Choleragen-stimulated Release of Guanyl Nucleotides from Turkey Erythrocyte Membranes*

(Received for publication, August 7, 1981, and in revised form, October 9, 1981)

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Choleragen stimulates adenylate cyclase by ADP ribosylating a guanyl nucleotide-binding regulatory protein (G/F). β-Adrenergic hormones also activate the adenylate cyclase of turkey erythrocytes, and it is currently believed that they do so in part by decreasing the affinity of G/F for factor GDP, an effect which is manifested by a hormone-stimulated release of guanyl nucleotides from the membranes. Since choleragen might also activate adenylate cyclase by a similar mechanism, the effect of toxin treatment on the release of guanyl nucleotides from turkey erythrocyte membranes was examined.

In the presence of NAD, choleragen was found to stimulate release of guanyl nucleotides from membranes which had been preloaded with radiolabeled GTP. No stimulation of release was observed with cAMP or when NAD was replaced by NADP, which does not serve as a substrate for choleragen-catalyzed ADP ribosylation. While either isoproterenol or choleragen can stimulate release of guanyl nucleotides from the membranes, the amount of guanyl nucleotide released in the presence of both isoproterenol and choleragen may stimulate adenylate cyclase, in part, by a mechanism similar to that of β-adrenergic agonists.

The abbreviations used are: G/F, the GTP-binding protein of the GTP-binding protein complex; App(NH)p, pyrophosphate; EGTA, ethylene glycol bis(beta-aminoethyl ether)-N,N',N''-tetraacetic acid; [32P]GTP, a 5'-GTP complex labeled with [32P]GTP; GDP, guanosine 5'-diphosphate; GTP, guanosine 5'-triphosphate; Gpp(NH)p, guanosine 5'-O-(3-deoxy-d-xylo-oct-4-ulosonoyl)diphosphate; PM, polyethyleneimine-cellulose thin layer chromatography sheets.

Several hormones are believed to activate adenylate cyclase by binding to receptors which interact with G/F and promote the replacement of bound GDP with GTP. Cassel and Selinger showed that isoproterenol stimulated release of tightly bound [3H]GDP from turkey erythrocyte membranes; the rate of release was correlated with the rate of activation of adenylate cyclase (12). As reported here, we have now found that choleragen in the presence of NAD increases the release of bound guanyl nucleotide from turkey erythrocyte membranes and this nucleotide appears to come from the same pool that is released by isoproterenol.

MATERIALS AND METHODS

App(NH)p, pyruvate kinase, phosphoenolpyruvate (sodium salt), and isoproterenol were purchased from Sigma; Gpp(NH)p was purchased from Boehringer Mannheim; choleragen and diethiothreitol were from Schwarz/Mann; propanolol-HCl was obtained from Ayerst Laboratories; [α-32P]GTP (10-30 Ci/mmol) and [β-32P]GTP (5-15 Ci/mmol) were obtained from New England Nuclear; polyethyleneimine-cellulose thin layer chromatography sheets were from Brinkmann.

Protein was measured by the method of Lowry et al. (13) using bovine serum albumin as standard. Just before addition to membranes, choleragen, 1 mg/ml, was activated by incubation for 10 min at 30°C in 50 mM glycine buffer, pH 8.0, containing 20 mM diethiothreitol.

Turkey erythrocyte membranes were prepared as described previously (14). The procedure used to determine release of bound guanyl nucleotide from membranes was very similar to that of Cassel and Selinger (12). Membranes were washed twice with 10 volumes of 50 mM Tris-HCl, pH 7.5, containing 0.1 mM EGTA, suspended in 50 mM Tris-HCl, pH 7.5 (hereinafter referred to as buffer), and incubated in buffer containing 0.1 mM KC1, 6 mM MgCl2, 0.1 mM EGTA, 1 mM diethiothreitol, 0.2 mM App(NH)p, 1 mM phosphoenolpyruvate, and pyruvate kinase, 75 units/ml, (total volume, 1.5 ml) for 2 min at 37°C (membrane protein concentration, 2-3 mg/ml). Isoproterenol and [α-32P]GTP or [β-32P]GTP (5-6000 cpm/pmol), each in 30 μl of H2O, were then added to final concentrations of 20 and 0.3 μM, respectively, followed after 2 min at 37°C by propranolol and unlabeled GTP (final concentrations, 10 and 100 μM, respectively). Membranes were sedimented by centrifugation (17,000 g, 5 min), washed three times with eight volumes of ice-cold buffer, and suspended in five volumes of ice-cold buffer containing 0.05 M KCl, 3 mM MgCl2, 0.05 mM EGTA, 0.5 mM diethiothreitol, 0.1 mM App(NH)p, 0.5 mM GTP, 0.5 mM phosphoenolpyruvate, and pyruvate kinase, 37 units/ml (~1 mg of membrane protein/ml). After incubation at 37°C for 10 min, membranes were sedimented by centrifugation and then washed with eight volumes of buffer. For all experiments except that shown in Table II, the basal (unstimulated) release of guanyl nucleotides was decreased by incubating membranes prior to the experimental period for 1 h at 37°C in the medium described above (~1 mg of protein/ml and subsequently washing the membranes with eight volumes of buffer. To quantify release of the bound nucleotide, samples of membranes prepared in this way (~0.2 mg of protein/ml) were added to buffer containing 5 mM MgCl2, 0.25 mM App(NH)p, 1 mM GTP, 0.3 mM Gpp(NH)p, and other additions as noted in tables (total volume, 0.4 ml). After incubation at 37°C for the indicated time, assays were terminated by the addition of 1.3 ml of ice-cold buffer followed by centrifugation (1000 × g, 20 min) and samples of supernatants were taken for radioassay. Data reported are means ± S.E. of values from triplicate incubations.

Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 257, No. 1, Issue of January 10, 1982
Printed in U.S.A.
To identify the nucleotide released by choleragen, membranes loaded with [α-³²P]GTP and washed as described above were incubated at 37 °C in the medium used for assay of nucleotide release containing 2 mM GDP and 2 mM NAD with or without choleragen, 250 μg/ml (0.5 mg of membrane protein in 1 ml). After 1 h, the samples were cooled to 0 °C and centrifuged at 17,000 × g for 15 min. Samples (100 μl) of supernatants were applied to polyethyleneimine-cellulose thin layer sheets. Guanyl nucleotides were separated by chromatography with 1.2 M LiCl. Membranes incubated with choleragen released 3,110 and those incubated without, 2,380 cpm/mg of protein/h. Of the radioactivity recovered from chromatograms of choleragen-treated and control samples, respectively, 13 and 12% co-migrated with GTP, 27 and 36% with GMP, and 60 and 52% with GDP. From these data, it was calculated that 85% of the labeled nucleotide released by choleragen was GDP.

RESULTS AND DISCUSSION

Membranes that had bound radiolabeled GTP in the presence of isoproterenol and had then been extensively washed as described under "Materials and Methods" released radiolabeled guanyl nucleotide at a relatively constant rate during incubation at 37 °C in the release assay medium; 40 to 50% of the bound nucleotide was released in 1 h. As reported by Cassel and Selinger (12), addition of isoproterenol produced an immediate increase in the rate of release, which returned to the basal rate in 2 to 3 min. Addition of choleragen and NAD also accelerated release but to a lesser degree (Fig. 1). In this case, the rate of release did not return to the basal rate for almost 90 min.

Addition of choleragen or NAD alone did not alter the release of guanyl nucleotide, whereas, in the experiment shown in Table I, when both were present release was increased from 0.33 to 0.45 pmol/mg of protein/h. When NADP, which does not serve as a substrate for choleragen (15), was substituted for NAD, no stimulation of release was observed (Table I), consistent with the view that choleragen enhances release by catalyzing the ADP ribosylation of a membrane protein.

Incubation of membranes with choleragen and NAD for 1 h (followed by washing) before addition of isoproterenol virtually abolished the effect of the β-adrenergic agonist on guanyl nucleotide release. In the experiment shown in Fig. 1, isoproterenol-stimulated release and cholera-stimulated release of guanyl nucleotides. Membranes that had bound [³²P]GTP were prepared and incubated at 37 °C for the indicated times for assay of guanyl nucleotide release as described under "Materials and Methods" and the further addition of 2 mM NAD. In one experiment, samples were incubated with or without 50 μM isoproterenol and with or without choleragen, 250 μg/ml, in another. The increment in nucleotide released induced by isoproterenol (●) or choleragen (○) is shown. Release in the absence of additions was 0.16 pmol/mg of protein/3 min in the experiment in which isoproterenol-stimulated release was measured and 1.00 pmol/mg of protein/3 h when choleragen-stimulated release was measured.

FIG. 1. Time course of isoproterenol-stimulated and cholera-stimulated release of guanyl nucleotides. Membranes that had bound [³²P]GTP were prepared and incubated at 37 °C for the indicated times for assay of guanyl nucleotide release as described under "Materials and Methods" and with further addition of 2 mM NAD. In one experiment, samples were incubated with or without 50 μM isoproterenol and with or without choleragen, 250 μg/ml, in another. The increment in nucleotide released induced by isoproterenol (●) or choleragen (○) is shown. Release in the absence of additions was 0.16 pmol/mg of protein/3 min in the experiment in which isoproterenol-stimulated release was measured and 1.00 pmol/mg of protein/3 h when choleragen-stimulated release was measured.

| Membranes incubated with | Addition during release | Nucleotide released | Effect of isoproterol |
|---------------------------|------------------------|---------------------|----------------------|
| NAD                       | None                   | 0.13 ± 0.024        | +0.12                |
| NAD plus choleragen       | Isoproterenol          | 0.25 ± 0.017        | +0.06                |
|                           | None                   | 0.12 ± 0.008        | +0.01                |
|                           | Isoproterenol          | 0.14 ± 0.010        | +0.02                |

Table I

| Additions      | Nucleotide released pmol/mg protein/h |
|----------------|--------------------------------------|
| None           | 0.33 ± 0.017                          |
| Choleragen, 250 μg/ml | 0.32 ± 0.016                      |
| NAD, 2 mM      | 0.33 ± 0.004                          |
| NAD plus choleragen | 0.45 ± 0.014                    |
| NADP, 2 mM     | 0.32 ± 0.017                          |
| NADP plus choleragen | 0.33 ± 0.006                    |

Table II

These findings suggest a mechanism of choleragen action whereby the toxin catalyzes ADP ribosylation of the G/F which results in a decreased affinity of the G/F for guanine nucleotides. Since, in the absence of hormones, the unmodified G/F is believed to bind GDP very tightly, the effect of toxin modification would be to stimulate release of this nucleotide, thus emptying the nucleotide-binding sites on this protein. GTP could then bind and activation of adenylate cyclase would ensue. This mechanism is consistent with the recent findings of Lad et al. (16), who demonstrated that pretreat-
ment of turkey erythrocyte membranes with choleragen decreases the time lag normally observed for activation of adenylate cyclase by the poorly hydrolyzable GTP analog, Gpp(NH)p. Therefore, choleragen may activate adenylate cyclase, at least in part, by a mechanism similar to that of β-adrenergic hormones; that is, both choleragen and β-adrenergic hormones may decrease the affinity of G/F for guanyl nucleotides, thus increasing the rate of release of GDP and freeing the site for subsequent binding of the activating ligand, GTP.

Acknowledgment—We thank D. Marie Sherwood for expert secretarial assistance.

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