After 10 min at 37°C, 1 ml of ice-cold water containing 1 mg BSA was added, followed by 0.3 ml of 100% TCA. After 10 min on ice, precipitated protein was sedimented by centrifugation and the supernatant was adjusted to pH 9 with 5 M NH_4OH. The sample was diluted to 10 ml with 0.25 M NH_4COOH, pH 9, and then applied to 1.5 ml of dihydroxyboronyl-Bio-Rex 70 equilibrated with 0.25 M NH_4COOH, pH 9 in a Bio-Rad Econo-column (Bio-Rad Laboratories). The column was washed with 10 ml of 0.25 M NH_4COOH, pH 9, and bound material was then eluted with 5 ml of H_2O. After lyophilization the sample was dissolved in 5 ml of buffer A containing AMP and ADP-ribose standards and then applied, as before, to the Mono Q column. Isocratic elution was with 10 mM Na_2SO_4 in buffer A. Fractions (0.33 ml) were collected and then counted with 5 ml of Pico-Fluor 15.

Inositol Phosphate Release and DNA Synthesis Assays

Phospholipids were labeled with [2-^{3}H]inositol and after appropriate incubations the total cellular [^{3}H]inositol phosphate content was determined by ion-exchange chromatography, as described previously (Staddon et al., 1991). DNA synthesis was assayed by [^{3}H]thymidine incorporation (Rozengurt et al., 1990).

Results

Pertussis Toxin and Cholera Toxin Catalyze the ADP-Ribosylation of Several Proteins in Membranes from Swiss 3T3 Cells

Incubation of Swiss 3T3 membranes with [^{32}P]NAD$^+$ followed by analysis of labeled material by SDS-PAGE and autoradiography revealed the endogenous labeling of several bands (Fig. 1 *C*). Addition of preactivated pertussis toxin, at 50 μg/ml, stimulated the labeling of 40- and 43-kD proteins (Fig. 1, *PTx*, *arrows*), and in addition ADP-ribosylated the S-1 (28 kD) and S-2/S-3 (23/22 kD) toxin subunits (Tamura et al., 1982). Addition of cholera toxin clearly ADP-ribosylated a 46-kD protein, presumably the α-subunit of G_s, and to a lesser extent a 40-kD protein that comigrated with that ADP-ribosylated by pertussis toxin (Fig. 1, *CTx*, *dots*). Thus, in membranes from Swiss 3T3 cells, pertussis toxin and cholera toxin can ADP-ribosylate more than one protein under the conditions of the assay. We attempted to establish which of these proteins are substrates for the toxins in intact cells.

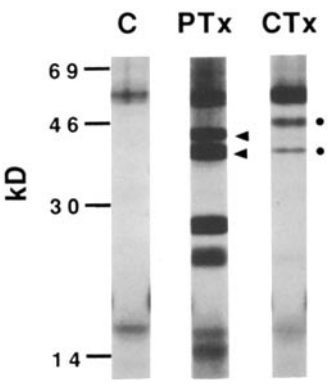

Figure 1. Pertussis toxin and cholera toxin catalyzed [^{32}P]-ADP-ribosylation of proteins in membranes from Swiss 3T3 cells. Membranes from Swiss 3T3 cells were prepared and ADP-ribosylated as described under Materials and Methods. The autoradiogram was derived from a 24-h exposure and shows proteins labeled under control conditions (*C*) and in the presence of pertussis toxin (*PTx*) or cholera toxin (*CTx*). The arrows depict the 40- and 43-kD pertussis toxin substrates and the dots depict the 40- and 46-kD cholera toxin substrates.

Pertussis Toxin–catalyzed ADP-Ribosylation in Intact Cells

To investigate ADP-ribosylation of toxin substrates in intact cells we explored the possibility of using [2-^{3}H]adenine to metabolically label the cellular NAD$^+$ pool, the substrate for ADP-ribosylation. [^{3}H]Adenine enters cells (Plagemann and Wohlheuter, 1980), including Swiss 3T3 cells (Becker et al., 1983), by carrier-mediated transport and is then phosphoribosylated to 5'-AMP. 5'-AMP in turn is phosphorylated to ATP which then enters the NAD$^+$ pool. We verified that [2-^{3}H]adenine is indeed incorporated into NAD$^+$ during the labeling of the Swiss 3T3 cells. After 16 h of incubation, the analysis of acid-soluble material revealed the incorporation of [2-^{3}H]adenine into NAD$^+$, ADP-ribose, AMP, ADP, and ATP (Table I). The ratio of counts incorporated into the NAD$^+$ and ATP pool after 16 h of labeling was approximately twice that of the ratio after 8 h but similar to that after 20 h of labeling (results not shown), indicating that isotopic equilibrium had been attained after 16 h.

The incorporation of radioactivity into high molecular weight material in cells incubated with [^{3}H]adenine for various times was analyzed by SDS-PAGE and fluorography. Treatment of these cells with pertussis toxin for 2 h before extraction caused the labeling of a 40-kD protein, but not that of a 43-kD protein (Fig. 2 *A*, *arrow*). The most prominent effect of pertussis toxin was observed after 16 h of labeling with [^{3}H]adenine but could be seen after 8 h of labeling.

The incubation of the cells with [^{3}H]adenine and without toxin treatment resulted in the incorporation of label into many bands that entered the gel (Fig. 2 *A*). Heating the gel at 95°C in the presence of 7% TCA for 45 min before fluorography had a striking effect: most of the label incorporated into material resolved by the gel was released except for that incorporated into the 40-kD protein in the cells incubated with pertussis toxin (Fig. 2 *B*). The basis of the effect of the acid treatment may be attributed to the depurination of RNA labeled in the cells by [2-^{3}H]ATP. In support of this, we have found that the inclusion of actinomycin D (4 μM) during a 6-h labeling with [^{3}H]adenine prevents

Table I. Analysis of Acid-soluble Radiolabeled Material Formed in Swiss 3T3 Cells Incubated with [2-^{3}H]Adenine

Metabolite	cpm/culture
NAD$^+$	$1.2 \pm 0.2 \times 10^6$
ADP-ribose	$5.2 \pm 1.1 \times 10^4$
AMP	$1.5 \pm 0.8 \times 10^5$
ADP	$1.9 \pm 0.3 \times 10^6$
ATP	$11.4 \pm 0.9 \times 10^6$

Confluent and quiescent cultures of Swiss 3T3 cells were incubated with 50 μCi/ml of [^{3}H]adenine for 16 h. Acid-soluble metabolites were assayed by chromatography, as described under Materials and Methods, for the incorporation of radioactivity into adenine-containing compounds. The values shown are the mean ± SD of four to five determinations involving three independent cell preparations. It was verified that the radioactivity coeluting from the Mono Q column in the flow through with standard NAD$^+$ was indeed counts incorporated into cellular NAD$^+$ by demonstrating that these counts coeluted with NADH after enzymatic reduction of the NAD$^+$ with glucose 6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (results not shown). NADH bound to the Mono Q column and was eluted at ~40 mM Na_2SO_4 using the gradients described under Materials and Methods. The ADP-ribose in the extracts is derived from both endogenous ADP-ribose and from NADH hydrolyzed during the acid extraction.

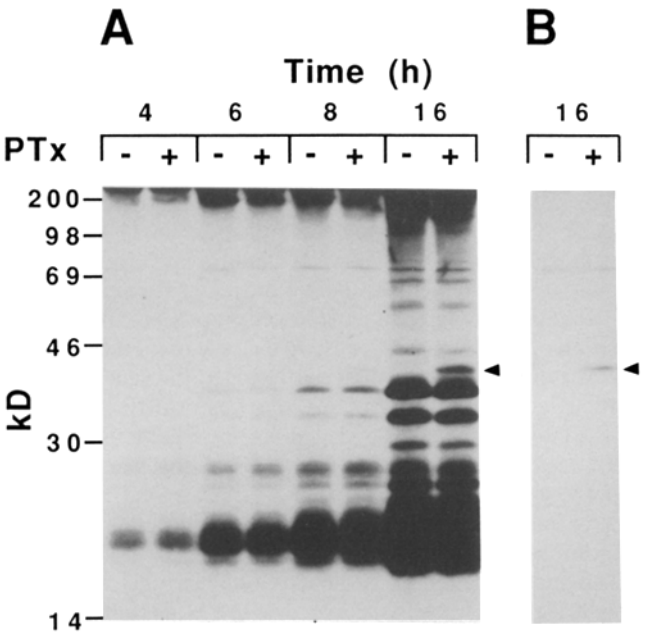

Figure 2. [2-^{3}H]adenine labeling of Swiss 3T3 cells: the effect of pertussis toxin. Confluent and quiescent cultures of Swiss 3T3 cells were incubated with 50 μCi/ml of [^{3}H]adenine for the times indicated and labeled material was analyzed by SDS-PAGE and fluorography, as described under Materials and Methods. The fluorogram in *A* was derived from a 7-d exposure and shows the kinetics of labeling of cellular material in the absence (−) or presence (+) of pertussis toxin (*PTx*). The incubation with pertussis toxin (100 ng/ml) was for 2 h before extraction of the cells. The fluorogram in *B* shows the effect of treatment of the 16-h portion of the gel shown in *A* with TCA at 95°C, as described under Materials and Methods. The arrow depicts the 40-kD pertussis toxin substrate.

the incorporation of label into the bands sensitive to the acid treatment (results not shown). Furthermore, these same bands are hydrolyzed by RNase A treatment of the cell extracts before electrophoresis. Thus, TCA treatment of the gel allows the clear discrimination of toxin-catalyzed labeling from the incorporation of label into other bands that occurs by endogenous cellular processes.

The basis of the pertussis toxin–catalyzed incorporation of label into the 40-kD protein was examined. Nicotinamide, a product of ADP-ribosyl transfer from NAD$^+$, inhibited the pertussis toxin–catalyzed labeling of the 40-kD protein in intact cells (Fig. 3). This action was not attributable to an inhibition of the formation of radiolabeled NAD$^+$ (results not shown). Nicotinamide at similar concentrations has been shown in vitro to inhibit pertussis toxin–catalyzed ADP-ribosylation (Katada and Ui, 1982). These observations suggest that the label incorporated into the 40-kD pertussis toxin substrate in intact cells may be ADP-ribose.

The label in the 40-kD protein pertussis toxin substrate was released by mercuric ions (Fig. 4, *inset*), consistent with a thioglycosidic linkage (Meyer et al., 1988; Jacobson et al., 1990). Radioactivity was similarly released by mercuric acetate from the pertussis toxin substrate after its extraction from the gel. All of the released radioactive material was retained by dihydroxy-boronyl-Bio-Rex 70 (results not shown), a resin that binds compounds containing two ribose groups (Alvarez-Gonzelez et al., 1983). Ion exchange chromatography, which distinguishes ADP-ribose from AMP, identified the retained material as ADP-ribose (Fig. 4). The radioactivity applied to the Mono Q column was quantitatively accounted for by the ADP-ribose peak. No radioactivity was detected in the ADP-ribose peak without mercuric acetate treatment (results not shown). Thus, [^{3}H]adenine

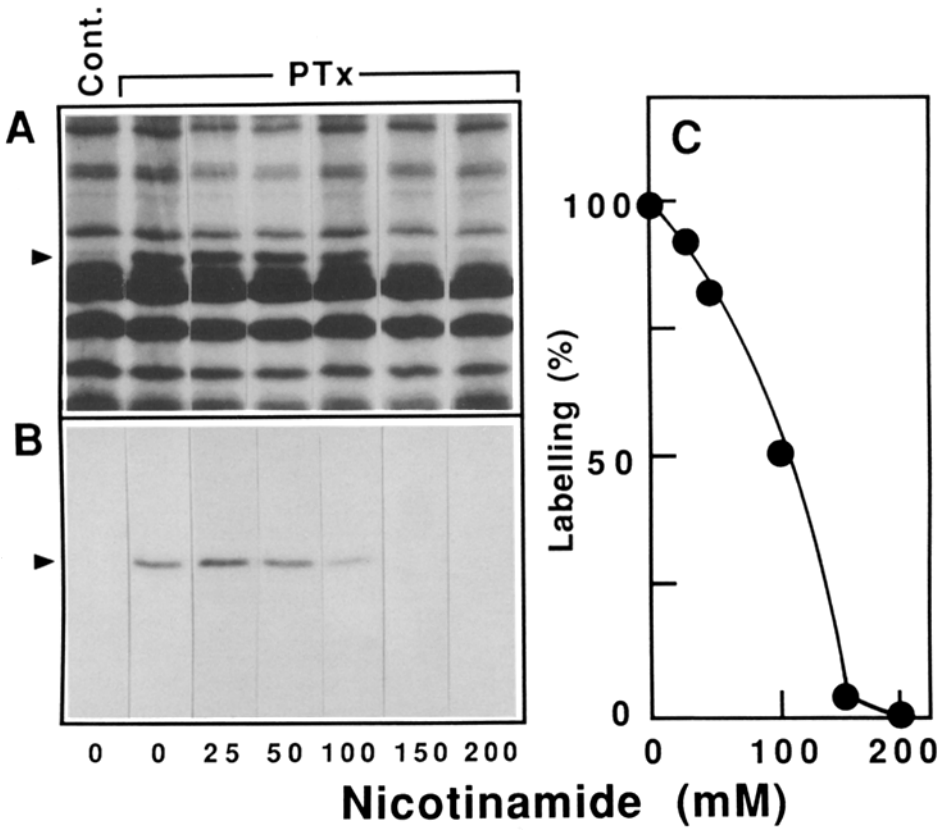

Figure 3. Nicotinamide inhibits the pertussis toxin–stimulated labeling of the 40-kD protein in intact cells. Confluent and quiescent Swiss 3T3 cells were labeled with 50 μCi/ml of [^{3}H]adenine for 16 h. The cells were then incubated for 2 h without (Cont.) or with 100 ng/ml of pertussis toxin (*PTx*) in the absence or presence of various concentrations of nicotinamide. The labeling of the 40-kD pertussis toxin substrate (*arrows*) was detected by fluorography of electrophoretically separated proteins, as described under Materials and Methods. The fluorogram is shown in *A* and the corresponding fluorogram after treatment of the gel with TCA at 95°C is shown in *B*. The amount of labeled 40-kD protein was quantified by densitometric scanning of the fluorogram in *B* and these data are presented in *C*. The data have been expressed as a percentage of the labeling achieved by pertussis toxin in the absence of nicotinamide.

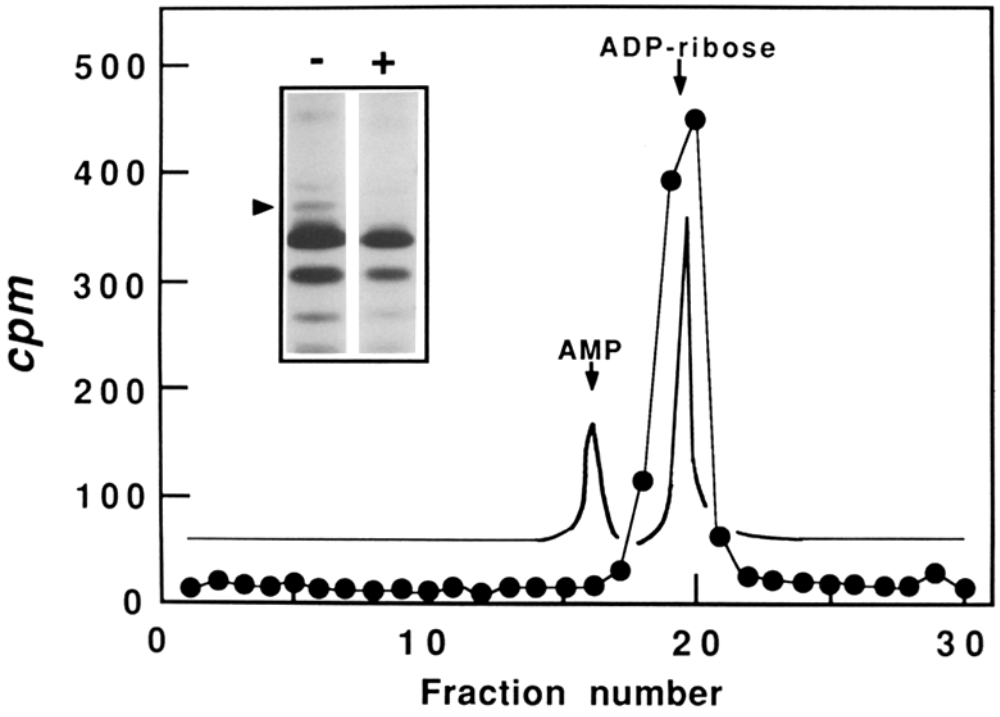

Figure 4. Hg^{2+} releases [^{3}H]-ADP-ribose from the 40-kD pertussis toxin substrate. Confluent and quiescent cultures of Swiss 3T3 cells were labeled with 50 μCi/ml of [^{3}H]adenine for 16 h and then treated with or without 100 ng/ml of pertussis toxin for 2 h. SDS lysates were analyzed by SDS-PAGE and fluorography, as described under Materials and Methods. The inset shows a fluorogram of the gel with samples analyzed from cells treated with pertussis toxin. The arrow indicates the 40-kD pertussis toxin substrate. The gel was fixed in acetic acid/methanol, as described under Materials and Methods, in the absence (−) or presence (+) of 10 mM mercuric acetate. The apparent slight decrease in the labeling of the bands other than the 40-kD pertussis toxin substrate is attributable to the lesser loading of material onto this particular lane, as confirmed by densitometric scanning of the gels before and after mercuric acetate treatment (results not shown). The analysis of radioactive material released by mercuric acetate was performed as follows. The pertussis toxin–labeled protein was extracted from the gel and incubated with 10 mM mercuric acetate, as described under Materials and Methods. The released radioactive material was chromatographed with 5′-AMP and ADP-ribose standards. The continuous lines show the UV absorbance (254 nm) of the standards and radioactivity is shown by the closed circles.

labeling together with acid treatment of the gel readily permits the detection in intact cells of pertussis toxin–catalyzed ADP-ribosylation of a 40-kD protein. Clearly, the 43-kD pertussis toxin substrate ADP-ribosylated in vitro is not a substrate for the toxin in intact 3T3 cells.

Dose-dependent Lag Period

Pertussis toxin acts after a lag period, reflecting the time required for cellular entry and activation of the toxin (Ui, 1990). Incubation of [2-^{3}H]adenine-labelled cells with pertussis toxin also demonstrates a lag period in the ADP-ribosylation of the 40-kD protein (Fig. 5, *A–C*). Furthermore, 0.1 ng/ml of pertussis toxin in an 8-h incubation was as effective as 100 ng/ml of the toxin in a 2-h incubation (Fig. 5 *D*). The lag period in the action of the toxin and the increase in its potency with time are clearly consistent with an expression of the catalytic activity of the toxin after its entry into the cells.

ADP-ribosylation of G_s in Intact Cells

In membranes from Swiss 3T3 cells the major cholera toxin substrate is a 46-kD protein (Fig. 1), the α-subunit of G_s (Pfeuffer and Helmreich, 1988; Birnbaumer et al., 1990). Similarly, in cells labeled with [^{3}H]adenine, cholera toxin stimulated the labeling of a 46-kD protein (Fig. 6 *A*). Labeling of the 40-kD protein weakly promoted by cholera toxin in vitro (Fig. 1) could not be detected in intact cells. The label in the [^{3}H]labeled protein was resistant to TCA at 95°C and the protein comigrated with in vitro ADP-ribosylated α_s (results not shown). The action of cholera toxin, like that of pertussis toxin, occurred after a lag period and its potency increased with time of incubation (Fig. 6, *B* and *C*). The cAMP elevating agents forskolin, prostaglandin E_1, that do not act by ADP-ribosylating α_s, and even 8-bromo cyclic AMP did not stimulate the labeling of the 46-kD protein (Fig. 6 *A*).

Irreversibility of Toxin Action

Swiss 3T3 cells were treated with or without pertussis toxin before the labeling of the cellular NAD^+ pool with [^{3}H]adenine. [^{3}H]ADP-ribosylated 40-kD protein did not appear in the pertussis toxin–pretreated cells (Fig. 7, see lanes *1* and *2*). The addition of the toxin at the end of the 16-h labeling period with [^{3}H]adenine stimulated the ADP-ribosylation of the 40-kD protein in the control cells, as expected (Fig. 7, see lanes *1* and *3*), but not in the pertussis toxin–pretreated cells (Fig. 7, see lanes *2* and *4*). Furthermore, cholera toxin stimulated the labeling of α_s in both the control cells and the pertussis toxin–pretreated cells (Fig. 7, see lanes *1* and *5*, *2* and *6*). The absence of radio-ADP-ribosylated 40-kD protein in the pertussis toxin pretreated cells is consistent with irreversible ADP-ribosylation of the protein before radiolabeling of the NAD^+ pool. The lack of effect of a subsequent addition of pertussis toxin to the pertussis toxin–pretreated cells is not attributable to impaired radiolabeling of the NAD^+ pool as cholera toxin was equally effective at ADP-ribosylating α_s in control and pertussis toxin–pretreated cells. These data are consistent with the irreversibil-

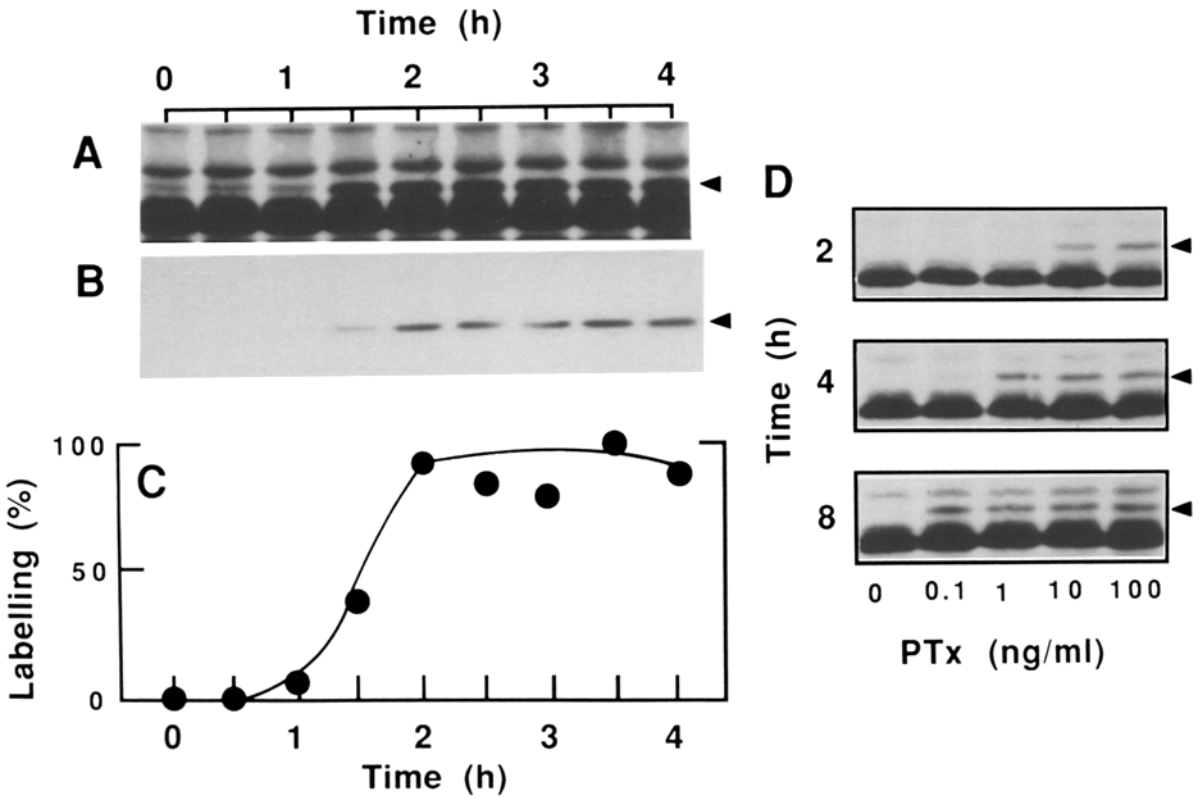

Figure 5. The action of pertussis toxin occurs after a lag period and its potency increases with time. Confluent and quiescent cultures of Swiss 3T3 cells were labeled with 50 μCi/ml of [^{3}H]adenine for 20 h, as described under Materials and Methods. Before extraction, the cells were treated with 100 ng/ml of pertussis toxin for the times indicated, and the fluorogram of electrophoretically analyzed SDS lysates is shown in *A*. The corresponding gel treated with TCA at 95°C is shown in *B*. The arrows indicate the 40-kD pertussis toxin substrate. Data from the densitometric scanning of the bands in *B* is shown in *C*. These data have been expressed as a percentage of the maximum labeling achieved by pertussis toxin. In *D*, the fluorograms were from gels not treated with TCA at 95°C and were derived from cells labeled for 16 h, as above, and then incubated for various further periods of time with pertussis toxin (*PTx*) at various concentrations to label the 40-kD protein (*arrows*).

ity of toxin-catalyzed ADP-ribosylation in intact cells, indicating that cells do not apparently possess the necessary glycohydrolases to remove ADP-ribose from the pertussis toxin substrate.

Is P. multocida Toxin an ADP-Ribosyltransferase?

P. multocida toxin is an extremely potent mitogen for Swiss 3T3 and other cultured mesenchymal cells (Rozengurt et al., 1990). This toxin activates phospholipase C as indicated by the stimulation of inositol phosphate release (Staddon et al., 1991) and activation of protein kinase C (Staddon et al., 1990). Intriguingly, the toxin enters the cells to elicit mitogenesis (Rozengurt et al., 1990) and activate phospholipase C (Staddon et al., 1990, 1991). The mechanism whereby *P. multocida* toxin activates phospholipase C is not known. However, the inositol phosphate species formed in response to *P. multocida* toxin and bombesin are remarkably similar (Staddon et al., 1991). The bombesin receptor is believed to couple to phospholipase C via a pertussis toxin–insensitive and yet to be identified G protein (Zachary et al., 1987; Erusalimsky et al., 1988; Fisher and Schonbrunn, 1988; Hasegawa-Sasaki et al., 1988; Cattaneo and Vicentini, 1989; Coffer et al., 1990; Sinnett-Smith et al., 1990; Plevin et al., 1990; Battey et al., 1991). Recently, a likely candidate for this G protein has been identified as G_q (Shenker et al., 1991; Smrcka et al., 1991; Taylor et al., 1991). As the deduced amino acid sequence of *P. multocida* toxin exhibits partial homology with that of other ADP-ribosylating toxins, including cholera and pertussis (Lax et al., 1990), a plausible hypothesis to explain the action of *P. multocida* toxin is that it ADP-ribosylates and thereby constitutively activates the 42-kD α-subunit of G_q that usually couples the bombesin receptor to phospholipase C. Initially, experiments attempting to detect ADP-ribosylation catalyzed by *P. multocida* toxin employed the in vitro conditions described in Fig. 1, using 10 μg/ml of toxin. ADP-ribosylation was not detected (results not shown), perhaps because the toxin requires activation (Rozengurt et al., 1990; Staddon et al., 1990, 1991). We therefore tested the hypothesis under conditions where the biological activity of the toxin can be unequivocally established, i.e., using intact cells and the methodology developed in the preceding sections.

Cultures of Swiss 3T3 cells were prelabeled with [2-^{3}H]-adenine and then incubated with *P. multocida* toxin or, for comparative purposes, pertussis toxin. After a 4-h incubation with 20 ng/ml of *P. multocida* toxin no increase in the labeling of a 42-kD protein was detected in fluorograms of gels that had not (Fig. 8 *A*) or had been treated with TCA at 95°C (Fig. 8 *B*). The action of pertussis toxin was clearly

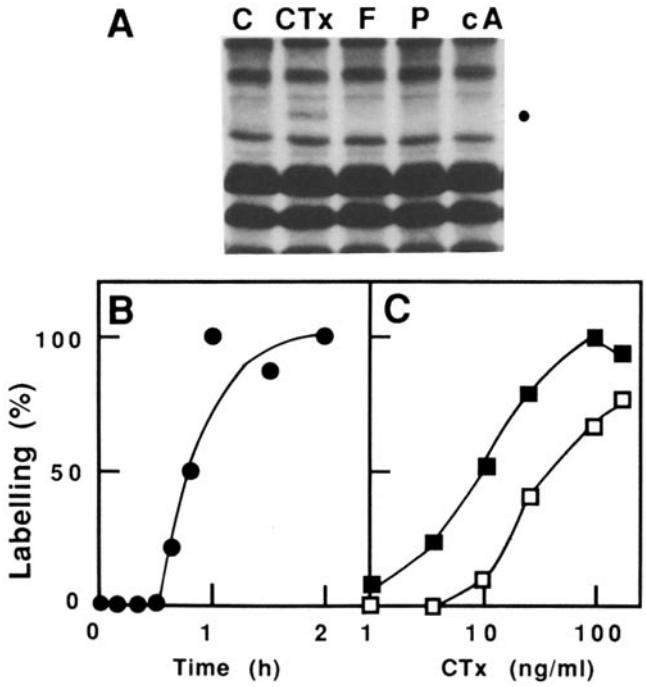

Figure 6. Cholera toxin stimulates the ADP-ribosylation of α_s in [^{3}H]adenine-labeled cells. Confluent and quiescent Swiss 3T3 cells were labeled for 16 h with 50 μCi/ml of [2-^{3}H]adenine as described under Materials and Methods. *A* shows the fluorogram of extracts derived from control cells (*C*) or cells treated for 1 h with either 100 ng/ml of cholera toxin (*CTx*), 25 μM forskolin (*F*), 500 ng/ml prostaglandin E_1 (*P*), or 2.5 mM 8-bromo-cyclic AMP (*cA*). The dot indicates the migration of the 46-kD cholera toxin substrate. In parallel with these incubations, other cultures were incubated in the absence of [2-^{3}H]adenine and treated exactly as above. The cells were extracted into 0.1 M HCl and radioimmunoassayed for cAMP (Rozengurt et al., 1987). The control value for the cellular cAMP content was 10 ± 1 pmol/mg cell protein (n = 4, mean ± SD): after treatment with cholera toxin, forskolin, and prostaglandin E_1, this was increased to 24 ± 1, 127 ± 23, and 24 ± 5 pmol/mg cell protein, respectively. *B* shows the increase in the labeling of the 46-kD protein, shown in *A*, in response to 100 ng/ml of cholera toxin as a function of time. The data were derived from the densitometric scanning of the fluorogram and have been expressed as a percentage of the maximum labeling achieved by cholera toxin. *C* shows the effects of incubation time on the increase in labeling of the 46-kD protein, shown in *A*, in response to various concentrations of cholera toxin. The open squares represent a 1-h incubation and the closed squares a 2-h incubation.

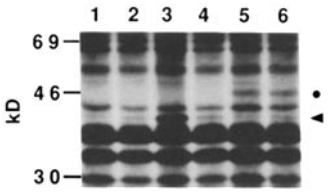

Figure 7. Pretreatment with pertussis toxin selectively blocks ADP-ribosylation of the 40-kD protein. Confluent and quiescent Swiss 3T3 cells were washed twice with 2 ml of DME/Waymouth medium (1:1) and then incubated for 2 h with 1 ml of this medium in the absence or presence of 100 ng/ml of pertussis toxin. The medium was then replaced by 1 ml of DME:Waymouth medium containing 50 μCi/ml of [^{3}H]adenine. After 16 h, the cells were incubated in the absence or presence of either 100 ng/ml pertussis toxin or 100 ng/ml cholera toxin for a further 2 h (the toxins were added directly to the labeled culture medium as 100-fold concentrated stocks). Cell lysates were analyzed by SDS-PAGE and fluorography, as described under Materials and Methods. The lanes show the labeling of extracts derived from cells treated as follows: (*1*) labeled with [^{3}H]adenine and receiving no additions of toxin; (*2*) treated with pertussis toxin before the incubation with [^{3}H]adenine; (*3*) labeled with [^{3}H]adenine and then incubated with pertussis toxin; (*4*) treated with pertussis toxin before the incubation with [^{3}H]adenine and then reincubated with pertussis toxin; (*5*) labeled with [^{3}H]-adenine and then incubated with cholera toxin; (*6*) treated with pertussis toxin before the incubation with [^{3}H]adenine and then incubated with cholera toxin. The arrow depicts the 40-kD pertussis toxin substrate and the dot the 46-kD cholera toxin substrate.

detected (Fig. 8, *A* and *B*). In fact, *P. multocida* toxin did not appear to stimulate the ADP-ribosylation of any protein (Fig. 8, *A* and *B*), including those in the 21-kD range of the gels (results not shown). RNase A was also used instead of TCA to hydrolyze labeled RNA from the extracts before electrophoresis, and did not reveal any *P. multocida* toxin-catalyzed ADP-ribosylation (results not shown). Similar negative results were obtained in incubations with 20 ng/ml of *P. multocida* toxin with samples taken every 30 min up to 4 h. Similarly, varying the dose of *P. multocida* toxin from 1 to 100 ng/ml in 4-h incubations did not reveal any stimulation of ADP-ribosylation. Increasing the time of fluorography by tenfold did not detect ADP-ribosylation (results not shown). It was also established that *P. multocida* toxin did not interfere with ADP-ribosylation per se, as cholera toxin clearly stimulated the labeling of α_s in either control or *P. multocida* toxin–pretreated cultures (Fig. 8 *C*). As another positive control, parallel cultures of cells were labeled with [2-^{3}H]inositol instead of [2-^{3}H]adenine: in these cells *P. multocida* toxin clearly stimulated inositol phosphate release (Fig. 8 *D*). Furthermore, with the cells used in these experiments the mitogenicity of the *P. multocida* toxin preparation was clearly verified (Fig. 8 *E*). These results suggest that *P. multocida* toxin does not act by ADP-ribosylation of G_q, at least as detected under conditions clearly revealing ADP-ribosylation catalyzed by pertussis and cholera toxin.

Discussion

Investigations into the pathological basis of bacterial infection have provided toxins that have been widely used as investigative tools in cell biology. However, a satisfactory approach to directly study toxin-catalyzed ADP-ribosylation in intact cells has not been described. We now demonstrate, using a new approach, pertussis toxin and cholera toxin–catalyzed ADP-ribosylation of specific substrate proteins in intact cells.

Pertussis toxin is known to ADP-ribosylate several G protein α subunits in membranes from a variety of tissues: the assignment of function to these proteins is a major problem (see Birnbaumer et al., 1990). In Swiss 3T3 cell membranes, we found that pertussis toxin ADP-ribosylated 40- and 43-kD proteins. In a previous study, employing different conditions to identify toxin substrates, pertussis toxin was described to ADP-ribosylate only a 40-kD protein in membranes from Swiss 3T3 cells (Murayama et al., 1983). In these cells, pertussis toxin interferes with several signaling processes, e.g., potentiation of cAMP accumulation by protein kinase C (Rozengurt et al., 1987; Millar and Rozengurt,

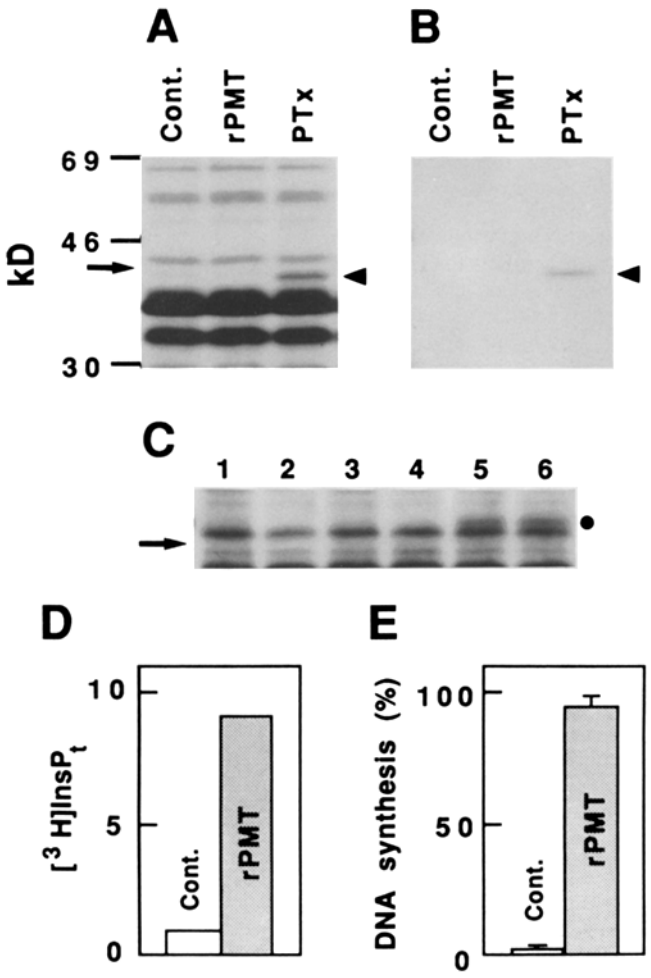

Figure 8. Does *P. multocida* toxin act by ADP-ribosylation? Confluent and quiescent Swiss 3T3 cells were labeled for 20 h with 50 μCi/ml of [2-^{3}H]adenine as described under Materials and Methods. Before extraction, the cells were incubated in the absence (*Cont.*) or presence of recombinant *P. multocida* toxin (*rPMT*, 20 ng/ml) or pertussis toxin (*PTx*, 100 ng/ml) for 4 h or 1 h, respectively. The SDS-lysates were analyzed by SDS-PAGE and shown are fluorograms of the gels before (*A*) and after (*B*) TCA treatment at 95°C. The arrowhead depicts the 41-kD pertussis toxin substrate and the long arrow indicates the migration expected of proteins with molecular mass of 42 kD. *C* shows part of a fluorogram derived from cells labeled, as above, with [2-^{3}H]adenine for 16 h. The cells were incubated for a further 4 h (lane *1*) or 5 h (lane *2*) or, in parallel, were incubated with 20 ng/ml of *P. multocida* toxin for 4 h (lane *3*) or 5 h (lane *4*). After the 4-h incubation in the absence or presence of *P. multocida* toxin, cholera toxin was added to these cultures to a final concentration of 100 ng/ml and the incubations were continued for a further hour. Lane *5* shows the effect of cholera toxin when added to control cultures and lane *6* shows the effect of cholera toxin when added to *P. multocida* toxin-treated cultures. The dot depicts the cholera toxin substrate and the arrow the expected migration of 42-kD proteins. *D* shows the stimulation of inositol phosphate release by the preparation of recombinant *P. multocida* toxin (*rPMT*) used in this study and in parallel with the cultures used to obtain the data presented in panels A–C. Inositol phosphate release was determined as described under Materials and Methods. [^{3}H]Inositol-labeled cultures were incubated in the absence (*Cont.*) or presence of 20 ng/ml recombinant *P. multocida* toxin (*rPMT*) for 4 h. LiCl was added to a final concentration of 20 mM for 20 min before extraction of the cells. The values are the means of duplicate incubations expressed relative to the control value, 329 cpm/sample. Similarly, the data depicted in *E* verify that the preparation of recombinant *P. multocida* toxin (*rPMT*, 20 ng/ml) was capable of stimulating DNA synthesis in the cultures. DNA synthesis was determined by measuring [^{3}H]thymidine incorporation as described under Materials and Methods. The values are the means ± SD of triplicate incubations and have been expressed relative to DNA synthesis in the presence of FBS (24.9 ± 1.4) × 10^{-3} cpm.

1988), bombesin induction of c-*myc* expression (Letterio et al., 1986; Millar and Rozengurt, 1988), arachidonic acid release stimulated by thrombin (Murayama and Ui, 1985), DNA synthesis (Letterio et al., 1986; Murayama and Ui, 1987; Zachary et al., 1987; Millar and Rozengurt, 1988; Taylor et al., 1988), and arachidonic acid release stimulated by the G protein activator mastoparan (Gil et al., 1991). The basis of the pertussis toxin sensitivity of these events is not known. It is conceivable that the 40- and 43-kD toxin substrates revealed in this study in vitro could play different roles in signal transduction. A greater understanding of the action of pertussis toxin could be achieved by establishing the relevance of in vitro catalyzed ADP ribosylation to that occurring in intact cells.

Rather than the indirect method of attempting to block in vitro ADP-ribosylation by pretreatment of intact cells with pertussis toxin (Ui, 1990), our approach has been a direct identification of proteins ADP-ribosylated by toxins in intact cells. As NAD^+ is membrane impermeant, it must either be injected into the cell or metabolically radiolabeled in order to study toxin-catalyzed ADP-ribosylation. Godeau et al. (1980) demonstrated the ADP-ribosylation of elongation factor 2 in *Xenopus* oocytes microinjected with [^{3}H]NAD^+ and the A subunit of diphtheria toxin. For metabolic labeling, [^{32}P]Phosphate has been used to study diphtheria toxin (Fendrick et al., 1989) and *C. botulinum* C2 toxin (Reuner et al., 1987) catalyzed ADP-ribosylation in intact cells, but this has required the purification of the known target proteins. Since [^{32}P]P_i also labels the cellular ATP pool, scarce [^{32}P]ADP-ribosylated proteins have to be discriminated from abundant [^{32}P]-phosphoproteins. Furthermore, this problem is exacerbated with toxins that can indirectly cause changes in protein phosphorylation. Indeed, cholera toxin and, in certain cell types, pertussis toxin indirectly increase the cellular cAMP content thus activating cAMP-dependent protein kinase (Pfeuffer and Helmreich, 1988; Ui, 1990). [^{3}H]Adenine, [^{3}H]adenosine, and [^{14}C]ribose have also been employed to label NAD^+ and hence to study ADP-ribosylation and polyADP-ribosylation as catalyzed by endogenous enzymes (Ueda et al., 1975; Carlson and Lazarides, 1983; Tanuma and Johnson, 1983; Ledford and Jacobs, 1986; Adolph et al., 1987; Aboul-Ela et al., 1988). The use of [2-^{3}H]adenine to label NAD^+ is more appropriate than [^{32}P]-P_i in cellular studies of ADP-ribosylation, but has not been employed, to our knowledge, to study the action of bacterial toxins in cultured cells.

The new procedure, described here, employs [2-^{3}H]adenine to metabolically radiolabeled NAD^+ and exploits the acid stability of ADP-ribose amino-acid linkages formed by toxins to allow the clear visualization of toxin-catalyzed labeling. Pertussis toxin, at concentrations affecting signaling in Swiss 3T3 cells (Zachary et al., 1987; Rozengurt et

al., 1987), caused the selective labeling of a 40-kD protein in a time- and dose-dependent manner. The incorporation of radioactivity into the 40-kD protein was blocked by nicotinamide. Furthermore, the incorporated radioactivity was released by mercuric acetate, consistent with a thioglycosidic linkage, and was identified as [^{3}H]ADP-ribose. Indeed, ADP-ribose incorporated into pertussis toxin G protein substrates in vitro is at a cysteine residue four amino acids from the COOH terminus (see Ui, 1990; Birnbaumer et al., 1990). Although pertussis toxin ADP-ribosylates two proteins in the membrane preparation, only one of these is ADP-ribosylated in the intact cells.

Cholera toxin has been shown to increase the cellular levels of cAMP and promote mitogenesis in Swiss 3T3 cells (Rozengurt et al., 1981). These actions have been attributed to the ADP-ribosylation of G_s. However, cAMP-independent actions of cholera toxin have been reported in which the role of ADP-ribosylation is not clear (see, e.g., Quershi et al., 1991). Indeed, it has been shown that cholera toxin ADP-ribosylates pertussis toxin substrates in vitro (see Birnbaumer et al., 1990; Ui, 1990) but the significance of this in intact cells remains to be established. In the present study we also demonstrate that cholera toxin can label a 40-kD protein in membranes from Swiss 3T3 cells. Using the procedure described here, cholera toxin, at concentrations that elicit increases in cAMP (Rozengurt et al., 1981), specifically ADP-ribosylates the 46-kD substrate, presumably α_s, in intact cells but not the 40-kD substrate in isolated membranes. The identification of toxin substrates in intact cells will assist, together with immunological and molecular biological approaches, in the elucidation of the complex role of these proteins in signal transduction. Furthermore, the approach described here will allow studies in intact cells of the actions of other toxin ADP-ribosyl-transferases.

The results reported here have other important implications. Some bacterial toxins are composed of a cellular binding domain and a domain with latent enzymatic activity. The binding domain serves to transfer the active domain into the cell allowing the expression of its activity (see, e.g., Middlebrook and Dorland, 1984; Neville and Hudson, 1986; Olsnes and Sandvig, 1988). Expression of the activity of the bacterial toxin in vitro (e.g., after treatment with reducing agents, mild detergents, proteases, etc.) may permit the detection of ADP-ribosyltransferase activity. However, if the basis of activation of a toxin is not understood, then an alternative method is needed to investigate the possibility that it is an ADP-ribosyltransferase. A cellular study, as described here, will allow the investigation of toxin-catalyzed ADP-ribosylation under conditions where the toxin is known to be biologically active.

We have applied the methodology developed here to investigate the possibility that *P. multocida* toxin acts by ADP-ribosylation. This toxin, an extremely potent mitogen for mesenchymal cells including Swiss 3T3 (Rozengurt et al., 1990), activates phospholipase C (Staddon et al., 1990, 1991), and its deduced amino acid sequence contains a homologous arrangement found in known toxin ADP-ribosyltransferases (Lax et al., 1990). Under conditions clearly demonstrating cholera and pertussis toxin–catalyzed ADP-ribosylation, we did not find ADP-ribosylation by *P. multocida* toxin of G_q or any other bands, implying that it must act by mechanisms other than ADP-ribosylation. It is possible that the lack of apparent ADP-ribosylation by *P. multocida* toxin is simply because the G protein substrate is not abundant enough to be detected. However, in membranes from bovine brain, at least, the G_q protein was detected by immunoblots that in parallel revealed pertussis toxin substrates (Shenker et al., 1991).

In conclusion, we have described a new approach to study toxin-catalyzed ADP-ribosylation. The procedure is simple and, for the first time, has allowed the identification of pertussis and cholera toxin substrates in intact cells. It permits an analysis of the relevance of ADP-ribosylation observed in vitro to that occurring in intact cells, an important consideration when attempting to correlate in vitro phenomena with cellular events. The procedures described here will also be of utility in studies of microbial toxins with unknown mechanisms of action.

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References

Aboul-Ela, N., E. L. Jacobson, and M. K. Jacobson. 1988. Labeling methods for the study of poly- and mono(ADP-ribose) metabolism in cultured cells. *Anal. Biochem.* 174:239–250.

Adolph, K. W. 1987. ADP-ribosylation of nuclear proteins labelled with [^{3}H]adenosine: changes during the HeLa cell cycle. *Biochim. Biophys. Acta.* 909:222–230.

Aktories, K., and I. Just. 1990. Botulinum C2 toxin. *In* ADP-ribosylating Toxins and G Proteins: Insights into Signal Transduction. J. Moss and M. Vaughan, editors. American Society for Microbiology, Washington D.C. 79–95.

Aktories, K., U. Braun, B. Habermann, and S. Rosener. 1990. Botulinum ADP-ribosyltransferase C3. *In* ADP-ribosylating Toxins and G Proteins: Insights into Signal Transduction. J. Moss and M. Vaughan, editors. American Society for Microbiology, Washington D.C. 97–115.

Alvarez-Gonzalez, R., H. Juarez-Salinas, E. L. Jacobson, and M. K. Jacobson. 1983. Evaluation of immobilized boronates for studies of adenine and pyridine nucleotide metabolism. *Anal. Biochem.* 135:69–77.

Battey, J. F., J. M. May, M. H. Corjay, H. Shapira, K. Kusano, R. Harkins, J. M. Wu, T. Slattery, E. Mann, and R. I. Feldman. 1991. Molecular cloning of the bombesin/gastrin-releasing peptide receptor from Swiss 3T3 cells. *Proc. Natl. Acad. Sci. USA.* 88:395–399.

Becker, M. A., P. Dicker, and E. Rozengurt. 1983. Mitogenic enhancement of purine base phosphoribosylation in Swiss mouse 3T3 cells. *Am. J. Physiol.* 244:C288–C296.

Birnbaumer, L., J. Abramowitz, and A. M. Brown. 1990. Receptor-effector coupling by G proteins. *Biochim. Biophys. Acta.* 1031:163–224.

Carlson, L., and E. Lazarides. ADP-ribosylation of the M_r 83,000 stress-inducible and glucose-regulated protein in avian and mammalian cells: modulation by heat shock and glucose starvation. 1983. *Proc. Natl. Acad. Sci. USA.* 80:4664–4668.

Cattaneo, M. G., and L. M. Vicentini. 1989. Differential mechanisms of inositol phosphate generation at the receptors for bombesin and platelet-derived growth factor. *Biochem. J.* 262:665–668.

Chamberlain, J. P. 1979. Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate. *Anal. Biochem.* 98:132–135.

Coburn, J., S. T. Dillon, B. H. Iglewski, and G. M. Gill. 1989*a*. Exoenzyme S of *Pseudomonas aeruginosa* ADP-ribosylates the intermediate filament protein vimentin. *Infect. Immunol.* 57:996–998.

Coburn, J., R. T. Wyatt, B. H. Iglewski, and G. M. Gill. 1989*b*. Several GTP-binding proteins, including $p21^{c-H-ras}$, are preferred substrates of *Pseudomonas aeruginosa* exoenzyme S. *J. Biol. Chem.* 264:9004–9008.

Coffer, A., I. Fabregat, J. Sinnett-Smith, and E. Rozengurt. 1990. Solubilization of the bombesin receptor from Swiss 3T3 cell membranes: functional association to a guanine nucleotide regulatory protein. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 263:80–84.

Collier, R. J. 1990. Diphtheria toxin: structure and function of a cytocidal protein. *In* ADP-ribosylating Toxins and G Proteins: Insights into Signal Transduction. J. Moss and M. Vaughan, editors. American Society for Microbiology, Washington, D.C. 3–19.

Dicker, P., and E. Rozengurt. 1980. Phorbol esters and vasopressin stimulate DNA synthesis by a common mechanism. *Nature (Lond.).* 287:607–612.

Downes, C. P., P. T. Hawkins, and R. F. Irvine. 1986. Inositol 1,3,4,5-

tetrakisphosphate is the probable precursor of inositol 1,3,4-trisphosphate in agonist-stimulated parotid gland. *Biochem. J.* 238:501–506.
Erusalimsky, J. D., I. Friedberg, and E. Rozengurt. 1988. Bombesin, diacylglycerols, and phorbol esters rapidly stimulate the phosphorylation of an M_r = 80,000 protein kinase C substrate in permeabilized 3T3 cells: effect of guanine nucleotides. *J. Biol. Chem.* 263:19188–19194.
Fendrick, J. L., and W. J. Iglewski. 1989. Endogenous ADP-ribosylation of elongation factor 2 in polyoma virus-transformed baby hamster kidney cells. *Proc. Natl. Acad. Sci. USA.* 86:554–557.
Fisher, J. B., and A. Schonbrun. 1988. The bombesin receptor is coupled to a guanine nucleotide-binding protein which is insensitive to pertussis and cholera toxins. *J. Biol. Chem.* 263:2808–2816.
Gil, J., T. E. Higgins, and E. Rozengurt. 1991. Mastoparan, a novel mitogen for Swiss 3T3 cells, stimulates pertussis toxin-sensitive arachidonic acid release without inositol phosphate accumulation. *J. Cell Biol.* 113:943–950.
Gill, D. M., and M. Woolakis. 1988. [^{32}P]ADP-ribosylation of proteins catalyzed by cholera toxin and related heat-labile enterotoxins. *Methods Enzymol.* 165:235–245.
Godeau, F., P. Boquet, S. Schorderet-Slatkine, M. Schorderet, and E.-E. Baulieu. 1980. Studies of microbial toxins in *Xenopus laevis* oocytes. *Exp. Cell Res.* 129:133–137.
Hasegawa-Sasaki, H., F. Lutz, and T. Sasaki. 1988. Pathway of phospholipase C activation initiated with platelet-derived growth factor is different from that initiated with vasopressin and bombesin. *J. Biol. Chem.* 263:12970–12976.
Jacobson, M. K., P. T. Loflin, N. Aboul-Ela, M. Migmuang, J. Moss, and E. L. Jacobson. 1990. Modification of plasma membrane protein cysteine residues by ADP-ribose in vivo. *J. Biol. Chem.* 259:10825–10828.
Katada, T., and M. Ui. 1982. ADP-ribosylation of the specific membrane protein of C6 cells by islet-activating protein associated with modification of adenylate cyclase. *J. Biol. Chem.* 257:7210–7216.
Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
Laskey, R. A., and A. D. Mills. 1975. Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. *Eur. J. Biochem.* 56:335–341.
Lax, A. J., and N. Chanter. 1990. Cloning of the toxin gene from *Pasteurella multocida* and its role in atrophic rhinitis. *J. Gen. Microbiol.* 136:81–87.
Lax, A. J., N. Chanter, G. Pullinger, T. Higgins, J. M. Staddon, and E. Rozengurt. 1990. Sequence analysis of the potent mitogenic toxin of *Pasteurella multocida*. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 227:59–64.
Ledford, B. E., and D. F. Jacobs. 1986. Translational control of ADP-ribosylation in eucaryotic cells. *Eur. J. Biochem.* 161:661–667.
Letterio, J. J., S. R. Coughlin, and L. T. Williams. 1986. Pertussis toxin-sensitive pathway in the stimulation of *c-myc* expression and DNA synthesis by bombesin. *Science (Wash. DC).* 234:1117–1119.
Meyer, T., R. Koch, W. Fanick, and H. Hilz. 1988. ADP-ribosyl proteins formed by pertussis toxin are specifically cleaved by mercury ions. *Biol. Chem. Hoppe-Seyler.* 369:579–583.
Middlebrook, J. L., and R. B. Dorland. 1984. Bacterial toxins: cellular mechanisms of action. *Microbiol. Rev.* 48:199–221.
Millar, J. B. A., and E. Rozengurt. 1988. Bombesin enhancement of cAMP accumulation in Swiss 3T3 cells: evidence for a dual mechanism of action. *J. Cell Physiol.* 137:214–222.
Moss, J., and M. Vaughan. 1988. ADP-ribosylation of guanyl nucleotide-binding proteins by bacterial toxins. *Adv. Enzymol.* 61:303–379.
Murayama, T., and M. Ui. 1985. Receptor-mediated inhibition of adenylate cyclase and stimulation of arachidonic acid release in 3T3 fibroblasts: selective susceptibility to islet activating protein, pertussis toxin. *J. Biol. Chem.* 260:7226–7233.
Murayama, T., and M. Ui. 1987. Possible involvement of a GTP-binding protein, the substrate of islet-activating protein, in receptor-mediated signaling responsible for cell proliferation. *J. Biol. Chem.* 262:12463–12467.
Murayama, T., T. Katada, and M. Ui. 1983. Guanine nucleotide activation and inhibition of adenylate cyclase as modified by islet-activating protein, pertussis toxin, in mouse 3T3 fibroblasts. *Arch. Biochem. Biophys.* 221:381–390.
Neville, D. M., and T. H. Hudson. 1986. Transmembrane transport of diphtheria toxin, related toxins and colicins. *Annu. Rev. Biochem.* 55:195–224.
Olsnes, S., and K. Sandvig. 1988. How protein toxins enter and kill cells. *In* Immunotoxins. A. E. Frankel, editor. Kluwer Academic Publishers, Norwell, MA. 39–73.
Pfeuffer, T., and E. J. M. Helmreich. 1988. Structural and functional relationships of guanosine triphosphate binding proteins. *Curr. Top. Cell. Regul.* 29:129–208.
Plagemann, P. G. W., and R. W. Wohlheuter. 1980. Permeation of nucleosides, nucleic acid bases, and nucleotides in animal cells. *Curr. Top. Membr. Transp.* 14:225–330.
Plevin, R., S. Palmer, S. D. Gardner, and M. J. O. Wakelam. 1990. Regulation of bombesin-stimulated inositol 1,4,5-trisphosphate generation in Swiss 3T3 fibroblasts by a guanine-nucleotide-binding protein. *Biochem. J.* 268:605–610.
Quershi, S. A., K. Alexandropoulos, L. K. Joseph, R. Spangler, and D. A. Foster. 1991. Cholera toxin induces expression of the immediate-early response gene JE via a cyclic AMP-independent signaling pathway. *Mol. Cell. Biol.* 11:102–107.
Reuner, K. H., P. Presek, C. B. Boschek, and K. Aktories. 1987. Botulinum C2 toxin ADP-ribosylates actin and disorganizes the microfilament network in intact cells. *Eur. J. Cell Biol.* 43:134–140.
Ribeiro-Neto, F. A. P., R. Mattera, J. D. Hildebrandt, J. Codina, J. B. Field, L. Birnbaumer, and J. D. Sekura. 1985. ADP-ribosylation of membrane components by pertussis and cholera toxin. *Methods Enzymol.* 109:566–572.
Rozengurt, E. 1986. Early signals in the mitogenic response. *Science (Wash. DC).* 234:161–166.
Rozengurt, E., A. Legg, G. Strang, and N. Courtney-Luck. 1981. Cyclic AMP: a mitogenic signal for Swiss 3T3 cells. *Proc. Natl. Acad. Sci. USA.* 78:4392–4396.
Rozengurt, E., M. Murray, I. Zachary, and M. Collins. 1987. Protein kinase C activation enhances cyclic AMP accumulation in Swiss 3T3 cells: Inhibition by pertussis toxin. *Proc. Natl. Acad. Sci. USA.* 84:2282–2286.
Rozengurt, E., T. Higgins, N. Chanter, A. J. Lax, and J. M. Staddon. 1990. *Pasteurella multocida* toxin: Potent mitogen for cultured fibroblasts. *Proc. Natl. Acad. Sci. USA.* 87:123–127.
Shenker, A., P. Goldsmith, C. G. Unson, and A. M. Spiegel. 1991. The G protein coupled to the thromoxane A_2 receptor in human platelets is a member of the novel G_q family. *J. Biol. Chem.* 266:9309–9313.
Sinnett-Smith, J., W. Lehmann, and E. Rozengurt. 1990. Bombesin receptor in membranes from Swiss 3T3 cells: binding characteristics, affinity labelling and modulation by guanine nucleotides. *Biochem. J.* 265:485–493.
Smrcka, A. V., J. R. Hepler, K. O. Brown, and P. C. Sternweiss. 1991. Regulation of polyphosphoinositide-specific phospholipase C activity by purified G_q. *Science (Wash. DC).* 251:804–807.
Staddon, J. M., N. Chanter, A. J. Lax, T. E. Higgins, and E. Rozengurt. 1990. *Pasteurella multocida* toxin, a potent mitogen, stimulates protein kinase C-dependent and -independent protein phosphorylation in Swiss 3T3 cells. *J. Biol. Chem.* 265:11841–11848.
Staddon, J. M., C. J. Barker, A. C. Murphy, N. Chanter, A. J. Lax, R. H. Michell, and E. Rozengurt. 1991. *Pasteurella multocida* toxin, a potent mitogen, increases inositol 1,4,5-trisphosphate and mobilizes Ca^{2+} in Swiss 3T3 cells. *J. Biol. Chem.* 266:4840–4847.
Tamura, M., K. Nogimori, S. Murai, M. Yajima, K. Ito, T. Katada, and M. Ui. 1982. Subunit structure of islet-activating protein, pertussis toxin, in conformity with the A-B model. *Biochemistry.* 21:5516–5522.
Tanuma, S., and G. S. Johnson. 1983. ADP-ribosylation of nonhistone high mobility group proteins in intact cells. *J. Biol. Chem.* 258:4067–4070.
Taylor, C. W., D. M. Blakely, A. N. Corps, M. J. Berridge, and K. D. Brown. 1988. Effects of pertussis toxin on growth factor-stimulated inositol phosphate formation and DNA synthesis in Swiss 3T3 cells. *Biochem. J.* 249: 917–920.
Taylor, S. J., H. Z. Chae, S. G. Rhee, and J. H. Exton. 1991. Activation of β1 isozyme of phospholipase C by α subunits of the G_q class of G proteins. *Nature (Lond.).* 350:516–518.
Todaro, G. J., and H. Green. 1963. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *J. Cell. Biol.* 17:299–313.
Ueda, K., A. Omachi, M. Kawaichi, and O. Hayaishi. 1975. Natural occurrence of poly(ADP-ribosyl) histones in rat liver. *Proc. Natl. Acad. Sci. USA.* 72:205–209.
Ui, M. 1990. Pertussis toxin as a valuable probe for G protein involvement in signal transduction. *In* ADP-ribosylating Toxins and G Proteins: Insights into Signal Transduction. J. Moss and M. Vaughan, editors. American Society for Microbiology, Washington D.C. 45–77.
Wick, M. J., and B. H. Iglewski. 1990. *Pseudomonas aeruginosa* exotoxin A. *In* ADP-ribosylating Toxins and G Proteins: Insights into Signal Transduction. J. Moss and M. Vaughan, editors. American Society for Microbiology, Washington D.C. 31–43.
Zachary, I., J. Millar, E. Nanberg, T. Higgins, and E. Rozengurt. 1987. Inhibition of bombesin-induced mitogenesis by pertussis toxin: dissociation from the phospholipase C pathway. *Biochem. Biophys. Res. Commun.* 146:456–463.