ADP Ribosylation of the Specific Membrane Protein of C6 Cells by Islet-activating Protein Associated with Modification of Adenylate Cyclase Activity*

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Islet-activating protein (IAP), one of the pertussis toxins, exerts dual actions on crude membrane preparations from rat C6 glioma cells; an $M_r = 41,000$ membrane protein was ADP-ribosylated while GTP (and GTP-dependent isoproterenol) activation of membrane adenylate cyclase was enhanced when membranes were incubated with IAP. Both actions of IAP were dependent on the incubation time and the concentrations of NAD and IAP, and were inhibited by nicotinamide; the one action was strictly paralleled by the other in magnitude. Tryptic digestion of the $M_r = 41,000$ protein was markedly influenced by the presence of guanyl-5'-yl $\beta$-$\gamma$-imidodiphosphate or NaF, the specific ligands of the regulatory component of the adenylate cyclase system. No ADP ribosylation occurred in the membranes prepared from intact C6 cells that had been incubated with IAP, suggesting that the IAP substrate had already been ADP-ribosylated by the intracellular NAD during incubation of the intact cells. Cholera toxin catalyzed ADP ribosylation of other proteins with $M_r = 45,000$ and 48,000/49,000 (doublet). It is concluded that IAP, added to intact cells or isolated membranes, causes unique modification of the receptor-adenylate cyclase coupling mechanism as a result of ADP ribosylation of the $M_r = 41,000$ protein which is presumably one of the subunits, other than the cholera toxin substrates, of the guanine nucleotide regulatory component of the cyclase system.

Materials—IAP was purified by our colleagues (Research Laboratories, Kakenyaku Kako Co., Shiga, Japan) from the 3-day culture supernatant of Bordetella pertussis (Tohama strain, Phase I) according to the procedure described elsewhere (1). Its stock solution was prepared by dissolving 1 mg of IAP in 1 ml of the vehicle consisting of 0.1 M potassium phosphate buffer (pH 7.0) and 2 M urea, and stored at 4 °C until use. The vehicle alone was used as control. Cholera toxin was purchased from Chemico-Research Institute (Kumamoto, Japan). [3H]methylated proteins (CFA-626) from Amersham, Corp., [14C]NAD (29 Ci/mmol) from New England Nuclear, Gpp(NH)p and GDP/BS from Boehringer Mannheim, f-isoproterenol, ATP, GTP, trypsin, and soybean trypsin inhibitor from Sigma. The sources of all other materials used are those described in the previous papers (5-12).

Cell Culture and Membrane Preparation—These were performed as described previously (10). The membrane preparation was suspended in 25 mM Tris-HCl containing 2.5 mM MgCl$_2$ (pH 7.5, referred to as the “membrane buffer” below) at a final concentration of 3 to 5 mg of protein per ml, and stored in liquid nitrogen before use. Membrane protein was determined by the method of Lowry et al. (13) using bovine serum albumin as standard.

Treatment of Membranes with IAP or Cholera Toxin—The membrane preparation (200-300 µg of protein) was incubated with IAP or

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cholera toxin (preactivated as below) for 10 min (or otherwise in Fig. 2) at 37 °C in 100 μl of the membrane buffer containing 1 mM ATP, 10 mM Tris-HCl, and 10 μM NAD. Other additions or modifications are noted in the figures and in Table I. Incubation was terminated by dilution with 1 ml of the ice-cold membrane buffer followed by centrifugation at 10,000 g for 3 min; the pellet was washed twice by repeating dilution and centrifugation. For radiolabeling, 10 μM [α-32P]NAD (5-10 Ci/mmol) was used instead of nonradioactive NAD.

Cholera toxin was preactivated shortly before use by incubation at 37 °C for 10 min with 0.5% sodium dodecyl sulfate and 2.5 mM dithiothreitol in 25 mM Tris-HCl (pH 7.5), a procedure that releases enzymatically active fragment A (14). Packed chicken erythrocytes were centrifuged at 10,000 g for 5 min at 0 °C. The final supernatant was used as a protein factor to promote endogenous nucleotides by incubation with 10% Norit A for 30 min at 100 °C. Aliquots (approximately 50 pg each) were subjected to dithiothreitol in 25 mM Tris-HCl, and 0.02% bromophenol blue, pH 6.8) and heated for 3 min to obtain the cytosol. The separated cytosol was then made free of ADP ribosylation catalyzed by cholera toxin (14).

Polyacrylamide Gel Electrophoresis and Autoradiography—Radiolabeled membranes were dissolved in a gel sample buffer (1% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 10% glycerol, 62.5 mM Tris-HCl, and 0.02% bromophenol blue, pH 6.8) and heated for 3 min at 100 °C. Aliquots (approximately 50 μg each) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (16) on slab gels (1-mm thick) which consist of a 12.5% separating gel and a 4.5% stacking gel. After electrophoresis, gels were stained with Coo massie brilliant blue (R-250), destained, dried, and exposed to Kodak X-Omat film for 24 to 48 h at ~80 °C. The radiolabeled band (M, ~41,000) of the dried gels was excised and counted for its 32P content (17). It was corrected for the background enhancement estimated from the control regions of gels. Molecular weight markers were [15N] methylated proteins: myosin (200,000), phosphorylase b (92,500), bovine serum albumin (66,000), ovalbumin (46,000), carboxic anhydrase (30,000), and lysozyme (14,300).

RESULTS

Differential Membrane Proteins ADP Ribosylated by IAP and Cholera Toxin—Fig. 1 shows the radiolabeled pattern of membrane proteins after incubation of C6 cell membranes with [α-32P]NAD in the presence of IAP and/or cholera toxin. The M, = 41,000 protein was the dominant labeled protein, but some other bands (M, = 36,000 and 29,000) were ADP ribosylated to a much lesser extent when membranes were incubated with IAP (lanes 3 and 4), in accord with our recent report (12). Cholera toxin treatment of membranes resulted in different labeling patterns; radioactivity was detected in the M, = 45,000 and M, = 48,000/49,000 doublet proteins (lanes 7 and 8). The cholera toxin-induced 32P incorporation into these proteins was enhanced by a chicken erythrocyte cytosol (lane 8 versus lane 7) which was without effect on IAP-induced radiolabeling (compare lane 3 with lane 4). The effects of IAP and cholera toxin were mutually additive in a sense that the protein bands labeled by IAP alone and those labeled by cholera toxin alone were both labeled when membranes were incubated in the presence of both toxins (lanes 9 and 10). Thus, different proteins were ADP ribosylated by IAP and cholera toxin.

Modification of Adenylate Cyclase by Treatment of Membranes with IAP or Cholera Toxin—It has lately become feasible to observe the direct action of IAP on the cell-free membrane preparations by including NAD and ATP in the reaction mixture (12). This direct action of IAP on membrane adenylate cyclase is compared with that of cholera toxin in Table I. Adenylate cyclase activity of control membranes (treated with neither IAP nor cholera toxin) increased when the assay medium was supplemented with isoproterenol, GTP, Gpp(NH)p, or NaF. The isoproterenol-induced increment was larger in the presence of guanine nucleotides than in their absence, indicating that the C6 cell membrane adenylate cyclase system comprises, in addition to the catalytic moiety, β-adrenergic receptors and the guanine nucleotide regulatory component.

The effect of IAP on membrane adenylate cyclase was directed to the enzyme response to a particular effector rather than its basal activity estimated in the absence of effectors; GTP activation of the cyclase was much larger in IAP-treated membranes than in control membranes, despite minimum difference in the basal activity between these membranes (Table I). The isoproterenol-induced increase in the cyclase...

**Fig. 1. Polyacrylamide gel analysis of radioactive products resulting from IAP- or cholera toxin-catalyzed ADP ribosylation of cell membranes.** Membranes were incubated with [α-32P]NAD and 0.2 mM GTP in the presence of IAP and/or preactivated cholera toxin, and then electrophoresed, as described under "Experimental Procedures." In lanes 2, 4, 6, and 8, and 10, incubation medium was supplemented with a chicken erythrocyte cytosol (nucleotide-free, final A, = 36). Lanes 1 and 2, control for IAP; 3 and 4, IAP (50 μg/ml); 5 and 6, control for cholera toxin; 7 and 8, cholera toxin (50 μg/ml); 9 and 10, IAP plus cholera toxin. Arrow A marks M, = 48,000/49,000 doublet; B, M, = 45,000; C, M, = 41,000; D, M, = 36,000; E, M, = 29,000 protein.

### Table I

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<th>Pretreatment of membranes with</th>
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<th>Cholera toxin</th>
<th>IAP plus cholera toxin</th>
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<td>-isP</td>
<td>+isP</td>
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<td>29 pmol cAMP/mg protein/min</td>
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<td>60.8</td>
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activity was also enhanced by IAP treatment only when GTP was simultaneously present. Gpp(NH)p- (and NaF-) induced activation was much less sensitive to IAP treatment than GTP-induced activation. As a result, the effect of Gpp(NH)p and NaF conversely suppressed, the cyclase activity in cholera toxin-treated cells (Table I). When membranes had been treated with both IAP and cholera toxin, membrane adenylate cyclase exhibited such enzymic properties as to be theoretically assessable by additive combination of the effect of each alone; the basal activity increased, GTP activation was markedly potentiated, and NaF became inhibitory. Thus, these two bacterial toxins, IAP and cholera toxin, exert their own unique influence on the adenylate cyclase system of C6 cell membranes in manners distinctly independent of each other. These different modifications of adenylate cyclase activity would reflect the fact shown in Fig. 1 that differential proteins were ADP ribosylated by the two toxins.

**Relationship between ADP Ribosylation of the M, = 41,000 Protein and Potentiation of GTP Activation of Adenylate Cyclase under the Influence of IAP**—If ADP ribosylation of the membrane protein catalyzed by IAP would be the mechanism underlying its unique action on membrane adenylate cyclase, these two parameters of IAP action should be altered in parallel with each other under a variety of conditions. We used GTP-dependent adenylate cyclase activity (i.e. the activity estimated in the presence of 10 µM GTP) as a quantitative measure of the action of IAP on the cyclase, since enhancement of GTP activation of the cyclase is the result characteristic of IAP treatment of membranes (see Table I).

Fig. 2 shows time courses and dose-response relationships for IAP actions. As time of exposure of membranes to 5 µg/ml of IAP in the presence of [α-32P]NAD was prolonged from 2 to 10 min, there were parallel progressive increases in both the 32P content of the M, = 41,000 protein band and GTP-dependent adenylate cyclase in the treated membranes (Fig. 2A). Similarly, parallel increases in both parameters were observed when the IAP concentration was raised from 0.1 to 25 µg/ml in the medium for the 10-min membrane incubation (Fig. 2B). The dose-response curves for both IAP actions were shifted to the left as time of incubation was prolonged from 10 to 30 min, although the maximal cyclase activity was reduced due to instability of the enzyme at 37 °C. The concentration of IAP required for the half maximal effect was around 5 µg and 0.5 µg per ml for the 10- and 30-min IAP treatments, respectively, regardless of which of the parameters was used as a measure of IAP action. Thus, the actions of IAP to cause ADP ribosylation of a protein and to enhance GTP activation of adenylate cyclase in C6 cell membranes appear to be both catalytic in a sense that the effects provoked by its minute amounts were amplified by prolongation of incubation time.

Fig. 3A shows effects of the NAD concentration on the IAP actions. Increasing the concentration of [α-32P]NAD caused progressive increases in both the 32P content of the M, = 41,000 protein and GTP-dependent cyclase activity, and both of these IAP actions reached a plateau at 10 µM NAD. Nicotinamide inhibited both actions of IAP in similar dose-dependent manners; the concentration of nicotinamide required to elicit the half-maximal inhibition was around 50 mM for either case.

The correlation of the IAP-induced increase in GTP-dependent adenylate cyclase with the 32P content of the M, = 41,000 IAP substrate is illustrated in Fig. 4, in which the values obtained in Figs. 2 and 3 are plotted. The degree of ADP ribosylation increased in direct proportion to the increment of GTP-dependent adenylate cyclase activity, as visualized by a linear regression (r = 0.97, p < 0.001) in Fig. 4. Thus, the action of IAP on membrane adenylate cyclase appears to be closely related to, and probably reflecting, ADP ribosylation of the M, = 41,000 IAP substrate under these conditions.

** Susceptibility of the Membrane Protein ADP Ribosylated by IAP to Tryptic Digestion as Affected by Guanine Nucleotides and Fluoride**—Membranes labeled with [α-32P]NAD in the presence of IAP were incubated with guanine nucleotides or NaF, ligands of the guanine nucleotide regulatory protein, and then subjected to tryptic digestion before being applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 5). Incubation of labeled membranes with these ligands did not affect the behavior of the labeled IAP substrate on polyacrylamide gels (panel B, lanes 1, 3, 5, and 7). In the absence of these ligands, trypsin treatment of labeled mem-

![Fig. 2. Time- and dose-dependent effects of IAP on ADP ribosylation of the M, = 41,000 protein and GTP activation of adenylate cyclase. Membranes were incubated with [α-32P]NAD in the presence of IAP, and then incorporation of 32P into the M, = 41,000 protein (●, △) and GTP-dependent adenylate cyclase activity (○, ○) were measured, as described under "Experimental Procedures." Incubation time with 5 µg/ml of IAP (△) and the concentration of IAP during 10-min (○) or 30-min (△) incubation (●) are shown on the abscissa. Each point represents the mean of duplicate observations.](image)

![Fig. 3. ADP ribosylation of the M, = 41,000 protein and GTP activation of adenylate cyclase with IAP in the presence of various concentrations of NAD or nicotinamide. Membranes were incubated with IAP (25 µg/ml) as described under "Experimental Procedures" except for changes in the [α-32P]NAD concentration (A) or for addition of nicotinamide (B). Membranes were then analyzed for the 32P content of the M, = 41,000 protein (●) and assayed for GTP-dependent adenylate cyclase activity (○). Each point represents the mean of duplicate estimates.](image)
M, digestion digestion conditions. This value is plotted against the $^{32}$P content of the labeled with [a-$^{32}$P]NAD in the presence of slab gel, and electrophoresed as described under "Experimental Procedures." The reaction mixture was solubilized, applied to a polyacrylamide buffer supplemented with guanine nucleotides and incubated for 200 pg/ml of trypsin for 5 min at 37 °C, which was terminated by the addition of 200 μg/ml of soybean trypsin inhibitor. The reaction mixture was solubilized, applied to a polyacrylamide slab gel, and electrophoresed as described under "Experimental Procedures." A, Coomassie blue staining: lane 1, no trypsin; 2, trypsin. B, autoradiogram: lanes 1 and 2, no addition; 3 and 4, 0.1 mM GTP; 5 and 6, 0.1 mM Gpp(NH)p; 7 and 8, 10 mM NaF. Lanes 1, 3, 5, 7, and 9, no trypsin; 2, 4, 6, 8, and 10, trypsin.

branes resulted in the partial digestion of membrane proteins (compare lane 2 with lane 1 in panel A). As regards labeled proteins, there was a marked decrease in the $^{32}$P content of the $\bar{M}_i = 41,000$ protein band after trypsic digestion; it was with generation of new labeled peptide bands with lower molecular weights (panel B, lane 2). This trypsic digestion pattern was profoundly affected by Gpp(NH)p and NaF; incubation of labeled membranes with these ligands caused generation of a specific $\bar{M}_i = 40,000$ tryptic fragment with a corresponding loss of the $\bar{M}_i = 41,000$ IAP substrate (panel B, lanes 6 and 8). GTP was much less effective than Gpp(NH)p in generating the $\bar{M}_i = 40,000$ protein (panel B, lane 4), probably because of its rapid hydrolysis by cellular nucleotide phosphohydrolases.

It has been previously demonstrated that GBP/S, a GDP analog that is resistant to hydrolysis and phosphorylation, is a competitive inhibitor of adenylate cyclase activated by GTP, Gpp(NH)p, or NaF in membranes of several cell types (18, 19). Indeed, GBP/S inhibited Gpp(NH)p activation of adenylate cyclase of C6 cell membranes in a competitive manner (data not shown). Fig. 6 shows that incubation of membranes with GBP/S reduced Gpp(NH)p-mediated formation of the $\bar{M}_i = 40,000$ tryptic fragment in a dose-dependent manner; as the concentration of GBP/S was increased, the ability of Gpp(NH)p to generate the $\bar{M}_i = 40,000$ fragment was decreased. GBP/S alone caused a slight decrease in the trypsic digestion of the $\bar{M}_i = 41,000$ labeled protein and little or no generation of the $\bar{M}_i = 40,000$ tryptic fragment. Thus, the membrane protein that is the substrate of IAP-catalyzed ADP ribosylation displayed altered sensitivity to trypsic digestion when it had been incubated with guanine nucleotides or NaF. These results are compatible with the idea that guanosine triphosphate and NaF interact with the IAP substrate protein to induce conformational changes which render different sites on the protein molecule susceptible to trypsic cleavage. It is very likely, therefore, that the $\bar{M}_i = 41,000$ IAP substrate would be a subunit of the guanine nucleotide regulatory component of the adenylate cyclase system.

Failure of IAP and Cholera Toxin to Catalyze ADP Ribosylation of Their Specific Substrates in Membranes Prepared from the Respective Toxin-treated Cells—Membrane adenylate cyclase is similarly modified regardless of whether the membranes as such are directly exposed to IAP (Table I) or the membranes are prepared from IAP-treated intact cells (10). Conceivably, the action of IAP on intact cells shares the same mechanism as its direct action on membrane preparations. If the IAP-specific substrate labeled in membranes is also the relevant substrate in intact cells, their labeling should be prevented or decreased in membranes prepared from IAP-treated cells. This possibility was studied in Fig. 7.

As control, a part of experiments in Fig. 1 was repeated in Fig. 7; membranes of cells that had been exposed to neither

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**Fig. 4.** Correlation of IAP-induced increments of GTP-dependent adenylate cyclase activity with the $^{32}$P content of the $\bar{M}_i = 41,000$ IAP substrate. GTP-dependent adenylate cyclase activity of IAP-treated membranes is expressed as a percentage of the activity of the nontreated membranes estimated under the same conditions. This value is plotted against the $^{32}$P content of the $\bar{M}_i = 41,000$ protein of the same membranes incubated with [a-$^{32}$P]NAD. Data are from Figs. 2A (●), 2B (▲), 3A (■), and 3B (●).

**Fig. 5.** Effects of guanine nucleotides and NaF on trypsic digestion of the $\bar{M}_i = 41,000$ IAP substrate. Membranes first labeled with [a-$^{32}$P]NAD in the presence of 25 μg/ml of IAP and washed were resuspended (2 mg of protein/ml) in the membrane buffer supplemented with guanine nucleotides or NaF to be further incubated for 10 min at 37 °C. Aliquots were then submitted to trypsic digestion (200 μg/ml of trypsin for 5 min at 37 °C), which was terminated by the addition of 200 μg/ml of soybean trypsin inhibitor. The reaction mixture was solubilized, applied to a polyacrylamide slab gel, and electrophoresed as described under "Experimental Procedures." A, Coomassie blue staining: lane 1, no trypsin; 2, trypsin. B, autoradiogram: lanes 1 and 2, no addition; 3 and 4, 0.1 mM GTP; 5 and 6, 0.1 mM Gpp(NH)p; 7 and 8, 10 mM NaF. Lanes 1, 3, 5, 7, and 9, no trypsin; 2, 4, 6, 8, and 10, trypsin.

**Fig. 6.** Gpp(S)/GBP competition of Gpp(NH)p-mediated formation of the $\bar{M}_i = 40,000$ tryptic fragment. Membranes were labeled by [a-$^{32}$P]NAD and IAP, treated with guanine nucleotides, and subjected to trypsic digestion and polyacrylamide gel electrophoresis, as in Fig. 5, except for the altered concentration (50 μg/ml) of trypsin and its inhibitor. Guanine nucleotide treatment was with 50 μM Gpp(NH)p in combination with increasing concentrations of Gpp(S). Lane 1, no trypsin; 2 to 8, trypsin; 1 to 4, no Gpp(NH)p; 4 to 8, Gpp(NH)p. Lanes 1, 2, and 4, no Gpp/S; 3 and 5 to 8, Gpp(S) (5, 10, 6, 50, 7, 250; 3 and 8, 1250 μM).
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Fig. 7. Effects of pretreatment of intact C6 cells with IAP or cholera toxin on the subsequent ADP ribosylation of membrane proteins. C6 cells were incubated with IAP (100 ng/ml) or cholera toxin (1 µg/ml) for 3 h at 37 °C (10). Membranes prepared from these cells were labeled with [α-32P]NAD in the presence of IAP (25 µg/ml) or preactivated cholera toxin (100 µg/ml) and submitted to polyacrylamide gel electrophoresis as in Fig. 1. A nucleotide-free chicken erythrocyte cytosol (final A260 = 24) and 0.2 mM GTP were included in the reaction mixture for labeling. Lanes 1 to 3, cells incubated with no addition; lanes 4 to 6, cells incubated with IAP; lanes 7 to 9, cells incubated with cholera toxin; lanes 10 to 12, cells incubated with IAP plus cholera toxin. Lanes 1, 4, 7, and 10, labeling with no addition; lanes 2, 5, 8, and 11, labeling with IAP; lanes 3, 6, 9, and 12, labeling with cholera toxin. Arrows A to E designate the protein bands with the same M, values as in Fig. 1.

Fig. 8. IAP-catalyzed ADP ribosylation of the membrane M, = 41,000 protein inversely related to GTP-dependent adenylate cyclase activity enhanced by IAP treatment of membrane donor cells. Washed membranes were prepared from C6 cells that had been incubated with IAP for 3 h (see Fig. 7). Some aliquots of the membranes were assayed for GTP-dependent adenylate cyclase activity, and others were further incubated with [α-32P]NAD and 25 µg/ml of IAP and then submitted to polyacrylamide gel electrophoresis as detailed under "Experimental Procedures." The 32P content of the M, = 41,000 protein band (○) and adenylate cyclase activity (●) were plotted against the concentrations of IAP present in the medium for intact cell incubation. Each point represents the mean of duplicate observations.

IAP nor cholera toxin were submitted to ADP ribosylation in the presence of IAP or cholera toxin (lanes 1 to 3). As compared with the control, prior treatment of cells with IAP markedly reduced the subsequent IAP-dependent ADP ribosylation without causing significant change in labeling of the cholera toxin substrates in membranes prepared therefrom (lanes 4 to 6). On the contrary, there was marked reduction in labeling of the cholera toxin substrates but no alteration in the IAP substrate labeling if membranes prepared from cholera toxin-treated cells were incubated with [α-32P]NAD (lanes 7 to 9). Moreover, when cells had been treated with both cholera toxin and IAP, neither IAP nor cholera toxin were able to catalyze significant ADP ribosylation of proteins in the resultant membranes. Reduced labeling of the cholera toxin substrates in membranes prepared from the toxin-treated cells confirms the previous reports (20, 21).

Thus, it is highly probable that incubation of intact cells with IAP (or cholera toxin) causes ADP ribosylation of the membrane IAP (or cholera toxin) substrates by intracellular nonradioactive NAD. The ADP-ribosyl bonding once formed in the substrate proteins is so stable that further exchange reaction would not take place with radioactive ADP-ribosyl moiety during the subsequent incubation of the isolated membrane in the presence of [32P]NAD. When intact cells were incubated with increasing concentrations of IAP, there was progressive inhibition of the subsequent labeling of the M, = 41,000 protein in membranes prepared therefrom (Fig. 8). The degree of inhibition was inversely correlated to the increment of GTP-dependent adenylate cyclase that reflects the IAP action on intact cells. It is very likely, therefore, that ADP ribosylation of membrane proteins catalyzed by IAP in intact cells is responsible for enhanced GTP activation of adenylate cyclase characteristic of membranes prepared from the IAP-treated cells.

DISCUSSION

In the present study, IAP-catalyzed ADP ribosylation of the membrane M, = 41,000 protein strictly paralleled the characteristic enhancement of GTP activation of adenylate cyclase under various conditions; there were similarly progressive increases in both parameters when the time of exposure of membranes to, and the concentration of, IAP were increased. The potency of IAP increased in either case as the time of the exposure was prolonged. Moreover, both actions of IAP were dependent on the concentration of NAD and inhibited by nicotinamide, another product of ADP ribosylation reaction, with a quite similar dose-response relationship. Thus, there was a highly significant correlation between the ADP ribosylation of the M, = 41,000 IAP substrate and the IAP-specific modification of membrane adenylate cyclase (Fig. 4). We are then led to a conclusion that IAP directly added to C6 cell membranes enhances GTP activation (and GTP-dependent isoproterenol activation) of adenylate cyclase through ADP ribosylation of the M, = 41,000 protein.

As to the physiological role of the IAP substrate protein with an M, = 41,000, the data in Figs. 5 and 6 have provided indirect but convincing evidence for its close relation to the guanine nucleotide regulatory component of the adenylate cyclase system, by showing that susceptibility of the labeled protein to tryptic digestion was profoundly affected by the specific ligands of the regulatory component such as Gpp(NH)p or GTP (18), was able to suppress Gpp(NH)p-mediated formation of the M, = 40,000 tryptic fragment, suggesting that altered susceptibility of the IAP substrate to trypsin may have resulted from a certain conformational change upon binding of active ligands to the substrate protein itself or the associated protein(s). The ADP-ribosylated cholera toxin substrate is one of the subunits of the guanine nucleotide regulatory oligomeric protein (20, 22–25). In view of the findings (26, 27) that tryptic digestion of the cholera toxin substrates is likewise affected by the presence of guanine nucleotides, it is highly probable that the IAP substrate is also one of the subunits of, or a protein closely related to, the guanine nucleotide regulatory component of the receptor-adenylate cyclase system.
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IAP exerted just the same effects on membrane adenylate cyclase whether it was added directly to isolated membranes (Table I) or to intact cells from which membranes were subsequently prepared (see Ref. 10). Following the aforementioned conclusion that IAP-catalyzed ADP ribosylation of the membrane $M_s = 41,000$ protein is responsible for its direct action on membrane adenylate cyclase, the next important question is if IAP added to intact cells also causes ADP ribosylation of the same membrane protein using intracellular NAD as substrate. This question was answered in the affirmative by the results in Figs. 7 and 8. The membrane fraction that was prepared from C6 cells incubated with submaximal concentrations of IAP underwent only partial ADP ribosylation upon subsequent incubation with excess IAP; the degree of the ADP ribosylation was inversely correlated with the GTP-dependent adenylate cyclase activity that should reflect the degree of IAP action on the intact cells (Fig. 8). ADP-ribosyl bonding once generated with the IAP substrate must be very stable, since no radioactivity was lost from the labeled membranes during the subsequent incubation with nonradioactive NAD and IAP (data not shown). Conceivably, a fraction of the total molecules of the IAP substrate in the membrane is labeled with endogenous NAD upon incubation of intact cells with submaximal concentrations of IAP, and the rest is labeled with [32P]NAD during subsequent incubation of the membrane prepared therefrom. Thus, the degree of IAP-catalyzed labeling of the $M_s = 41,000$ protein occurring upon incubation of membranes from IAP-treated cells should be inversely correlated to the degree of ADP ribosylation that has occurred during IAP treatment of intact cells; it should be nearly null when intact cells have been treated with the saturating concentrations of IAP as really observed in Fig. 7. It is reasonable, therefore, to conclude that enhancement of GTP activation of membrane adenylate cyclase caused by IAP treatment of intact C6 cells is also due to ADP ribosylation of the $M_s = 41,000$ protein.

The IAP action on C6 cell membranes bears striking similarities to those of the action of cholera toxin in that both are capable of modifying adenylate cyclase responses through ADP ribosylation of membrane proteins. The IAP-catalyzed ADP ribosylation reaction is, however, different from the cholera toxin-catalyzed one in the following respects. First, the substrate proteins for cholera toxin-catalyzed ADP ribosylation are distinct from the IAP substrates. In C6 cell membranes, the $M_s = 45,000$ and $48,000/49,000$ (doublet) proteins were ADP ribosylated in the presence of activated cholera toxin (Fig. 1). In the case of other cell types, the major cholera toxin substrates are the proteins with an $M_s = 42,000$ for pigeon (14, 22, 23), turkey (28), and human (24, 29) erythrocytes, for human skin fibroblasts (21, 30) and for rat adipocytes (31), an $M_s = 45,000$ for S49 lymphoma (20, 26, 32) and for rabbit liver (33), an $M_s = 47,000$ for human fibroblasts (30), an $M_s = 52,000$ for S49 (20, 26), HTC-1 (20), thymus (34), rabbit liver (33), and cultured fibroblasts (25, 35), and an $M_s = 55,000$ for S49 (32). Most of these proteins have been shown to be the specific proteins responsible for the cholera toxin-induced modification of membrane adenylate cyclase or one of the subunits of the regulatory component of the cyclase system. Thus, the fact that the substrates for ADP ribosylation are different for IAP and cholera toxin may form a molecular basis for the sharp difference in adenylate cyclase responses between IAP- and cholera toxin-treated membranes shown in Table I.

Second, cholera toxin-induced ADP ribosylation is greatly accelerated by a cytosol factor of avian erythrocytes (14, see also Fig. 1) and GTP (23, 30, 34, 36), while these factors are without effect on the IAP-catalyzed reaction. Third, prior activation of cholera toxin, a procedure adapted for releasing the active fragment $A_1$ from the toxin, was essential for the direct action of the toxin on membranes to be observed; the holotoxin per se, which is effective on intact cells, failed to cause ADP ribosylation of membrane proteins when the cells were disrupted. In contrast, IAP was effective on C6 cell membranes directly without such a prior treatment as to cause subunit dissociation or fragmentation. Our recent studies have shown that the IAP molecule consists of five dissimilar peptides, one of which, tentatively referred to as the SI subunit with an $M_s = 28,000$, was as effective as the native IAP molecule when added directly to membrane preparations, although its biological activity was totally missing when added to intact C6 and other cells. Moreover, the SI subunit did, but the native IAP did not, catalyze hydrolysis of NAD to ADP-ribose and nicotinamide in the absence of cellular components. Presumably, the SI peptide, just like the $A_1$ protomer of cholera toxin (37, 38), is the enzymatically active subunit that catalyzes the transfer of ADP-ribose from NAD to the membrane protein, whereas the other peptides in the IAP molecule would appear to be similar to the B subunits of cholera toxin (37, 38) in that they are required for the SI subunit to gain access to the $M_s = 41,000$ protein in intact cells. It would be possible, therefore, to assume that the biologically active component, such as the SI subunit, is rapidly released through proteolysis or the disulfide bond reduction during incubation of the whole molecule of IAP with cell membranes.

In summary, IAP added to C6 cells caused drastic modification of adenylate cyclase responses to receptor agonists or guanine nucleotides through ADP ribosylation of the membrane $M_s = 41,000$ protein, which appeared to be closely related to, or one of the subunits of, the guanine nucleotide regulatory component of the receptor-cyclase system. Since the $M_s = 41,000$ protein is not identical with any of the cholera toxin substrates, the manner in which IAP modifies C6 cell adenylate cyclase responses is distinct from cholera toxin-induced modification. Following the treatment of cells with IAP, the activation of adenylate cyclase, or the increase in the cellular cAMP content, via $\alpha$-adrenergic, glucagon, or prostaglandin E receptors was enhanced in rat heart (9), C6 glioma (10), 3T3 fibroblast, and NG108-15 cell cultures, whereas the inhibition of the cyclase, or the decrease in the cellular cAMP, via $\alpha$-adrenergic, cholinergic muscarinic, adenosine, or opiate receptors was abolished or attenuated in rat ilea (5, 6, 8, 11), heart (9), and NG108-15 cell cultures. A possibility is currently under investigation that these IAP-specific modifications of the receptor-adenylate cyclase coupling in various cell types also may result from ADP ribosylation of the membrane protein responsible for receptor-mediated regulation of adenylate cyclase.

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