Interconversion of GRP78/BiP
A NOVEL EVENT IN THE ACTION OF PASTEURRELLA MULTOCIDA TOXIN, BOMBESIN, AND PLATELET-DERIVED GROWTH FACTOR*

(Received for publication, May 22, 1992)

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Incubation of Swiss 3T3 cells with [2-3H]adenine, as in other cell types, reveals the ADP-ribosylation of GRP78 (the 78-kDa glucose-regulated protein, also known as BiP, the immunoglobulin heavy chain-binding protein), a resident endoplasmic reticulum protein that assists in the processing of proteins destined for secretion or cell surface expression. Here we show that Pasteurella multocida toxin, a potent growth factor for cultured fibroblasts, decreases the ADP-ribosylation of GRP78/BiP to 16 ± 6% of the control value (n = 25). The action of the toxin occurred after a lag period, was blocked by lysosomotrophic agents, and potentiated by increased incubation time (ED50 4 ng/ml and 1 ng/ml in 4 and 8 h, respectively), thus indicating that the toxin enters the cells to act. Bombesin and platelet-derived growth factor (PDGF) similarly decreased the ADP-ribosylation of GRP78/BiP (ED50 0.5 nM and 2.5 ng/ml, respectively) but acted more rapidly than the toxin. Signaling pathways activated by the toxin, bombesin, and PDGF had effects on the ADP-ribosylation of GRP78/BiP. Thus, activation of protein kinase C alone by phorbol 12,13-dibutyrate was partially effective, and down-regulation of protein kinase C attenuated but did not block the action of the toxin, bombesin, and PDGF. Agents that mobilize Ca2+ from the endoplasmic reticulum (A23187, ionomycin, and thapsigargin) caused a decrease in the ADP-ribosylation of GRP78/BiP that was similar in magnitude to that achieved by the toxin, bombesin, and PDGF, implicating a role for inositol 1,4,5-trisphosphate-mediated Ca2+ mobilization in the action of the mitogenic agents. The Ca2+ dependence decrease in the ADP-ribosylation of GRP78/BiP may represent its conversion from an inactive to an active state.

It is increasingly accepted that most stress proteins act as molecular chaperones, assisting in the folding, assembly, and disassembly of proteins in the cell (for review see Gething and Sambrook, 1992). GRP78/BiP is an abundant and highly conserved member of the stress protein family that resides within the lumen of the ER (Munro and Pelham, 1986, 1987; Pelham, 1989; Gething and Sambrook, 1992). GRP78/BiP is believed to bind transiently to nascent proteins as they are translocated into the ER (Vogel et al., 1990), facilitating the attainment of the correct conformation of these proteins destined for surface expression or secretion (Pelham, 1989; Flynn et al., 1989, 1991; Welch et al., 1991; Gething and Sambrook, 1992). Release of GRP78/BiP from bound protein is achieved by the hydrolysis of ATP (Munro and Pelham, 1986), which can be transported into the ER (Clairmont et al., 1992).

It is known that GRP78/BiP is post-translationally modified by ADP-ribosylation (Carlsson and Lazarides, 1983; Munro and Pelham, 1986; Hendershot et al., 1988; Leno and Ledford, 1989) and phosphorylation (Hendershot et al., 1988). A variety of treatments can increase the ADP-ribosylation of GRP78/BiP, including cycloheximide addition or amino acid starvation (Carlsson and Lazarides, 1983; Leno and Ledford, 1989, 1990; Freiden et al., 1992). Conversely, treatments which cause an increase in underglycosylated proteins can cause a decrease in the ADP-ribosylation of GRP78/BiP (Carlsson and Lazarides, 1983; Freiden et al., 1992). However, it is not known if ADP-ribosylation of GRP78/BiP can be regulated by physiological signals rather than stressful stimuli.

Quiescent Swiss 3T3 cells, a useful model system to study cell regulation (Rozengurt, 1986), can be induced to recommence cell proliferation by a variety of factors, including bombesin (Rozengurt and Simnett-Smith, 1983), PDGF (Ross et al., 1986) and Pasteurella multocida toxin (Rozengurt et al., 1990b). Bombesin (see Rozengurt et al., 1990a) and PDGF (see Cantley et al., 1991) act by binding to their cognate receptors on the outer surface of the cell, whereas P. multocida toxin enters the cells to act (Rozengurt et al., 1990b). By different mechanisms, all three agents stimulate the phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate, resulting in Ca2+ mobilization and protein kinase C activation. The occupied bombesin receptor (Zachary and Rozengurt, 1987; Battey et al., 1990) couples via a G protein to activate phospholipase C (Erusalimsky et al., 1988; Coffer et al., 1990; for review see Rozengurt et al., 1990a). The tyrosine kinase activity of the activated PDGF receptor results in the tyrosine phosphorylation and activation of phospholipase C-γ1 (Meisenhelder et al., 1989; Kim et al., 1991). The stimulation of phospholipase C by P. multocida toxin requires the cellular entry and processing of the toxin (Rozengurt et al., 1990b; Staddon et al., 1990; 1991a).

In the present study we report that P. multocida toxin, bombesin, and PDGF induce a dramatic decrease in the ADP-ribosylation of GRP78/BiP. This decrease is an early event in the action of these mitogenic agents and implies communication among signaling events, protein processing, and mitogenesis.

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The abbreviations used are: GRP78, 78-kDa glucose-regulated protein; BiP, immunoglobulin heavy chain-binding protein; ER, endoplasmic reticulum; PDGF, platelet-derived growth factor; mAb, monoclonal antibody; DMEM, Dulbecco’s modified Eagle medium; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

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**EXPERIMENTAL PROCEDURES**

**Materials**—[2-3H]Adenine (15–25 Ci/mmol), [3H]adenosine (20–25 Ci/mmol), L-[35S]methionine (>1,000 Ci/mmol), 45CaCl2 (10–40 mCi/mg) and PDEG (c-stim recombinant) were from Amersham Corp. Ionomycin and A23187 were from Calbiochem. Bombesin, phorbol 12,13-dibutyrate, thapsigargin, and [Arg8]vasopressin were from Sigma. Fura2/AM was(191,981,1255,1071)

J. Council, Compton, U. K.) and was prepared as described previously (Chanter et al., 1986; Lux and Chanter, 1990). The rat anti-BiP mAb used in the immunoprecipitation (Bole et al., 1986) was from Dr. L. J. Hendershot (St. Jude Children’s Research Hospital, Memphis TN). For the Western blot analysis, a rat mAb against the C terminus of BiP was used and was a gift from Dr. L. J. Hendershot, Dr. I. Haas (Institut für Genetik, Köln, Germany) and Dr. J. Kearney (University of Alabama at Birmingham, AL).

**Cell Culture**—Stock cultures of Swiss 3T3 cells (Todaro and Green, 1963) were propagated as described (Dicker and Rozengurt, 1980). For experimental purposes, 10<sup>4</sup> cells were subcultured in 35-mm Nunc dishes in 2.5 ml of DMEM containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. After 6 days at 37 °C under a humidified atmosphere of 10% CO<sub>2</sub>, 90% air, the cells were confluent and arrested in the G<sub>1</sub>/G<sub>0</sub> phase of the cell cycle (Dicker and Rozengurt, 1980). Cultures of Rat-1 cells were propagated as described (Chanter et al., 1986).

**Metabolic Labeling and Sample Preparation**—The cultures were washed twice with 2 ml of DMEM:Weymouth’s medium (1:1) and then incubated in 1 ml of this medium containing 50–100 μCi of lyophilized [3H]adenine, as indicated. The cultures were labeled for 16 h. Addition of growth factors then were made to the cultures and the incubations continued at 37 °C. After the required times the incubation medium was rapidly replaced with 125 μl of Laemmli sample buffer (Laemmli, 1970). The lysed cells were scraped from the dishes and immediately heated at 100 °C for 4 min. For two-dimensional gel electrophoresis, the cells were similarly extracted but into 100 μl of buffer comprised of 0.5% (w/v) SDS, 20 mM Tris/HCl, 1 mM EDTA, and 0.1% (v/v) β-mercaptoethanol, pH 8.0. After heating, the samples were rapidly frozen, lyophilized, and then dissolved in 100 μl of buffer comprised of 9.5 M urea, 4% (v/v) Nonidet P-40, 2% (w/v) Ampholines, and 0.1 M dithiothreitol.

For [35S]methionine labeling, the cultures were washed twice with 2 ml of ice-cold DMEM containing 1 mg/ml L-methionine and then incubated with 1 ml of this medium containing 50 μCi of L-[35S]methionine. After 1 h of labeling, the cultures were washed twice with 2 ml of ice-cold PBS (0.14 M NaCl, 5 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>), and the sample preparation was as described above for two-dimensional gel electrophoresis.

**Immunoprecipitation**—All procedures were at 4 °C. The radiolabeled cultures were washed twice with 2 ml of PBS and then solubilized with 0.5 ml of 1% (v/v) Nonidet P-40 in 0.15 M NaCl, 50 mM Tris/HCl, pH 7.4. After 10 min, the lysates were scraped from the dishes and then centrifuged at 100,000 × g for 15 min. The supernatant was incubated for 2 h in the absence or presence of the anti-BiP mAb, and the immune complex was then collected using protein A agarose. After 1 h, the beads were washed three times with buffer comprised of 1% (w/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (v/v) SDS, 0.5 M NaCl, 50 mM Tris/HCl, pH 7.4, and then once with PBS. Immunoprecipitated protein was solubilized by the addition of Laemmli sample buffer followed by heating for 5 min at 100 °C. The solubilized proteins were analyzed by SDS-PAGE.

**Gel Electrophoresis**—SDS-PAGE (12.5% acrylamide) and two-dimensional gel electrophoresis (employing pH 5-7 Ampholines in the first dimension) were as described previously (Laemmli, 1970; O’Farrell, 1975). Treatment of the gels with 7% trichloroacetic acid at 0.25% (v/v) SDS, 20 mM Tris/HCl, 1 mM EDTA, and 0.1% (v/v) β-mercaptoethanol, pH 8.0. After heating, the gels were scanned to quantitate labeled protein in terms of peak height (see Staddon et al., 1991b).

**Western Blot Analysis**—The samples for immunoblotting were subjected to SDS-PAGE using a 12.5% gel which was then equilibrated in buffer comprised of 39 mM glycerine, 48 mM Tris, 0.05% (w/v) SDS, and 20% (v/v) CH<sub>3</sub>OH. Transfer to nitrocellulose (Hybond-C Extra, Amersham) was at 0.8 mA/cm<sup>2</sup> for 1 h. The blots were blocked by a 2-h incubation in PBS containing 5% (w/v) dried milk and 0.1% (v/v) Tween 20. The blots were then probed with a rat mAb directed against the C terminus of GRP78/BiP in PBS containing 0.1% Tween 20. After 1 h at 22 °C the filters were washed three times for 20 min in PBS containing 0.1% Tween 20. The filters were then incubated for 1 h at 22 °C with a 1:5,000 dilution of rabbit anti-rat IgG coupled to horseradish peroxidase (ICN immunobiologicals) in PBS containing 0.1% Tween 20. After washing the filters, immunoreactive bands were detected by enhanced chemiluminescence (ECL, Amersham).

**Ca<sup>2+</sup> Mobilization**—Equilibration of the cells with 45CaCl<sub>2</sub> and the determination of their 45CaCl<sub>2</sub> content after rapid and exhaustive washing with medium containing EGTA was as described by Lopez-Rivas and Rozengurt (1984). The concentration of cytoplasmic free Ca<sup>2+</sup> ([Ca<sup>2+</sup>]i) was determined from the fluorescence of cells loaded with Fura2, as described by Millar and Rozengurt (1990).

**PHATP Determination**—As described above, cells were labeled with [3H]adenine, and after the required additions the extracts were chromatographically analyzed for [3H]ATP, as described previously (Staddon et al., 1991b).

**RESULTS**

**P. multocida Toxin Decreases the ADP-ribosylation of GRP78/BiP in Intact Swiss 3T3 Cells**—Confluent and quiescent cultures of Swiss 3T3 cells were metabolically labeled with [3H]adenine, lysed, and analyzed by SDS-PAGE. Fluorography revealed the incorporation of [3H]adenine into many bands (Fig. 1A). However, depurination of RNA by incubating the gels with 7% trichloroacetic acid at 95 °C for 45 min resulted in a single labeled band that migrated as a protein of approximately 70 kDa (Fig. 1B). RNase A treatment of the samples prior to electrophoresis has the same effect as the acid incubation (results not shown). The labeling of the 70-kDa protein by [3H]adenine occurred slowly, achieving a steady state after 12 h (Fig. 1C).

**P. multocida toxin had a striking effect on the [3H]adenine labeling of the 70-kDa band**: after a 4-h incubation at 29 ng/ml the labeling was greatly reduced (Fig. 1, A and B). In 23 independent experiments, the labeling was reduced to 16 ± 5%.
6% (mean ± S.E., range 6–23%) of the control value. A similar effect of the toxin was obtained with Swiss 3T3 cells labeled with [2-3H]adenine instead of [2-3H]adenosine (results not shown). Under identical conditions, cholera and pertussis toxins stimulated the ADP-ribosylation of their respective 46- and 40-kDa G protein α subunit substrates (Fig. 1, A and B), as described previously (Staddon et al., 1991b). However, these two ADP-ribosyl transferases had no effect on the labeling of the 70-kDa band. We decided to attempt to identify the 70-kDa band and investigate the significance of the decrease in its labeling in the action of P. multocida toxin.

As the incubation of other cell types with [3H]adenine results in the incorporation of label into GRP78 (Carlsson and Lazarides, 1983; Munro and Pelham, 1986; Hendershot et al., 1988; Leno and Ledford, 1989, 1990), we determined whether the [3H]adenine-labeled 70-kDa band in Swiss 3T3 cells could be identified as GRP78.

Two-dimensional gel electrophoresis of [2-3H]adenine-labeled material from control cells revealed a focused spot with an apparent molecular mass of 70 kDa and a pl of approximately 5 (Fig. 2). Treatment of the cells with P. multocida toxin resulted in a large decrease in labeling of the focused material (Fig. 2), indicating that it was identical to that in the band observed in the analysis by one-dimensional gel electrophoresis (Fig. 1, A and B). The adenine-labeled spot comigrated with a [35S]methionine-labeled protein (Fig. 2) with electrophoretic characteristics of GRP78 (Welch et al., 1983; Munro and Pelham, 1986; Hendershot et al., 1988). The synthesis of GRP78 is known to result in the incorporation of label into GRP78/BiP (Carlsson and Lazarides, 1983; Munro and Pelham, 1986; Hendershot et al., 1988, 1990), we determined whether the [3H]adenine-labeled 70-kDa band in Swiss 3T3 cells could be identified as GRP78.

As the incubation of other cell types with [3H]adenine results in the incorporation of label into GRP78 (Carlsson and Lazarides, 1983; Munro and Pelham, 1986; Hendershot et al., 1988) and the incorporation of adenine was blocked by nicotinamide (Ledford and Jacobs, 1986). Similarly, in Swiss 3T3 cells labeled with [2-3H]adenine for 16 h a subsequent incubation for 2 h in the presence of 200 μM nicotinamide caused the loss of label from GRP78/BiP (results not shown). These observations are consistent with the incorporation of adenine into GRP78/BiP in the form of ADP-ribose.

P. multocida Toxin Enters the Cells to Act—P. multocida toxin is known to enter the cells to stimulate both DNA synthesis (Rozengurt et al., 1990b) and polyphosphoinositide hydrolysis (Staddon et al., 1990, 1991a). Several lines of evidence indicate that P. multocida toxin likewise enters the cells to elicit the decrease in ADP-ribopectase of GRP78/BiP. (i) The decrease in labeling occurred after a lag period of 1–1.5 h (Fig. 3A), similar to that for the activation of

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**Fig. 2. Identification of the [2-3H]adenine-labeled protein as GRP78/BiP.** Confluent and quiescent cultures of Swiss 3T3 cells were labeled with 100 μCi/ml [2-3H]adenine for 20 h and treated in the absence (C) or presence of 20 ng/ml of recombinant P. multocida toxin (rPMT) for 4 h prior to extraction. The extracts were subjected to two-dimensional gel electrophoresis, pl increasing to the right, followed by fluorography. The arrows indicate the [3H]adenine-labeled spot. Other cultures were incubated in DMEM for 16 h in either the presence of glucose (Cont.), the absence of glucose (–Glc), the presence of 10 mM deoxyglucose (DOG), or the presence of 0.5 μg/ml tunicamycin (Tun). They were then labeled with [35S]methionine, extracted, and analyzed by two-dimensional gel electrophoresis followed by fluorography. The arrow indicates the [35S]methionine-labeled protein that comigrates with the [2-3H]adenine-labeled spot. The depicted reference proteins are actin (A, 42 kDa) and vimentin (V, 58 kDa). Extracts from cells labeled with [2-3H]adenine as above were immunoprecipitated in the absence (–) or presence (+) of the monoclonal antibody raised against GRP78/BiP. Immunoprecipitates were analyzed by one-dimensional SDS-PAGE and fluorography. The arrow indicates the migration of GRP78/BiP.

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**Fig. 3. Characteristics of the action of P. multocida toxin.** Confluent and quiescent cultures of Swiss 3T3 cells were labeled with [2-3H]adenine for 20 h. The labeling of GRP78/BiP was detected by SDS-PAGE followed by trichloroacetic acid treatment and fluorography and quantified by densitometry. In panel A the cells were treated with 20 ng/ml of P. multocida toxin for various times prior to extraction. In panel B the cells were incubated with various concentrations of toxin for 4 (closed circles) or 8 h (open circles). In panel C the cells were incubated in the absence (C) or presence of either n-methylamine/HCl, pH 7.4 (M, 10 mM), ammonium chloride (N, 10 mM), or chloroquine (Q, 100 μM) in the absence (open bars) or presence (closed bars) of 20 ng/ml toxin for 4 h. The values shown are the means ± S.D. of the values obtained from five replicate incubations. In panel D the cells were incubated in the presence of 20 ng/ml P. multocida toxin for 4 h, and methyamine (10 mM final concentration) was added either with the toxin or at various periods of time after toxin addition.
polyphosphoinositide hydrolysis (Staddon et al., 1990, 1991a).

(ii) The potency of the toxin increased with the time of incubation (EDso 1 and 4 ng/ml for 8- and 4-h incubations, respectively; Fig. 3B) and was similar to that required for the stimulation of both DNA synthesis (Rozenburg et al., 1990b) and polyphosphoinositide hydrolysis (Staddon et al., 1990, 1991a).

(iii) The lysosomotropic agents methylene, ammonium chloride, and chloroquine completely blocked the ability of the toxin to decrease the labeling of GRP78/BiP (Fig. 3C).

(iv) The inhibitory action of methylene was dependent upon the time of its addition: when added at increasing times up to 1.5 h after the toxin it became progressively less inhibitory, and when added 1.5 h after the toxin it had no effect (Fig. 3D). Thus, the toxin appears to enter the cells via an endosomal/lysosomal pathway where it is processed and then released into the cytosol in an active form.

The decrease in the [3H]adenine labeling of GRP78/BiP by treatment with P. multocida toxin is not confined to Swiss 3T3 cells. Addition of the toxin to quiescent cultures of Rat-1 cells, which are also very sensitive to the growth promoting effects of P. multocida toxin (Higgins et al., 1992), decreased the labeling of GRP78/BiP by 80% after 4 h of incubation (Fig. 4). These effects were not restricted to cell lines. Treatment with the toxin (20 ng/ml) also decreased the labeling of GRP78/BiP in tertiary cultures of mouse embryo fibroblasts (40% after a 4-h incubation).

**PDGF and Bombesin Similarly Decrease the ADP-ribosylation of GRP78/BiP**—Next we examined whether the decrease in ADP-ribosylation of GRP78/BiP could be induced by other potent growth-promoting factors, such as bombesin and PDGF, which bind to specific cell surface receptors to activate phospholipase C. Bombesin and PDGF caused a large decrease in the ADP-ribosylation of GRP78/BiP (Fig. 5, A and B). This decrease occurred much more rapidly than that in response to the toxin: it was significant after 15 min and maximal after 30–60 min. Furthermore, the magnitude of the decrease in ADP-ribosylation of GRP78/BiP in response to the toxin, bombesin, and PDGF was comparable. The half-maximal effects of bombesin and PDGF were achieved at 0.5 nM and 2.5 ng/ml, respectively, and maximal effects were achieved at 3–10 nM and 10–25 ng/ml (Fig. 5, C and D), concentrations similar to those required for the stimulation of polyphosphoinositide hydrolysis and cell proliferation (Mendoza et al., 1986; Zachary et al., 1986; Mehmet et al., 1990).

The lysosomotropic agent methylene, which completely blocked the ability of P. multocida toxin to decrease the ADP-ribosylation of GRP78/BiP (Fig. 3C), did not block the effect of bombesin and PDGF (Fig. 5, A and B). The selectivity of the action of methylene clearly indicates that its inhibition of the action of P. multocida toxin reflects the inhibition of the activation of the toxin rather than an inhibition of the mechanisms that lead to a decrease in ADP-ribosylation of GRP78/BiP.

Further experiments have shown that vasopressin (20 nM, 1-h incubation), which also activates polyphosphoinositide hydrolysis but not as potently as P. multocida toxin, bombesin, and PDGF (Nanberg and Rozenburg, 1988; Issandou and Rozenburg, 1990) decreased the ADP-ribosylation of GRP78/BiP to 57 ± 11% of the control value (mean ± S.D. of five independent experiments). Whereas P. multocida toxin, bombesin, and PDGF are mitogenic when added alone to Swiss 3T3 cells, vasopressin is only mitogenic in the presence of other factors, e.g. insulin (Dicker and Rozenburg, 1980; Rozenburg and Sinnett-Smith, 1983).

**Evidence for Interconversion of GRP78/BiP**—The apparent decrease in the ADP-ribosylation of GRP78/BiP may reflect either the removal of label from a fixed amount of protein (i.e. interconversion between ADP-ribosylated and non-ADP-ribosylated forms) and/or secretion/degradation of the labeled protein. The following observations indicate that the decrease in ADP-ribosylation of GRP78/BiP is caused by interconversion of the protein. After treatment of the cells with P. multocida toxin, bombesin, and PDGF we failed to detect ADP-ribosylated GRP78/BiP in the culture medium (results not shown). As in other cell types (Carlsson and Lazarides, 1983; Ledford and Jacobs, 1986; Freiden et al., 1992), the addition of cycloheximide caused an increase in the ADP-ribosylation of GRP78/BiP in Swiss 3T3 cells (Table 1).
Confluent and quiescent cultures of Swiss 3T3 cells were labeled with 50 μCi/ml [2-3H]adenine for 20 h. The labeling of GRP78/BiP was detected by SDS-PAGE followed by trichloroacetic acid treatment and fluorography and quantified by densitometry. The values shown are expressed relative to control and are the means ± S.E. of the number of replicates indicated in parentheses. Prior to extraction, the cultures were incubated with 10 nM bombesin or 25 ng/ml PDGF for 1 or 2 h. Cycloheximide (100 μM) was added for 1 h to control cultures or cultures that had been preincubated with bombesin or PDGF for 1 h.

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<thead>
<tr>
<th>GRP78/BiP labeling</th>
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<tr>
<td>Control</td>
<td>100 ± 21 (8)</td>
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<tr>
<td>Bombesin 1 h</td>
<td>17 ± 3 (4)</td>
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<tr>
<td>2 h</td>
<td>8 ± 1 (4)</td>
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<tr>
<td>PDGF 1 h</td>
<td>19 ± 8 (4)</td>
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<tr>
<td>2 h</td>
<td>12 ± 2 (4)</td>
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<tr>
<td>Cycloheximide alone</td>
<td>225 ± 28 (8)</td>
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<tr>
<td>Cycloheximide + bombesin</td>
<td>185 ± 32 (4)</td>
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<tr>
<td>Cycloheximide + PDGF</td>
<td>206 ± 28 (4)</td>
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Significantly, in either bombesin- or PDGF-treated cultures the subsequent addition of cycloheximide caused an increase in the ADP-ribosylation of GRP78/BiP (Fig. 6, upper panel). Further aliquots of the same extract were electrophoresed in parallel and then electrotransferred to nitrocellulose filters. The filters were probed in the absence (results not shown) or presence (lower panel) of antibody raised to the C terminus of GRP78/BiP. Immunoreactive bands were detected by anti-rat IgG coupled to horseradish peroxidase and ECL (Amersham). The arrow in the lower panel indicates the primary antibody-dependent reactivity of a band that comigrated with the [3H]ADP-ribosylated band in the upper panel.

Fig. 6. Effect of P. multocida toxin, bombesin, and PDGF on the ADP-ribosylation and cellular content of GRP78/BiP. Confluent and quiescent cultures of Swiss 3T3 cells were labeled with [2-3H]adenine for 20 h. Prior to extraction, the cells were incubated with either 10 nM bombesin for 1 h, 25 ng/ml PDGF for 1 h, or 20 ng/ml P. multocida toxin for 4 h. Aliquots of the extracts were analyzed by SDS-PAGE followed by trichloroacetic acid treatment and fluorography, revealing the decrease in ADP-ribosylation of GRP78/BiP (upper panel). Further aliquots of the same extract were electrophoresed in parallel and then electrotransferred to nitrocellulose filters. The filters were probed in the absence (results not shown) or presence (lower panel) of antibody raised to the C terminus of GRP78/BiP. Immunoreactive bands were detected by anti-rat IgG coupled to horseradish peroxidase and ECL (Amersham). The arrow in the lower panel indicates the primary antibody-dependent reactivity of a band that comigrated with the [3H]ADP-ribosylated band in the upper panel.

To test directly the hypothesis that mitogens promote an interconversion from ADP-ribosyl-GRP78/BiP to the unmodified state, cultures were labeled with [3H]adenine and treated in the absence or presence of P. multocida toxin, bombesin, or PDGF. Lysates from these cultures were separated by SDS-PAGE and electrotransferred to filters. Under conditions resulting in P. multocida toxin-, bombesin- and PDGF-induced decreases in ADP-ribosylation of GRP78/BiP (Fig. 6, upper panel), the cellular content of GRP78/BiP remained constant as revealed by immunoblotting (Fig. 6, lower panel). This observation clearly supports the notion that the observed decrease in ADP-ribosylation of GRP78/BiP reflects the removal of ADP-ribose from a fixed amount of cellular protein.

Signaling Pathways and the ADP-ribosylation of GRP78/BiP—The stimulation of polyphosphoinositide hydrolysis leads to the activation of protein kinase C and the inositol 1,4,5-trisphosphate-mediated release of an ER store of Ca2+ (Nishizuka, 1986; Berridge, 1987). Since P. multocida toxin, bombesin, and PDGF induced a striking decrease in ADP-ribosylation of GRP78/BiP the role of these two signaling pathways in effecting this response was explored. The activation of protein kinase C alone, as elicited by phorbol dibutyrate, only caused a slight decrease (23%) in the ADP-ribosylation of GRP78/BiP but could not elicit the much larger decreases caused by P. multocida toxin (75%), bombesin (67%), or PDGF (61%; Fig. 7). The mechanism of action of P. multocida toxin, bombesin, and PDGF was further examined by studying their effects in cells depleted of protein kinase C. Pretreatment of the cells with phorbol dibutyrate for 40 h is known to down-regulate protein kinase C in Swiss 3T3 cells (Rojzberg-Pena and Rozengurt, 1984). In these down-regulated cells the magnitude of the decrease in ADP-ribosylation of GRP78/BiP as elicited by P. multocida toxin (39%), bombesin (49%), or PDGF (34%) was approximately half of that observed in control cells (Fig. 7), i.e. attenuated but not blocked. Thus, the action of P. multocida toxin, bombesin, and PDGF is not completely dependent upon the activation of protein kinase C.

A possible role for the mobilization of the ER Ca2+ pool in the decreased ADP-ribosylation of GRP78/BiP was explored by the use of Ca2+ ionophores. Low concentrations (100 nM) of A23187 and ionomycin caused a decrease in the cellular content of Ca2+ that was comparable in magnitude to that elicited by bombesin (Fig. 8A). Furthermore, the addition of bombesin to the ionophore-treated cultures did not cause a further mobilization of Ca2+ (results not shown), implying that common pools of Ca2+ were mobilized by ionophore and inositol 1,4,5-trisphosphate. Other studies have shown that

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<th>Mitogens and GRP78/BiP</th>
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<tr>
<td>Control</td>
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<td>Bombesin</td>
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<td>PDGF</td>
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<td>Cycloheximide alone</td>
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<td>Cycloheximide + bombesin</td>
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Fig. 7. Partial effect of protein kinase C activation. Confluent and quiescent cultures of Swiss 3T3 cells were incubated in the absence of presence of phorbol dibutyrate (800 nM final concentration). After 24 h the cultures were washed twice with 2 ml of DMEM and were incubated for a further 20 h with 1 ml of DMEM:Weymouth’s medium (1:1) containing 50 μCi of [2-3H]adenine in the absence (open bars) or continued presence of 800 nM phorbol dibutyrate (closed bars). The labeling of GRP78/BiP was detected by SDS-PAGE followed by trichloroacetic acid treatment and fluorography and quantified by densitometry. Prior to extraction, control cells were incubated with 200 nM phorbol dibutyrate (PBb) for 1 h, and control or pretreated cells were incubated with recombinant P. multocida toxin (rPMT, 20 ng/ml for 4 h), bombesin (Bom., 10 nM for 2 h), or PDGF (25 ng/ml for 2 h). Values obtained after a 2-h incubation with phorbol dibutyrate were not significantly different from those from a 1-h incubation (results not shown). The values shown are the mean ± S.E. of several independent experiments: phorbol dibutyrate, n = 12; rPMT, PDGF, and bombesin, n = 7.
these low concentrations of A23187 caused a transient increase in the concentration of cytoplasmic free Ca\(^{2+}\) that was comparable to that elicited by bombesin (Rozengurt and Sinnett-Smith, 1988).

When tested for their effects on the labeling of GRP78/BiP, it was found that ionomycin and A23187 caused a decrease in ADP-ribosylation of GRP78/BiP that was comparable in magnitude to that achieved by P. multocida toxin (Fig. 8B). The effect of the Ca\(^{2+}\) ionophores was maximal after 30 min. These data indicate that the mobilization of ER Ca\(^{2+}\) may lead to a decrease in the ADP-ribosylation of GRP78/BiP. To examine this possibility further we tested the effect of thapsigargin, which, as a potent and specific inhibitor of the ER Ca\(^{2+}\)-ATPase (Takemurua et al., 1989; Thastrup et al., 1990), causes the net release of Ca\(^{2+}\) from the ER. In Swiss 3T3 cells, thapsigargin reduced the "Ca\(^{2+}\)" content of the "Ca\(^{2+}\)"-equilibrated cells (Fig. 8A), consistent with the release of Ca\(^{2+}\) from the ER and its subsequent efflux from the cells. The magnitude of the depletion in the "Ca\(^{2+}\)" content of the cells was comparable to that induced by bombesin. In parallel experiments, 100 nM thapsigargin caused a decrease in the adenine labeling of GRP78/BiP (Fig. 8B). In 1-h incubations, thapsigargin at 10 nM reduced the ADP-ribosylation of GRP78/BiP to 46% of the control value, whereas 1 nM thapsigargin was ineffective. At the concentrations used in this study, A23187, ionomycin, and thapsigargin did not alter the cellular content of ATP (Table II).

**DISCUSSION**

The results presented here demonstrate that the potent mitogens P. multocida toxin, bombesin, and PDGF cause a striking decrease in the ADP-ribosylation of GRP78/BiP. This protein, the only member of the stress protein family that has been reported to be post-translationally modified by ADP-riboseylation (Welch et al., 1991), was identified in the present study by one- and two-dimensional PAGE, by its response to cellular perturbations known to induce its synthesis (e.g. glucose starvation or addition of deoxyglucose or tunicamycin), and by immunoprecipitation and Western blotting. P. multocida toxin decreased the ADP-ribosylation of GRP78/BiP after a lag period (1.5 h), and its effect was selectively blocked by lysosomotrophic agents, suggesting that the toxin enters the cell via an endosomal/lysosomal pathway to act. Other responses induced by P. multocida toxin, including polyphosphoinositide breakdown, Ca\(^{2+}\) mobilization, activation of protein kinase C, and commitment to DNA synthesis, exhibit similar characteristics (Rozengurt et al., 1990b; Staddon et al., 1990, 1991a). Our results also show that bombesin and PDGF induced a similar but more rapid decrease in the ADP-ribosylation of P. multocida toxin, bombesin, and PDGF decreased the ADP-ribosylation of GRP78/BiP at the potent concentrations that induce cell proliferation. Thus, the conversion of GRP78/BiP from an ADP-ribosylated state to an unmodified state is shown to be an early event in the stimulation of cell proliferation.

The ADP-ribosylated and unmodified forms of GRP78/BiP are functionally different. Hendershot et al. (1988) presented evidence that only the unmodified form of GRP78/BiP is functional, i.e. capable of binding to ER proteins. Furthermore, unmodified GRP78/BiP exists either complexed to proteins or as a free monomer, whereas ADP-ribosylated GRP78/BiP exists as an inactive oligomer (Freiden et al., 1992). Consequently, the P. multocida toxin-, bombesin- and PDGF-induced decrease in ADP-ribosylation of GRP78/BiP may represent the conversion of GRP78/BiP from an inactive to an active state.

It is known that growth factors induce a rapid increase in gene expression and that several of these immediate-early genes encode secretory proteins (Bravo, 1990). The increase in the amount of protein that needs to be processed by GRP78/BiP could recruit GRP78/BiP from the inactive pool. Indeed, increasing the cellular content of underglycosylated proteins, which remain trapped in the ER, causes a decrease in the ADP-ribosylation of GRP78/BiP (Carlsson and Lazarides, 1983; Freiden et al., 1992). Conversely, cycloheximide addition or amino acid starvation causes a dramatic increase in the ADP-ribosylation of GRP78/BiP (Carlsson and Lazarides, 1983; Leno and Ledford, 1989, 1990; Freiden et al., 1992; Table I), which is explained by an increase in the amount of free and therefore ADP-ribosylatable GRP78/BiP as a consequence of the lack of entry of new proteins into the ER and the completion of the processing of existing ER proteins (Freiden et al., 1992). This can account for the ability of cycloheximide to reverse the decrease in ADP-ribosylation induced by mitogenic stimulation (Table I). Thus, the availability of functional GRP78/BiP may be regulated through its interconversion to match the cell's requirement for the processing of secreted proteins.
Manipulation of some of the signaling pathways employed by *P. multocida* toxin, bombesin, and PDGF had effects on the ADP-ribosylation of GRP78/BiP. The activation of protein kinase C appears to be partly responsible for the ability of *P. multocida* toxin, bombesin, and PDGF to decrease the ADP-ribosylation of GRP78/BiP. However, the decrease in ADP-ribosylation of GRP78/BiP induced by *P. multocida* toxin, bombesin, and PDGF was much greater than that achieved by the activation of protein kinase C alone. Furthermore, the ADP-ribosylation of GRP78/BiP was still decreased, although to a lesser extent, by these agents in protein kinase C down-regulated cells. Thus, it is likely that other signals also play a role in determining the magnitude of the response to these agents.

In Swiss 3T3 cells the inositol 1,4,5-triphosphate-mediated mobilization of Ca²⁺ from intracellular stores induces a net flux of Ca²⁺ out of the cell. The store of Ca²⁺ that is mobilized is not well defined, and it is believed to be part of the ER (see Tsien and Tsien, 1990). The finding that the mobilization of ER Ca²⁺ by A23187, ionomycin, and thapsigargin decreases the ADP-ribosylation of GRP78/BiP suggests that Ca²⁺ mobilization may be involved in the mechanism of action of *P. multocida* toxin, bombesin, and PDGF. Since GRP78/BiP is a resident ER protein (Munro and Pelham, 1987), it is possible that the ER Ca²⁺ content in itself is a mechanism that determines the availability of active GRP78/BiP. It should be noted that A23187 has been shown to induce the expression of GRP78/BiP (Drummond et al., 1987) but after a lag period of a few hours, i.e. at times later than the effects of A23187, ionomycin, and thapsigargin observed in the present study. Indeed, the cellular content of GRP78/BiP after treatment with bombesin, PDGF, and *P. multocida* toxin was not increased during the decrease in ADP-ribosylation (Fig. 6). GRP78/BiP is known as a major Ca²⁺-binding protein of the ER (Macer and Koch, 1988), and decreasing the ER Ca²⁺ content promotes the dissociation of GRP78/BiP-protein complexes (Suzuki et al., 1991).

In conclusion, the results presented here demonstrate for the first time that potent growth factors such as *P. multocida* toxin, bombesin, and PDGF cause a striking interconversion of GRP78/BiP, a major ER chaperone.