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GTP STABILIZATION OF ADENYLATE CYCLASE ACTIVATED AND ADP-RIBOSYLATED BY CHOLERAGEN

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SUMMARY

Choleragen activates adenylate cyclase in human skin fibroblasts by catalyzing the ADP-ribosylation of the 42,000 and 47,000 dalton guanyl nucleotide-binding regulatory components (G) of adenylate cyclase. The ADP-ribose linkage to 42,000 and 47,000 dalton proteins was stable at 30°C for 1 h with or without GTP, whereas GTP was required to stabilize activity of the G proteins. In human erythrocytes, choleragen catalyzed the ADP-ribosylation of only a 42,000 dalton G. The ADP-ribosyl-protein linkage was stable for 1 h at 30°C whether or not GTP was present, despite a rapid loss of G activity in the absence of GTP. Inactivation of choleragen-activated G in both the human fibroblast and human erythrocyte is, therefore, not secondary to the de-ADP-ribosylation of specifically labeled G subunits.

INTRODUCTION

Choleragen activates adenylate cyclase by catalyzing the ADP-ribosylation of one or more subunits of the regulatory component (G)[1] of adenylate cyclase (1-11). Specific choleragen substrates have been characterized in several tissues, including pigeon erythrocyte (3,4), human erythrocyte (5), mouse S49 lymphoma cell (6), rat fat cell (7), mouse 3T3 cell (8), rabbit liver (9), rat liver (10), and human skin fibroblast (11). Two criteria have been employed to establish that a protein(s), ADP-ribosylated by choleragen, is a subunit of G. In some cases, e.g., the erythrocyte, a ^{32}P-ADP-ribosyl protein of M_r = 42,000, copurifies with G activity (5); in others, e.g., the S49 cell and human fibroblast, choleragen treatment of intact cells prevents the subsequent choleragen-catalyzed ADP-ribosylation of specific proteins in isolated

[1] Abbreviations used are: G, the guanyl nucleotide-binding regulatory component of adenylate cyclase; CHAPS, 3-[3-(cholamidopropyl)dimethylammonio]-1-propane sulfonate; cyc$^-$, the cyc$^-$ variant of the murine S49 lymphoma cell.

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particulate fractions (6,11). ^{32}P-ADP-ribosylation of peptides of 42,000 and 47,000 daltons in human fibroblast membranes was prevented by incubation of the cells with choleragen (11).

GTP enhances the choleragen-catalyzed ADP-ribosylation of numerous proteins (12) and, in particular, is required for demonstration of ADP-ribosylation of the cyclase regulatory component in all systems described above (3-11, 13). GTP is also required for activation of adenylate cyclase by choleragen (14-16) as well as for expression of maximal catalytic activity of the cyclase previously activated by toxin (17). After activation by choleragen, brain adenylate cyclase is unstable at 30°C unless GTP is present (17). Hudson et al. (18) observed that the GTP analog, guanosine-5'-O-(3-thiotriphosphate) altered the susceptibility of the 42,000 dalton pigeon erythrocyte G to tryptic digestion. Several enzymes capable of degrading ADP-ribosyl groups, notably phosphodiesterases, are present in mammalian cells (19,20). To investigate whether the inactivation of cyclase that was prevented by GTP was due to removal of ADP-ribose (actually AMP because of the locus of the radioactive label) from G, we studied two systems known to contain proteins specifically ADP-ribosylated by choleragen, human skin fibroblasts which contain both G and the catalytic subunit of adenylate cyclase, and human erythrocytes. As the erythrocytes contain G but lack catalytic subunit (21,22), its effect on the stability of ADP-ribose-G could also be evaluated.

MATERIALS AND METHODS

Materials. Choleragen was purchased from Schwarz-Mann; phosphoenolpyruvate, pyruvate kinase, thymidine, GTP, and ATP were from Sigma; [α-^{32}P]NAD and [α-^{32}P]ATP were from New England Nuclear; NAD was from P-L Biochemicals. CHAPS (3-[3-(cholamidopropyl)dimethylammonio]-1-propane sulfonate) was prepared as described (23).

Cells. Human skin fibroblasts, grown as previously described (24), were washed twice with Dulbecco's phosphate-buffered saline, harvested by scraping, and sedimented at 1000 x g. After homogenization in 50 mM glycine, pH 8.0, and centrifugation for 20 min at 20,000 x g, the crude particulate fraction was suspended in 50 mM glycine, pH 8.0.

Fresh heparinized blood was obtained from the NIH Blood Bank through the courtesy of Dr. Harvey Klein. After washing extensively with 150 mM NaCl con-

taining 10 mM Tris(Cl^-), pH 7.5, erythrocytes were lysed in 100 volumes of 10 mM Tris(Cl^-), pH 7.5. Membranes were collected by centrifugation at 11,000 x g for 10 min, washed twice with 150 mM KCl containing 10 mM EDTA, washed twice with 50 mM glycine, pH 8.0, and finally suspended in 50 mM glycine, pH 8.0.

Cyc^- S49 lymphoma cells, obtained from Dr. Alfred G. Gilman, were grown in Dulbecco's minimal essential medium containing 10% heat-inactivated horse serum. Membranes were prepared as described by Ross et al. (25) without the sucrose density gradient step.

Assays. Adenylate cyclase activity was assayed for 20 min at 30°C in a total volume of 0.1 ml containing 0.25 mM [α-^{32}P]ATP (10-20 cpm/pmol), 50 mM HEPES, pH 8.0, 1 mM EDTA, bovine serum albumin (0.1 mg/ml), 0.5 mM theophylline, 0.1 mM ascorbate, 2 mM phosphoenolpyruvate, 0.85 units of pyruvate kinase, and other additions as described in the figure legends; [^{32}P]cAMP was isolated by the method of Salomon et al. (26). Protein was determined by the method of Lowry et al. (27). Choleragen was activated before addition to membrane preparations as previously described (12).

ADP-Ribosylation. Two different procedures were used for ADP-ribosylation of membrane proteins. In Method I, conditions were similar to those previously shown to be optimal for activation of brain adenylate cyclase by choleragen (14,17). Membranes were incubated for 30 min at 30°C in 40 mM glycine (pH 8.0), 5 mM $MgCl_2$, 5 mM phosphoenolpyruvate, 55 units/ml of pyruvate kinase, 50 µM GTP, 250 µM [α-^{32}P]NAD, and 200 µg/ml of activated choleragen. The reaction was terminated with 10 volumes of 50 mM glycine buffer, pH 8.0, after which the membranes were washed twice and suspended in the same buffer. Samples of labeled membranes were incubated further as described in figure legends before proteins were precipitated with 10% trichloroacetic acid and prepared for electrophoresis. When fibroblast membranes were incubated under these conditions, choleragen caused a sevenfold increase in adenylate cyclase activity (from 4.6 to 34 pmol/min/mg protein) and numerous proteins were ^{32}P-ADP-ribosylated (Fig. 1, lane 1). Prior exposure of intact fibroblasts to choleragen did not apparently prevent the labeling of any bands, however, the specific choleragen substrates of M_r = 42,000 and 47,000 were not clearly distinguishable (Fig. 1, lane 2).

Specific labeling of these peptides was demonstrable when ^{32}P-ADP-ribosylation was carried out using Method II (11). Membranes were incubated for 30 min at 30°C in 50 mM potassium phosphate, pH 7.5, containing 20 mM thymidine, 4 mM GTP, 20 µM [α-^{32}P]NAD, and activated choleragen (250 µg/ml). Following incubation, membranes were washed with glycine buffer as in Method I and incubated further as described in figure legends. Labeled proteins were precipitated with 10% trichloroacetic acid, and subjected to electrophoresis. Proteins of M_r = 42,000 and 47,000 were the major labeled components (Fig. 1, lane 3). Exposure of cells to choleragen prevented the subsequent ^{32}P-ADP-ribosylation of these proteins (Fig. 1, lane 4). The effectiveness of these procedures in demonstrating specific labeling of the 42,000 and 47,000 dalton peptides is most likely due to the use of only 20 µM NAD. With higher concentrations of NAD, usually used for activation of cyclase, the background radioactivity is increased to an extent that obscures the 42,000 and 47,000 dalton bands (data not shown).

Electrophoresis and Radioautography. Trichloroacetic acid-precipitated particulate fractions were solubilized in 1% sodium dodecyl sulfate and subjected to electrophoresis on 8% acrylamide slab gels according to the method of Laemmli (28). Kodak X-Omat R film was exposed to dried gels for 2-4 days.

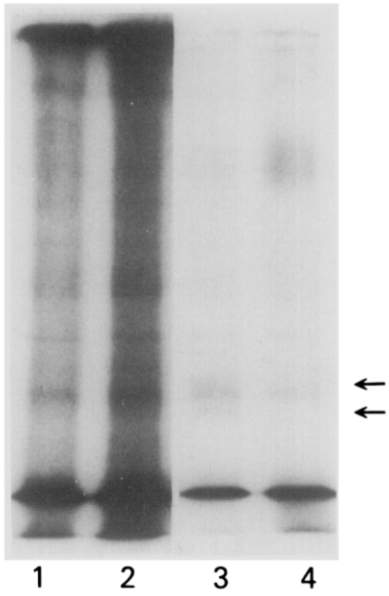

Figure 1: Comparison of conditions for optimal cyclase activation with optimal labeling conditions on the ^{32}P-ADP-ribosylation of fibroblast membrane proteins by choleragen. Lanes 1 and 2. Fibroblast membranes (0.09 mg protein) were ^{32}P-ADP-ribosylated in a total volume of 35 µl by Method I, except that the reaction was terminated with 10% trichloroacetic acid and no further incubation was performed; the specific activity of [α-^{32}P]NAD was 1700 cpm/pmol. Precipitated proteins were solubilized in 1% sodium dodecyl sulfate and subjected to electrophoresis and radioautography. Lane 1, control cells; lane 2, cells incubated 3 h at 37°C with 10 µg choleragen in 10 ml medium prior to membrane preparation. Lanes 3 and 4. Fibroblast membranes (0.09 mg protein) were ^{32}P-ADP-ribosylated by Method II in a total volume of 100 µl. The reaction was terminated and samples prepared for electrophoresis as above. Lane 3, control cells; lane 4, cells incubated with choleragen. Proteins of M_r = 42,000 and 47,000 are indicated by arrows.

RESULTS AND DISCUSSION

Incubation of fibroblast membranes with choleragen under conditions previously shown to label specifically proteins of 42,000 and 47,000 daltons (11) resulted in a sixfold activation of adenylate cyclase (Fig. 2, zero time). On incubation of the activated cyclase at 30°C without GTP, there was a rapid loss of activity; when GTP was present, cyclase activity was stable for at least 1 h (Fig. 2). This GTP effect is similar to that previously reported for the brain adenylate cyclase (17). When fibroblast particulate proteins, ^{32}P-ADP-ribosylated by choleragen using Method II, were incubated for 1 h at 30°C, however, no loss of radioactivity from the 42,000 or 47,000 dalton proteins was observed whether or not GTP was present (Fig. 3). The decrease in

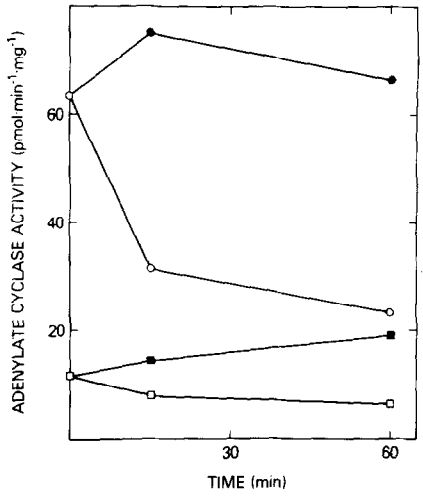

Figure 2: Effect of GTP on stability of adenylate cyclase activity of human skin fibroblast membranes following activation by choleragen. Fibroblast membranes (0.5 mg protein) were incubated using Method II with unlabeled NAD in a total volume of 2.4 ml; when choleragen was omitted, an equivalent volume of activation buffer was substituted. Membranes were then washed twice with 10 volumes of 50 mM glycine, pH 8.0, and incubated for the indicated time at 30°C with or without 100 μM GTP, 10 mM $MgCl_2$, 200 mM NaCl, 1 mM phosphoenolpyruvate, and 0.4 units of pyruvate kinase before assay of adenylate cyclase. Assays contained 24 μg of membrane protein. Just prior to assay, GTP, $MgCl_2$, NaCl, phosphoenolpyruvate, and pyruvate kinase were added to those samples incubated without it. Final concentrations in assay were 100 μM GTP and 6 mM $MgCl_2$. □,■ - no choleragen during the first incubation; ○,● - with choleragen; ○,□ - no GTP during the second incubation, ●,■ with GTP.

cyclase activity, therefore, did not result from the de-ADP-ribosylation of these proteins.

Since fibroblasts contain the catalytic subunit of cyclase as well as G, it was not clear whether the stability of the ADP-ribosylated G and/or GTP-dependent stabilization of choleragen-activated cyclase required the presence of catalytic subunit. To investigate this possibility, human erythrocyte membranes, which contain G but lack the catalytic subunit, were activated with choleragen. When activated membranes were solubilized and then reconstituted with cyc^- membranes (which contain catalytic subunit but lack G), cyclase activity was 11 times that of cyc^- membranes reconsituted with extracts of erythrocyte membranes not activated with choleragen (Fig. 4, zero time). Incubation of choleragen-activated erythrocyte membranes at 30°C without GTP resulted in a loss of the capacity to reconstitute cyclase activity, which was

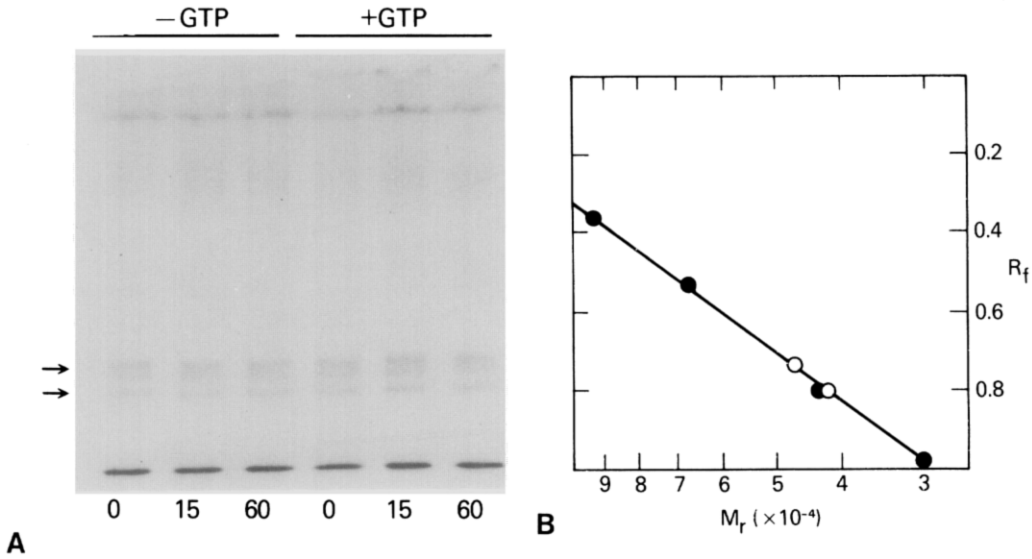

Figure 3: Effect of GTP on stability of fibroblast proteins ADP-ribosylated by choleragen. A. A portion of the fibroblast membranes used in Fig. 2 was simultaneously ^{32}P-ADP-ribosylated by Method II in the presence of choleragen; the ^{32}P-NAD specific activity was 2500 cpm/pmol. Washed membranes were then incubated with (+ GTP) or without (- GTP) GTP, $MgCl_2$, NaCl, phosphoenolpyruvate, pyruvate kinase as described in Fig. 2. At the indicated times (in minutes), incubations were terminated with 10% trichloroacetic acid; precipitated proteins were prepared for electrophoresis as in Methods. Labeled proteins of 42,000 daltons and 47,000 daltons in the radioautograph are indicated by the arrows. B. Molecular weights of standard (●) and ADP-ribosylated (○) proteins are plotted vs. relative mobility. Standards were phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), and carbonic anhydrase (30,000).

partially prevented when GTP was present during the 1 h incubation (Fig. 4). Radioactivity in the 42,000 dalton erythrocyte G, ^{32}P-ADP-ribosylated by choleragen under identical conditions (Method I), was stable to incubation at 30°C whether or not GTP was present, despite the decrease in ability to reconstitute cyclase activity (Fig. 5). Thus, it appears that the stability of ADP-ribosyl-G requires neither catalytic subunit nor GTP.

In human erythrocyte membranes, several proteins in addition to the 42,000 dalton protein were ^{32}P-ADP-ribosylated under the conditions of Method I (Fig. 5). Certain of these, notably peptides of 50,000 and 87,000 daltons, lost radioactivity during incubation of membranes at 30°C. There is no evidence that either of these peptides is associated with erythrocyte G (5,29) and the

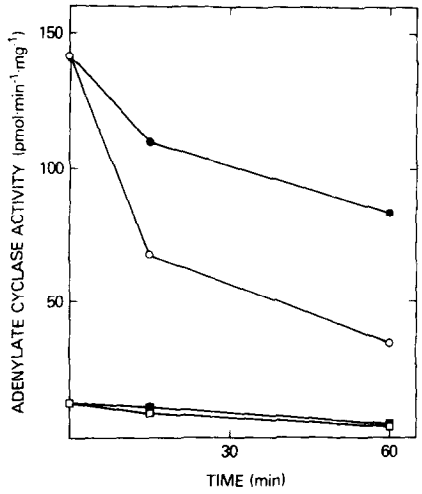

Figure 4: Effect of GTP on stability of G activity of human erythrocyte membranes. Human erythrocyte membranes (6.7 mg protein) were incubated as in Method I with unlabeled NAD in a total volume of 0.96 ml; activation buffer was used when choleragen was omitted. After incubation, membranes were washed twice with 10 volumes of 50 mM glycine, pH 8.0, and samples (0.25 mg protein) were incubated further at 30°C with or without 150 μM GTP, 15 mM $MgCl_2$, 0.15 mM phosphoenolpyruvate, and 0.25 units of pyruvate kinase. After incubation for the time indicated, membranes were solubilized 15 min at 2°C with 0.5% CHAPS, after which GTP, $MgCl_2$, NaCl, phosphoenolpyruvate, and pyruvate kinase were added to those samples incubated without it; cyc^- membranes (2 mg protein) were added to all samples and, after 30 min at 2°C, adenylate cyclase activity was assayed. The cyc^- membranes alone had an activity of 0.48 pmol/min/mg protein. Symbols are as in Fig. 2.

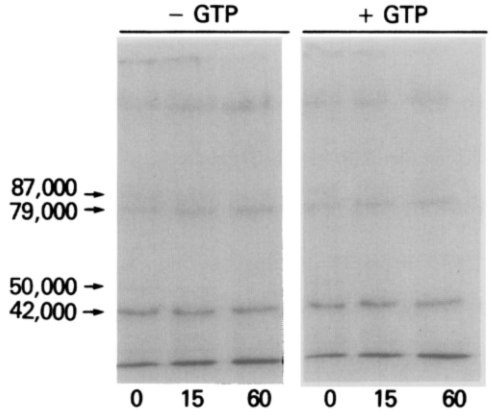

Figure 5: Effect of GTP on stability of ADP-ribosyl-G from human erythrocyte membranes. Erythrocyte membranes were ^{32}P-ADP-ribosylated with choleragen by Method I in parallel to the first incubation of Fig. 4; the specific activity of ^{32}P-NAD was 340 cpm/pmol. After washing, membranes were incubated further with (+ GTP) or without (- GTP) GTP, $MgCl_2$, NaCl, phosphoenolpyruvate, and pyruvate kinase as described in Fig. 4. At the indicated times (in minutes), incubations were terminated with 10% trichloroacetic acid; precipitated proteins were subjected to electrophoresis and radioautography. Molecular weight standards were as described in Fig. 3.

loss of label was not detectably influenced by GTP. Thus, although ADP-ribosyl moieties on some proteins modified by choleragen may be cleaved by endogenous enzymes, perhaps phosphodiesterases, which are widespread in animal tissues (19,20), others, e.g., those on G, are seemingly less susceptible to degradation or removal. Whatever the reason for the stability of the ADP-ribosyl groups on G, it may explain the apparently irreversible nature of choleragen activation of adenylate cyclase in many types of cells.

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