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Biochemistry

Enhancement of choleragen ADP-ribosyltransferase activities by guanyl nucleotides and a 19-kDa membrane protein

(adenylate cyclase/cholera toxin)

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ABSTRACT **Choleragen activates adenylate cyclase by catalyzing, in the presence of NAD, the ADP-ribosylation of $G_{s\alpha}$, the stimulatory guanyl nucleotide-binding protein of the cyclase system. Kahn and Gilman [Kahn, R. A. & Gilman, A. G. (1986) *J. Biol. Chem.* 261, 7906–7911] identified another guanyl nucleotide-binding protein termed ADP-ribosylation factor (ARF) that stimulated this reaction. It was proposed that the toxin substrate is an ARF-$G_{s\alpha}$ complex and that ARF may have a physiological role in regulation of $G_{s\alpha}$ activity. We have found that purified ARF from bovine brain enhances not only the ADP-ribosylation of $G_{s\alpha}$ but also $G_{s\alpha}$-independent choleragen-catalyzed reactions. These are (*i*) ADP-ribosylation of agmatine, a low molecular weight guanidino compound; (*ii*) ADP-ribosylation of several proteins unrelated to $G_{s\alpha}$; and (*iii*) auto-ADP-ribosylation of the toxin A_1 peptide. These reactions, as well as the ADP-ribosylation of ARF itself, were stimulated by GTP or stable GTP analogues such as guanyl-5′-yl imido-$\beta\gamma$-diphosphate and guanosine 5′-*O*-[γ-thio]triphosphate; GDP and guanosine 5′-*O*-[β-thio]diphosphate were inactive. These observations are consistent with the conclusion that ARF interacts directly with the A subunit of choleragen in a GTP-dependent fashion thereby enhancing catalytic activity manifest as transfer of ADP-ribose to $G_{s\alpha}$ and other proteins, to the toxin A_1 peptide, or to agmatine. It is tempting to speculate that ARF may be involved in regulating one or another of the ADP-ribosyltransferases found in animal cells.**

Choleragen activates adenylate cyclase by catalyzing the ADP-ribosylation of a regulatory component of the cyclase complex (1). The adenylate cyclase system is composed of stimulatory and inhibitory receptors coupled through guanyl nucleotide-binding proteins, termed G_s and G_i, respectively, to a catalytic unit that converts ATP to cAMP (1, 2). G_s and G_i are heterotrimers composed of α, β, and γ subunits (1, 3). The α subunits bind and hydrolyze GTP (1). G_s and G_i are activated when GTP or a nonhydrolyzable GTP analogue—(e.g., guanylyl imidodiphosphate (p[NH]ppG), guanosine 5′-*O*-[γ-thio]triphosphate (GTP[γ-S])—is bound (1). Activation is believed to result from dissociation of the α and $\beta\gamma$ components (1, 4). Hydrolysis of GTP to GDP is associated with inactivation and subunit reassociation (1). Choleragen-catalyzed ADP-ribosylation of G_s α subunit ($G_{s\alpha}$) inhibits GTP hydrolysis (5) and stabilizes an active $G_{s\alpha}$·GTP dissociated from guanyl nucleotide-binding protein $\beta\gamma$ subunit ($G_{\beta\gamma}$) (6). ADP-ribosylation of $G_{s\alpha}$ is promoted by GTP and protein factors from both membrane and cytosolic fractions (6–16). Kahn and Gilman purified a membrane protein, termed ADP-ribosylation factor (ARF), that enhanced choleragen-catalyzed ADP-ribosylation of purified $G_{s\alpha}$ (15). The effect of ARF was dependent on GTP and it was shown that ARF is a guanyl nucleotide-binding protein (15, 16). It was proposed that ARF complexes with $G_{s\alpha}$ and thereby promotes the ability of $G_{s\alpha}$ to serve as a toxin substrate, leading to the speculation that ARF might have a physiological role in the regulation of $G_{s\alpha}$ activity (6).

In addition to catalyzing the ADP-ribosylation of G_s, choleragen exhibits other related enzymatic activities that presumably reflect its ability to activate the high-energy ribosyl-nicotinamide bond of NAD (17, 18). The toxin catalyzes (*i*) the hydrolysis of NAD (19), (*ii*) the ADP-ribosylation of arginine, and other simple guanidino compounds such as agmatine (20, 21), (*iii*) the modification of proteins unrelated to $G_{s\alpha}$ (presumably due to the presence of one or more accessible arginines) (22, 23), and (*iv*) the auto-ADP-ribosylation of the choleragen A_1 peptide (24, 25):

(*i*) NAD + HOH → ADP-ribose + nicotinamide + H^+
(*ii*) NAD + guanidino-R → ADP-ribose-guanidino-R + nicotinamide + H^+
(*iii*) NAD + protein → ADP-ribose-protein + nicotinamide + H^+
(*iv*) NAD + choleragen-A_1 → ADP-ribose-choleragen-A_1 + nicotinamide + H^+.

While investigating the ADP-ribosylation of purified $G_{s\alpha}$, we observed that ARF also enhanced the modification of other proteins as well as the auto-ADP-ribosylation of the choleragen A_1 peptide. Further studies established, as reported here, that ARF enhanced the activity of the toxin in each of the four reactions outlined above and thus does not act specifically to facilitate the ADP-ribosylation of $G_{s\alpha}$.

MATERIALS AND METHODS

Materials. DEAE-Sephacel and Blue Sepharose CL-6B were purchased from Pharmacia Fine Chemicals; Ultrogel AcA 44 and Ultrogel AcA 54 were from LKB (Bromma, Sweden); choleragen and choleragen A subunit were from List Biochemical Laboratories (Campbell, CA); [α-^{32}P]NAD (20–40 Ci/mmol; 1 Ci = 37 GBq) was from New England Nuclear; [*carbonyl*-^{14}C]NAD (30–50 Ci/mol) was from Amersham; GTP, ATP, NAD, and L-α-dimyristoyl phosphatidylcholine were from Sigma; guanosine 5′-*O*-[β-thio]diphosphate (GDP[β-S]), GTP[γ-S], and p[NH]ppG were from Boehringer Mannheim; adenylyl imidodiphosphate (p[NH]ppA) was from ICN; and GDP was from P-L Biochemicals. Heptylamine-Sepharose was prepared as described by Sternweis *et al.* (26).

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Abbreviations: GDP[β-S], guanosine 5′-*O*-[β-thio]diphosphate; GTP[γ-S], guanosine 5′-*O*-[γ-thio]triphosphate; p[NH]ppG, guanylyl imidodiphosphate; p[NH]ppA, adenylyl imidodiphosphate; G_s and G_i, stimulatory and inhibitory guanyl nucleotide-binding proteins of adenylate cyclase, respectively; $G_{s\alpha}$, α subunit of G_s; T_α and $T_{\beta\gamma}$, α and $\beta\gamma$ subunit of transducin; ARF, ADP-ribosylation factor.

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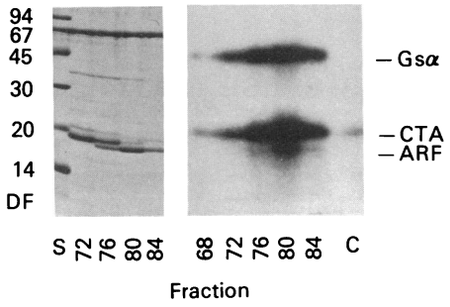

FIG. 1. Purification of ARF. After purification through the hydroxylapatite step (see *Materials and Methods*), the ARF preparation was chromatographed on Ultrogel AcA 54. (*Left*) $NaDodSO_4$ gel electrophoresis of column fractions as numbered. S, molecular mass standards: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), α-lactalbumin (14 kDa). (*Right*) Autoradiograph showing proteins ADP-ribosylated by choleragen in the presence of indicated fractions. C, column buffer control; CTA, choleragen A_1 peptide. Choleragen (25 μg) was incubated with G_s (0.2 μg), 250 μM GTP, 1 mM dimyristoyl phosphatidylcholine, and 20 μl of column fraction or buffer for 60 min at 30°C. DF, dye front.

Purification of Proteins. ARF was purified by the procedure of Kahn and Gilman (16) from bovine brain membranes prepared as described by Sternweis *et al.* (26). Briefly, bovine cortex (170 g) was homogenized (Polytron) in 680 ml of buffer A (20 mM Tris·HCl, pH 8.0/1 mM EDTA/1 mM dithiothreitol/1 mM NaN_3) containing 10% sucrose and 0.5 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged and the pellet was dispersed (Teflon homogenizer) in 680 ml of homogenizing buffer. After centrifugation, the pellet was washed once more in the same way. The final pellet (6.9 g of protein) dispersed in 510 ml of buffer A was added to 510 ml of buffer A containing 2% sodium cholate, stirred for 1 hr at 4°C and centrifuged (140,000 × *g*, 1 hr). The clear supernatant (732 ml) was supplemented with 10 μM $AlCl_3$/6 mM $MgCl_2$/10 mM NaF, and chromatographed sequentially on DEAE-Sephacel, Ultrogel AcA 44, and heptylamine-Sepharose. Fractions with ARF activity were pooled, concentrated, and applied to a column (1.2 × 93 cm) of Ultrogel AcA 54 equilibrated and eluted with buffer A containing 0.25 M sucrose, 0.1 M NaCl, and 5 mM $MgCl_2$. Active fractions were pooled and applied to a column of hydroxylapatite, which was eluted with a linear gradient (0–300 mM) of potassium phosphate. Active fractions were concentrated

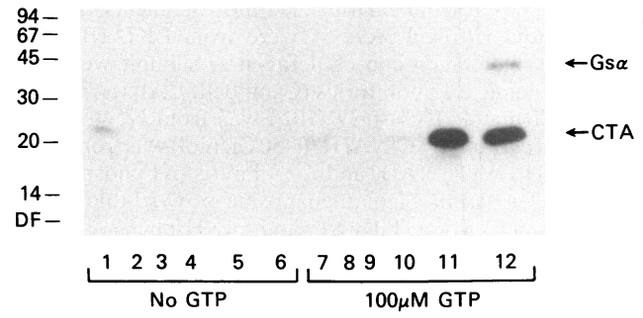

FIG. 2. Effect of GTP and ARF on ADP-ribosylation of $G_{s\alpha}$ by choleragen. ADP-ribosylation assays containing choleragen (25 μg), ARF (1.1 μg), G_s (0.2 μg), and/or 100 μM GTP as indicated were incubated for 60 min at 30°C before proteins were separated by electrophoresis. All assays contained 1 mM dimyristoyl phosphatidylcholine. Lanes: 1–6, no GTP; 7–12, 100 μM GTP; 1 and 7, choleragen; 2 and 8, ARF; 3 and 9, G_s; 4 and 10, choleragen and G_s; 5 and 11, choleragen and ARF; 6 and 12, choleragen, G_s, and ARF. DF, dye front.

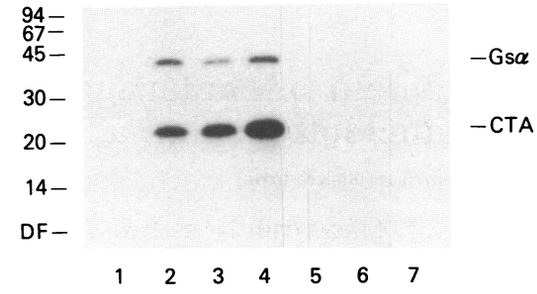

FIG. 3. Effect of nucleotides on ADP-ribosylation by choleragen in the presence of ARF. Assays contained choleragen (25 μg), ARF (1.1 μg), G_s (0.2 μg), 1 mM dimyristoyl phosphatidylcholine, and the indicated nucleotide (10 μM). Lanes: 1, no nucleotide; 2, GTP; 3, p[NH]ppG; 4, GTP[γ-S]; 5, GDP; 6, GDP[β-S]; 7, p[NH]ppA. DF, dye front.

and chromatographed again on Ultrogel AcA 54. Samples of fractions subjected to $NaDodSO_4$/15% polyacrylamide gel electrophoresis and assay of ARF activity established that activity was associated with a peptide of ≈19 kDa (Fig. 1). These fractions were pooled and used for the experiments described here.

G_s was purified from rabbit liver membranes essentially as described by Sternweis *et al.* (26). Transducin was extracted from bovine rod outer segments with 100 μM GTP (27) and subunits were separated using Blue Sepharose CL-6B (28).

Assay of ARF Activity. Stimulation of choleragen-catalyzed ADP-ribosylation of $G_{s\alpha}$ was used to assess ARF activity during purification. Assays (total vol, 100 μl) contained choleragen (usually 25 μg), 10 μM [^{32}P]NAD (≈2 μCi), G_s (usually 0.2 μg), 1 mM dimyristoyl phosphatidylcholine, 5 mM $MgCl_2$, ARF preparation, and other additions as indicated. After incubation at 30°C for 60 min, 2 ml of cold 7.5% trichloroacetic acid and 10 μg of bovine serum albumin were added, and samples were kept on ice for 30 min. Precipitated proteins were pelleted by centrifugation, dissolved in 1% $NaDodSO_4$/5% mercaptoethanol (65°C, 10 min), and subjected to electrophoresis in 15% polyacrylamide gels by the

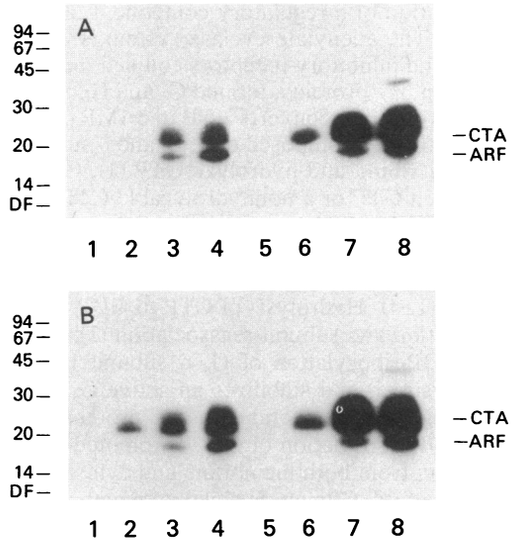

FIG. 4. Effect of ARF on ADP-ribosylation catalyzed by choleragen (*A*) or its A subunit (*B*). Assays contained 100 μM GTP with the indicated amounts of ARF and choleragen or A subunit. (*A*) Lanes 1–4, 10 μg of choleragen; lanes 5–8, 25 μg of choleragen. (*B*) Lanes 1–4, 2.5 μg of A subunit; lanes 5–8, 6.2 μg of A subunit. (*A* and *B*) Lanes: 1 and 5, no ARF; 2 and 6, 0.6 μg of ARF; 3 and 7, 1.1 μg of ARF; 4 and 8, 2.8 μg of ARF. DF, dye front.

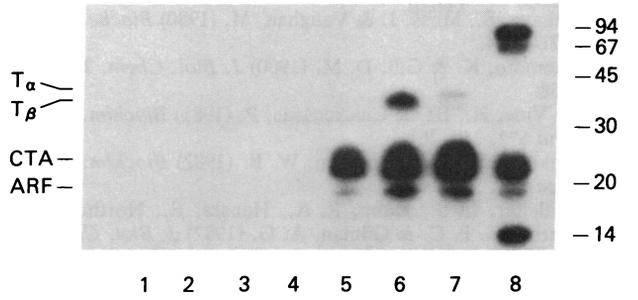

FIG. 5. Effect of ARF on ADP-ribosylation of proteins catalyzed by choleragen. Assays contained 25 μg of choleragen and 100 μM GTP. Lanes: 1–4, no ARF; 5–8, 1.1 μg of ARF; 1 and 5, no added protein; 2 and 6, 5 μg of $T_{\beta\gamma}$; 3 and 7, 3.8 μg of T_α; 4 and 8, mixture of standard proteins (4 μg of phosphorylase b, 5 μg of bovine serum albumin, 8.8 μg of ovalbumin, 5 μg of carbonic anhydrase, 5 μg of soybean trypsin inhibitor, 7.3 μg of α-lactalbumin).

method of Laemmli (29). Gels were exposed to Kodak X-Omat AR film.

Enzyme Assays. NAD:agmatine ADP-ribosyltransferase activity was assayed as described (19, 20) in a total vol of 300 μl, containing 50 mM potassium phosphate (pH 7.5), 10 mM agmatine, 100 μM [*carbonyl*-^{14}C]NAD (≈36,000 cpm), 30 μg of ovalbumin, 5 mM $MgCl_2$, 100 μM GTP, and 20 mM dithiothreitol with or without purified ARF as indicated. Reactions, run in duplicate, were initiated with choleragen (2.5 μg) or choleragen A subunit (1 μg). After 60 min at 30°C, two 0.1-ml samples were transferred to columns (0.5 × 2 cm) of AG 1-x2, which were washed four times with 1.2 ml of water. Eluates containing [*carbonyl*-^{14}C]nicotinamide were collected for radioassay. NAD glycohydrolase activity was assayed as described for ADP-ribosyltransferase activity, except that agmatine was omitted (19, 20).

RESULTS

The protein composition of fractions from Ultrogel AcA 54 gel permeation chromatography of ARF is shown in Fig. 1 (*Left*). One band at 19 kDa comigrated with ARF activity, as reflected by the enhancement of choleragen-catalyzed ADP-ribosylation of $G_{s\alpha}$ (*Right*). Fractions containing ARF increased the auto-ADP-ribosylation of the choleragen A_1 peptide (*Right*) and ARF was itself ADP-ribosylated. (ADP-ribosylated A_1 and ARF were better separated in most other experiments; e.g., see Figs. 4 and 5.)

As noted by Kahn and Gilman (16), in the absence of GTP, ADP-ribosylation of $G_{s\alpha}$ (as well as auto-ADP-ribosylation of the toxin A subunit) was minimal with or without ARF. In the presence of GTP, ARF enhanced modification of $G_{s\alpha}$ and dramatically increased auto-ADP-ribosylation (Fig. 2). In the presence of ARF, p[NH]ppG and GTP[γ-S], like GTP, enhanced labeling of $G_{s\alpha}$ and the choleragen A_1 subunit, whereas GDP, GDP[β-S], and p[NH]ppA, each at 10 μM, were ineffective (Fig. 3).

ARF enhanced activity of the choleragen A subunit as well as that of the holotoxin in a concentration-dependent fashion (Fig. 4). Increased auto-ADP-ribosylation of the A_1 peptide was clearly dependent on ARF concentration and the ADP-ribosylated toxin A_1 peptide was well separated from ARF (Fig. 4). ADP-ribosylation of several other proteins was also increased by ARF. These included the β subunit of transducin (T_β), and to a lesser degree T_α, as well as phosphorylase b, serum albumin, and α-lactalbumin (Fig. 5).

The ADP-ribosylation of agmatine by choleragen A subunit was increased by ARF in a GTP-dependent reaction (Table 1). GTP[γ-S] and p[NH]ppG were also effective, whereas p[NH]ppA, ATP, GDP, and GDP[β-S] were inactive (Table 2). The stimulatory effect of ARF on the NAD:agmatine ADP-ribosyltransferase activity was specific; ovalbumin at 300 times the ARF concentration did not affect activity (data not shown). In the NAD-agmatine ADP-ribosyltransferase assay, release of [*carbonyl*-^{14}C]nicotinamide from [*carbonyl*-^{14}C]NAD is used as an index of activity. Auto-ADP-ribosylation and ADP-ribosylation to ARF also contribute to nicotinamide release, but the increase (≈2 nmol in Table 2) caused by 1 μg (≈53 pmol) of ARF with 1 μg (≈34 pmol) of choleragen A subunit could not be accounted for by these reactions. In the absence of agmatine, the release of [*carbonyl*-^{14}C]nicotinamide from [*carbonyl*-^{14}C]NAD is considerably slower. This was also enhanced by ARF (data not shown), but it is difficult to assess just how much of the increase reflects increased NAD glycohydrolase activity since, in this case, the contribution of auto-ADP-ribosylation and ADP-ribosylation of ARF to nicotinamide release could be significant.

Table 1. Effect of ARF and GTP on NAD:agmatine ADP-ribosyltransferase activity of choleragen A subunit

ARF	Transferase activity, nmol per μg per 60 min	
	No GTP	100 μM GTP
None	1.00	0.83
1 μg	1.03	2.66
3 μg	—	3.52

Assays contained 1 μg of choleragen A subunit with other additions as indicated.

DISCUSSION

It has been proposed that ARF, in the presence of GTP, enhances the choleragen-catalyzed ADP-ribosylation of $G_{s\alpha}$ by forming an ARF–$G_{s\alpha}$ complex, thereby increasing the availability of the arginine in $G_{s\alpha}$ that is modified by the toxin (15). The data reported here demonstrate, however, that ARF can interact with the toxin independent of $G_{s\alpha}$ and in doing so increases the catalytic activity of the A_1 peptide in three $G_{s\alpha}$-independent reactions. It was found that, in addition to stimulating choleragen-catalyzed ADP-ribosylation of $G_{s\alpha}$, ARF enhances the auto-ADP-ribosylation of the toxin A_1 peptide, the ADP-ribosylation of agmatine, and the hydrolysis of NAD to ADP-ribose and nicotinamide. ARF was itself ADP-ribosylated and it will be important to ascertain the effect of this modification on its interaction with GTP and/or its function. Although $G_{s\alpha}$ is also a guanyl nucleotide-binding protein, our data are most consistent with the conclusion that a primary effect of guanyl nucleotides in enhancing choleragen-catalyzed ADP-ribosylation results from their interaction with ARF. In the choleragen-catalyzed reactions that are

Table 2. Effect of nucleotides on NAD:agmatine ADP-ribosyltransferase activity of choleragen A subunit in the presence of ARF

Addition (100 μM)	Transferase activity, nmol per μg per 60 min
None	1.09
GTP	3.16
p[NH]ppG	2.88
GTP[γ-S]	2.67
GDP	1.28
GDP[β-S]	1.16
p[NH]ppA	1.20
ATP	1.07

Assays contained 1 μg of choleragen A subunit and 1 μg of ARF.

independent of $G_{s\alpha}$, it is clear that the activating species is ARF·GTP.

The positive allosteric effect of ARF on the toxin A_1 peptide does not exclude an ARF–$G_{s\alpha}$ interaction. It does, however, introduce the possibility of an alternative hypothesis, wherein the primary interaction of ARF is with the toxin. It seems not unlikely that association of ARF with A_1 enhances its catalytic activity toward the major intracellular target $G_{s\alpha}$, just as it does toward other unrelated proteins and alternative ADP-ribose acceptors such as agmatine. If, as it now appears, ARF does not specifically facilitate choleragen-catalyzed ADP-ribosylation of $G_{s\alpha}$, there is no compelling reason to believe that these two guanyl nucleotide-binding proteins interact functionally in the cell and it becomes necessary to seek another role for ARF. Why choleragen activity should be stimulated by an endogenous guanyl nucleotide-binding protein is unclear. It is tempting to speculate that ARF may be involved in regulating one or another of the cellular NAD:arginine ADP-ribosyltransferases.

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