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

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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 [2-3H]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, [3H]-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 [3H]ADP-ribose from the labeled protein by mercuric acetate. Cholera toxin catalyzed the [3H]-labeling of a 46-kD protein in the [2-3H]adenine-labeled cells. Pretreatment of the cells with pertussis toxin before the labeling of NAD+ with [2-3H]adenine blocked [2-3H]ADP-ribosylation catalyzed by pertussis toxin, but not that by cholera toxin. Thus, labeling with [2-3H]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.
riboylation 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

[2-3H]adenine (20 Ci/mmol), [14C]methylated molecular weight markers, cyclic AMP ([3H]) assay kits and [adenylate-32P]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). Recombiant P. multocida toxin was prepared as described previously (Lex 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, 103 cells were subcultured in 35-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% CO2, the cell cultures were confluent and arrested in the G1/G0 phase of the cell cycle (Dicker and Rozengurt, 1980).

ADP-Ribosylation of Membranes

Swiss 3T3 cell membranes were prepared (Simnett-Smith et al., 1990) and suspended at 3 mg of protein/ml in 50 mM Hepes/Na, 1 mM EGTA, 5 mM MgCl2, 1 mg/ml bacitracin, 1 mg/ml soybean trypsin inhibitor, 10 µg/ml aprotinin and 50 µM PMSE, 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 7.3, comprising 0.33 M Tris/HC1, 3.3 mM thymidine, 16.7 mM DTT, and 167 µM [32P]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, the precipitated protein was sedimented by centrifugation. The pellet was dissolved in SDS-sample buffer (Laemmli, 1970) comprising: 62.5 mM Tris/HC1, 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 [3H]Adenine

All incubations were at 37°C under a humidified atmosphere of 10% CO2, 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 [3H]adenine. To preserve the pH of the incubation medium, additions were rapidly made to the dishes under an atmosphere of CO2.

For the analysis of the incorporation of [3H]adenine into acid-soluble material, the incubation 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 (0.075 ml) of the upper phase containing the neutralized extract was diluted to 5 ml with 0.3 M NH4COOH, pH 9. Dibutyryl cAMP was then added at the following concentrations: 0.25 µM NAD+ and ADP-ribose (Alvarez-Gonzalez et al., 1983), was used to separate these metabolites from unincorporated [3H]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 NH4COOH, 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 NH4COOH, pH 9, which was then combined with the initial effluent to give the adenine nucleotide fraction. To remove unincorporated [3H]adenine, the column was washed three times with 5 ml of 0.25 M NH4COOH, pH 9. NAD+ and ADP-ribose were then eluted from the column with 10 ml of H2O.

The samples were lyophilized and then dissolved in 10 ml of buffer A comprising 10 mM Hapes/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 Na2SO4 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 Na2SO4 in buffer A was increased at a rate of 5 mM/min to elute ATP, 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 [3H]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 H2O and then incubated in H2O 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 [3H]-labeled protein derived from the equivalent of the cultures of five 35-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 rinsed for two further 5-min periods in water. The gel was then homogenized in six times the gel volume of 50 mM NH4HCO3 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 NH4HCO3, 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 H2O) 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 H2O 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 NH4OH. The sample was diluted to 10 ml with 0.25 M NH4COOH, pH 9, and then applied to 1.5 ml of dihydroyxynoborol-Bio-Rex 70 equilibrated with 0.25 M NH4COOH, pH 9 in a Bio-Rad Econocolumn (Bio-Rad Laboratories). The column was washed with 10 ml of 0.25 M NH4COOH, pH 9, and bound material was then eluted with 5 ml of H2O. 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 Na2SO4 in buffer A. Fractions (0.33 ml) were collected and then counted with 5 ml of Picofluor 15.

Inositol Phosphate Release and DNA Synthesis Assays

Phospholipids were labeled with [2-3H]inositol and after appropriate incubations the total cellular [3H]inositol phosphate content was determined by ion-exchange chromatography, as described previously (Staddon et al., 1991). DNA synthesis was assayed by [3H]thymidine incorporation (Rosenburg 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 [32P]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 Gβ, 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.

Table 1. Analysis of Acid-soluble Radiolabeled Material Formed in Swiss 3T3 Cells Incubated with [2-3H]Adenine

| Metabolite | cpm/culture |
|------------|-------------|
| NAD+       | 1.2 ± 0.2 x 10^6 |
| ADP-ribose | 5.2 ± 1.1 x 10^6 |
| AMP        | 1.5 ± 0.8 x 10^6 |
| ATP        | 1.9 ± 0.3 x 10^6 |
| ADP        | 3.0 ± 0.3 x 10^6 |

Confluent and quiescent cultures of Swiss 3T3 cells were incubated with 50 μCi/ml of [3H]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 Na2SO4 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-^3H]adenine labeling of Swiss 3T3 cells: the effect of pertussis toxin. Confluent and quiescent cultures of Swiss 3T3 cells were incubated with 50 ^Ci/ml of [^3H]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 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-Gonzalez 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, [^3H]adenine 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.

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Figure 4. Hg²⁺ releases [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 [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 [2-3H]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 Gₛ 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 Gₛ (Pfeuffer and Helmreich, 1988; Birnbaumer et al., 1990). Similarly, in cells labeled with [3H]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 labeling in the [3H]labeled protein was resistant to TCA at 95°C and the protein comigrated with in vitro ADP-ribosylated αₛ (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 E₁, that do not act by ADP-ribosylating αₛ, 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 [3H]adenine. [3H]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 [3H]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 αₛ in both the control cells and the pertussis toxin–pretreated cells (Fig. 7, see lanes 5 and 3, 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 αₛ in control and pertussis toxin–pretreated cells. These data are consistent with the irreversible-
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 [3H]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).

Is P. multocida Toxin an ADPRibosyltransferase?
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 G4 (Shenker et al., 1991; Smirka 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 G4 that usually couples the bombesin receptor to phospholipase C. Initially, attempts 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 [2-3H]-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...
of P. multocida toxin with samples taken every 30 min up
by tenfold did not detect ADP-ribosylation (results not
shown). Increasing the time of fluorography 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, in either control or P. multocida toxin-pretreated cultures (Fig. 8 C). As another positive control, parallel cultures of cells were labeled with [2-3H]adenine instead of [2-3H]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 Gα, 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,
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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 [2-3H]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-lysatess 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 [2-3H]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. [3H]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 [3H]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.
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 [3H]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 Uj, 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\textsubscript{\alpha}. However, cAMP-independent actions of cholera toxin have been reported in which the role of ADP-ribosylation is not clear (see, e.g., Quereshi et al., 1991). Indeed, it has been shown that cholera toxin ADP-ribosylates pertussis toxin substrates in vitro (see Birnbaumer et al., 1990; Uj, 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 40-kD substrate, presumably \alpha\textsubscript{\text{II}}, 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-ribosyltransferases.

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; Olness 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 \textit{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 \textit{P. multocida} toxin of \textit{G\alpha} 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 \textit{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 \textit{G\alpha} 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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