Mechanism of Activation of Cholera Toxin by ADP-Ribosylation Factor (ARF): Both Low- and High-Affinity Interactions of ARF with Guanine Nucleotides Promote Toxin Activation

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ABSTRACT: Activation of adenylyl cyclase by cholera toxin A subunit (CT-A) results from the ADP-ribosylation of the stimulatory guanine nucleotide binding protein ($G_{s\alpha}$). This process requires GTP and an endogenous guanine nucleotide binding protein known as ADP-ribosylation factor (ARF). One membrane (mARF) and two soluble forms (sARF I and sARF II) of ARF have been purified from bovine brain. Because the conditions reported to enhance the binding of guanine nucleotides by ARF differ from those observed to promote optimal activity, we sought to characterize the determinants influencing the functional interaction of guanine nucleotides with ARF. High-affinity GTP binding by sARF II (apparent K_D of ~70 nM) required Mg^{2+}, DMPC, and sodium cholate. sARF II, in DMPC/cholate, also enhanced CT-A ADP-ribosyltransferase activity (apparent EC_{50} for GTP of ~50 nM), although there was a delay before achievement of a maximal rate of sARF II stimulated toxin activity. The delay was abolished by incubation of sARF II with GTP at 30 °C before initiation of the assay. In contrast, a maximal rate of activation of toxin by sARF II, in 0.003% SDS, occurred without delay (apparent EC_{50} for GTP of ~5 μM). High-affinity GTP binding by sARF II was not detectable in SDS. Enhancement of CT-A ADP-ribosyltransferase activity by sARF II, therefore, can occur under conditions in which sARF II exhibits either a relatively low affinity or a relatively high affinity for GTP. The interaction of GTP with ARF under these conditions may reflect ways in which intracellular membrane and cytosolic environments modulate GTP-mediated activation of ARF.

Cholera toxin activates adenylyl cyclase by catalyzing the ADP-ribosylation of $G_{s\alpha}$,[1] the stimulatory guanine nucleotide binding protein of the adenylyl cyclase system (Cassel & Pfeuffer, 1978; Gill & Meren, 1978; Johnson et al., 1978; Northup et al., 1980; Richter, 1987; Casey & Gilman, 1988). Certain membrane-bound and soluble factors, in association with GTP, have been found to enhance the cholera toxin mediated ADP-ribosylation of $G_{s\alpha}$ and/or augment the activation of adenylyl cyclase (Enmoto & Gill, 1980; Le Vine & Cuatrecasas, 1981; Pinkett & Anderson, 1982; Schleifer et al., 1982; Gill & Meren, 1983; Kahn & Gilman, 1984, 1986; Gill & Coburn, 1987; Tsai et al., 1987, 1988b; Noda et al., 1988). One of these factors, referred to as ADP-ribosylation factor (ARF), was purified from rabbit liver membranes (Kahn & Gilman, 1984). ARF, purified from bovine brain membranes, was itself found to be a GTP-binding protein; dimyristoylphosphatidylcholine (DMPC), Mg^{2+}, and NaCl were required for high-affinity binding of GTPγS to ARF (Kahn & Gilman, 1986).

Recently, we purified one membrane (mARF) and two soluble (sARF I, sARF II) forms of ARF from bovine brain. These are ~19-kDa proteins (by SDS–PAGE) with very similar properties (Tsai et al., 1987, 1988b). These ARF proteins enhanced the ADP-ribosylation of several proteins unrelated to $G_{s\alpha}$ and auto-ADP-ribosylation of the cholera toxin A_1 peptide (Tsai et al., 1987, 1988b). These results are consistent with a direct effect of ARF on toxin, rather than on $G_{s\alpha}$, as was originally proposed (Kahn & Gilman, 1984, 1986). ARF activity required GTP or an analogue (Tsai et al., 1987, 1988b) and was enhanced by very low, nondenaturing concentrations of SDS (Noda et al., 1988). ARF activity did not require DMPC or NaCl, which had been reported necessary for the high-affinity binding of GTPγS by mARF (Kahn & Gilman, 1986). However, DMPC, in the presence of cholate, enhanced ARF stimulation of the ADP-ribosylation of $G_{s\alpha}$ by cholera toxin (Tsai et al., 1988b).

To investigate the basis for these apparent disparities, we sought to determine how conditions favoring high-affinity GTP binding affected ARF stimulation of CT-A directly and, similarly, how conditions selected for maximal ARF activity affected high-affinity binding of GTP by ARF. As reported here, enhancement of CT-A ADP-ribosyltransferase activity by sARF II is unusual in that it can occur under conditions in which different species of sARF exhibit a relatively low or high affinity for GTP, with different time courses of sARF II activation under the two conditions. These interactions of GTP with ARF in vitro may reflect the way in which intracellular membrane and cytosolic environments modulate GTP-mediated activation of ARF.

Experimental Procedures

Materials. GTP, NAD, agmatine, and dimyristoylphosphatidylcholine (DMPC) were purchased from Sigma; GTPγS was from Boehringer Mannheim; the A subunit of cholera toxin (CT-A) was from List Biologicals; sodium do-

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[1] Abbreviations: G_s, stimulatory guanine nucleotide binding protein of the adenylate cyclase system; $G_{s\alpha}$, α subunit of G_s; mARF and sARF, membrane-bound and soluble ADP-ribosylation factors, respectively; CT, cholera toxin; CT-A, A subunit of cholera toxin; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); DMPC, dimyristoylphosphatidylcholine; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

decyl sulfate (SDS) and AG 1-X2 anion-exchange resin were from Bio-Rad; sodium cholate was from Calbiochem; nitrocellulose filters (25-mm diameter) were from Millipore Corp.; [^{3}H]GTP (38 Ci/mmol), [γ-^{32}P]GTP (1300 Ci/mmol), and [^{32}P]NAD (20–40 Ci/mmol) were from Dupont–New England Nuclear; and [*adenine*-^{14}C]NAD (300 Ci/mmol) was from Amersham Corp.

Purification of ARF and $G_{s\alpha}$. sARF II was purified from the supernatant fraction of homogenized bovine cerebral cortex by sequential chromatography on CM-Sepharose (pH 7.0), CM-Sepharose (pH 5.3), hydroxylapatite, and Ultrogel AcA 54 (Tsai et al., 1988b). $G_{s\alpha}$ was purified from rabbit liver membranes essentially as described by Sternweis et al. (1981).

Assays. Binding of nucleotides to sARF II was determined by a modification (Kahn & Gilman, 1986) of a rapid filtration technique (Northup et al., 1982). sARF II was incubated at 30 °C in 20 mM HEPES (pH 8)/1 mM EDTA/1 mM dithiothreitol (DTT)/6 mM $MgCl_2$/[^{3}H]GTP [(6–8) × 10^5 cpm, concentration as indicated] with sodium cholate, DMPC, and/or NaCl as indicated (total volume 100 μL). At the indicated time, samples (50 μL) were transferred to nitrocellulose filters in a rapid-filtration manifold (Millipore) followed by washing 5 times, each with 2 mL of 25 mM Tris-HCl (pH 8)/100 mM NaCl/10 mM $MgCl_2$/1 mM DTT. After being dried (heat lamp), filters were dissolved in 8 mL of liquid scintillation fluid for radioassay. Data, corrected for quenching caused by filters, are presented as means of values from triplicate assays. Binding data were analyzed by using the LIGAND computer program to estimate the affinity of sARF II for nucleotide (Munson & Rodbard, 1980). In the present study, high-affinity GTP binding to sARF II occurred with a stoichiometry of 0.09–0.30 (usually about 0.15) mol/mol. It is possible that the remainder of the sARF II represents a species that is able to interact functionally with GTP, albeit with an affinity too low to be measured in the binding assay.

GTP hydrolysis was assayed by a modification (Kanaho et al., 1984) of the procedure of Brandt et al. (1983). sARF II was incubated at 30 °C in 20 mM Tris-HCl, pH 8, 20 mM potassium phosphate, pH 7.5, or 20 mM HEPES, pH 8, containing 1 mM EDTA/6 mM $MgCl_2$/1 mM DTT/5 μM [γ-^{32}P]GTP [(0.5–1.0) × 10^6 cpm] with DMPC, sodium cholate, NaCl, NAD, agmatine, CT-A, ovalbumin, and/or SDS as indicated (total volume 100 μL). At the indicated time, 500 μL of ice-cold 50 mM potassium phosphate, pH 7, containing 12% activated charcoal was added to each tube. After vortexing and centrifugation (1000*g*, 15 min), a sample (200 μL) was removed for radioassay. The zero-time blank was less than 5% of the total. Assays were carried out in triplicate or quadruplicate. Under these conditions, no hydrolysis of GTP by sARF II was detected (data not shown).

Activation of CT-A by sARF II was measured by using the NAD:agmatine ADP-ribosyltransferase assay (Moss & Stanley, 1981; Tsai et al., 1987; Noda et al., 1988). Assays (total volume 300 μL, unless otherwise indicated) contained 100 μM [*adenine*-^{14}C]NAD (60000 cpm), 10 mM agmatine, 20 mM DTT, sARF II (1 μg), CT-A (1 μg), and other additions as indicated with either (1) 50 mM potassium phosphate (pH 7.5)/5 mM $MgCl_2$/ovalbumin (0.1 mg/mL)/0.003% SDS or (2) 20 mM HEPES (pH 8)/1 mM EDTA/6 mM $MgCl_2$/3 mM DMPC/0.2% sodium cholate. After incubation at 30 °C for the indicated time, a sample (50 μL) was transferred to a column of AG 1-X2 which was washed 5 times with 1 mL of water. The eluates, which contained [^{14}C]ADP-ribosylagmatine, were collected for radioassay. Data presented are means of values from triplicate assays,

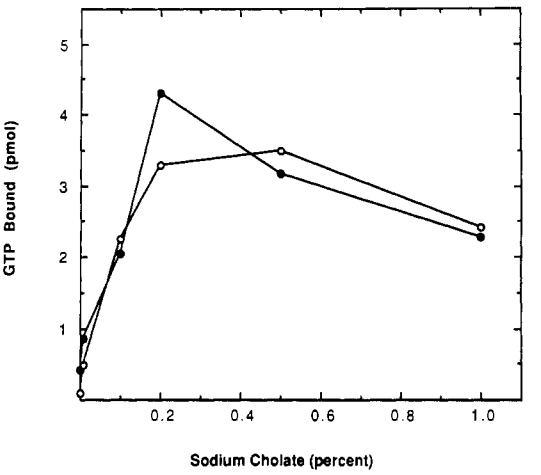

FIGURE 1: Effects of NaCl and sodium cholate on GTP binding by sARF II. sARF II (720 ng, 35 pmol) was incubated (2 h, 30 °C) in 100 μL of 20 mM HEPES (pH 8)/1 mM EDTA/6 mM $MgCl_2$/1 mM DTT/330 nM [^{3}H]GTP (565000 cpm) with the indicated concentration of sodium cholate without (●) or with (○) 400 mM NaCl before assay of bound GTP as described under Experimental Procedures.

unless otherwise noted. In some experiments, CT-A was activated [15 min at 30 °C in 20 mM DTT/50 mM Tris-HCl (pH 7.5)/1 mM EDTA/200 mM NaCl] immediately prior to use in the assay. In some experiments, GTP and/or CT-A was incubated with sARF II before initiation of the transferase assay. In all experiments, the concentrations of sARF II and CT-A in the transferase assay were each 1 μg/300 μL.

ARF-stimulation of the ADP-ribosylation of $G_{s\alpha}$ by CT was determined as described (Tsai et al., 1988b). Assays (total volume 100 μL) containing 25 mM potassium phosphate, pH 7.4, 25 μg of CT previously activated with 30 mM DTT, the indicated concentrations of GTP, 5 mM $MgCl_2$, 20 mM thymidine, $G_{s\alpha}$ (0.2 μg, in 0.3% cholate), 10 μM [^{32}P]NAD (2 μCi) and sARF II (1.3 μg) with or without 1 mM DMPC, were incubated for 60 min at 30 °C before precipitation with trichloroacetic acid, SDS–PAGE, and autoradiography.

RESULTS

Requirements for High-Affinity Binding of Guanine Nucleotides by sARF II. Binding of GTP by sARF II required sodium cholate in addition to Mg^{2+} and DMPC; NaCl (0–800 mM) had no significant effect on GTP binding with or without sodium cholate (Figure 1, Table I). Binding was apparently maximal with a combination of 3 mM DMPC and 0.2% sodium cholate (Figure 2). The apparent K_D for GTP under these conditions (Figure 3) was ~70 nM. Scatchard analysis of these data was consistent with the existence of a single class of binding sites exhibiting relatively high affinity for GTP.

Components of the ADP-Ribosyltransferase Assay Do Not Affect High-Affinity Binding of GTP by sARF II. To permit correlation between the conditions known to enhance sARF II stimulation of CT-A ADP-ribosyltransferase activity and the conditions required for high-affinity GTP binding by sARF II, it was necessary to examine whether components of the ADP-ribosyltransferase assay affected binding of GTP by sARF II. In the presence of DMPC and cholate, there was little or no effect of NAD, agmatine, or increased DTT on the extent of GTP binding (Table II). Enhancement of the ADP-ribosyltransferase activity of cholera toxin by sARF II is reported to require GTP or an analogue (Tsai et al., 1988b) and be increased severalfold by low concentrations of SDS (Noda et al., 1988). In the present study, GTP binding to sARF II under conditions demonstrated to elicit maximal

Table I: Effect of NaCl, DMPC, and Detergent on GTP Binding by sARF II[a]

cholate	DMPC	NaCl	SDS	GTP bound (pmol/assay)
−	−	−	−	0.073 ± 0.031
−	−	+	−	0.230 ± 0.009
−	+	−	−	0.230 ± 0.021
−	+	+	−	0.120 ± 0.020
−	+	−	+	0.126 ± 0.031
−	−	+	+	0.018 ± 0.007
+	−	−	−	0.426 ± 0.003
+	−	+	−	0.494 ± 0.042
+	+	−	−	3.86 ± 0.05
+	+	+	−	2.71 ± 0.15
+	+	−	+	3.57 ± 0.01
+	−	+	+	0.221 ± 0.116

[a] sARF II (500 ng, 24 pmol) was incubated (4 h, 30 °C) in 100 μL of 20 mM HEPES (pH 8)/1 mM EDTA/6 mM $MgCl_2$/400 nM [^{3}H]GTP (680 000 cpm) with other additions as indicated before assay of bound GTP. Data are means ± SEM of duplicate assays. Cholate, 0.2% sodium cholate; DMPC, 3 mM; NaCl, 400 mM; SDS, 0.003%.

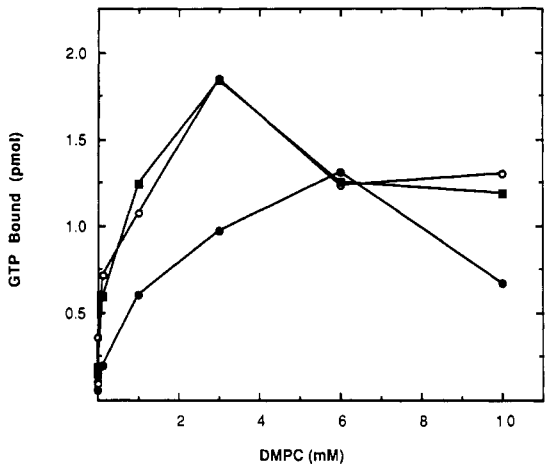

FIGURE 2: Effects of DMPC and sodium cholate on GTP binding by sARF II. sARF II (480 ng, 23 pmol) was incubated (2 h, 30 °C) in 100 μL of 20 mM HEPES (pH 8)/1 mM EDTA/6 mM $MgCl_2$/1 mM DTT/300 nM [^{3}H]GTP (565 000 cpm) with the indicated concentration of DMPC and 0.1% (●), 0.2% (○), or 0.5% (■) sodium cholate before assay of bound GTP.

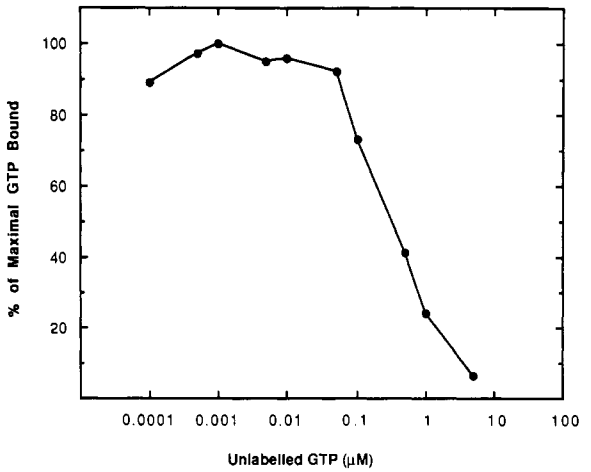

FIGURE 3: Competition by GTP for [^{3}H]GTP binding by sARF II. sARF II (550 ng, 27 pmol) was incubated (4 h, 30 °C) in 100 μL of 20 mM HEPES (pH 8)/1 mM EDTA/6 mM $MgCl_2$/1 mM DTT/3 mM DMPC/0.2% sodium cholate/400 nM [^{3}H]GTP (680 000 cpm) and the indicated concentration of unlabeled GTP before assay of bound GTP. Scatchard analysis performed by using the LIGAND computer program (Munson & Rodbard, 1980) yielded an apparent K_D of 70 nM GTP and maximal GTP binding of 8 pmol, representing 0.30 mol of GTP bound by 1 mol of sARF II.

Table II: Effect of Transferase Assay Components on GTP Binding by sARF II[a]

additions to binding assay					
DMPC/ cholate	DTT (mM)	NAD/AG	CTA	SDS	GTP bound (pmol/assay)
−	20	+	+	+	0.031 ± 0.033
+	20	+	+	−	0.450 ± 0.011
+	1	+	+	−	0.370 ± 0.023
+	1	+	−	−	0.390 ± 0.012

[a] sARF II (100 ng, 4.8 pmol) was incubated (4 h, 30 °C) in 100 μL of 20 mM HEPES (pH 8)/1 mM EDTA/6 mM $MgCl_2$/400 nM [^{3}H]GTP (680 000 cpm) with other additions as indicated before assay of bound GTP. Data are means ± SD of values from triplicate assays. DMPC/cholate, 3 mM DMPC/0.2% sodium cholate; NAD/AG, 100 μM NAD and 10 mM agmatine; CT-A, 100 ng of A subunit of cholera toxin; SDS, 0.003%. Assays with SDS also contained ovalbumin, 0.1 mg/mL.

sARF II stimulation of cholera toxin ADP-ribosyltransferase activity (0.003% SDS) was minimal (Table II and data not shown).

Effects of Detergents and Phospholipid on sARF II Enhancement of CT-A ADP-Ribosyltransferase Activity. In the presence of 0.003% SDS, half-maximal activation of toxin ADP-ribosyltransferase activity by sARF II was observed with ~5 μM GTP (Figure 4A). In contrast, the EC_{50} for GTP under conditions that supported maximal binding (i.e., 3 mM DMPC/0.2% sodium cholate) was ~50 nM (Figure 4B). With cholate alone (Figure 4C), the EC_{50} for GTP was intermediate between that found in the presence of cholate plus DMPC and that in SDS. DMPC and cholate also lowered the apparent EC_{50} for GTP in sARF II stimulation of the ADP-ribosylation of $G_{s\alpha}$ by CT-A (Figure 5), although the auto-ADP-ribosylation of the toxin A_1 subunit was decreased. These observations are consistent with the contention that specific phospholipid/detergent interactions are necessary to permit relatively high-affinity binding of GTP to sARF II, but are not required for activation of toxin by sARF II.

Effects of Detergents and Phospholipid on the Rate of sARF II Enhancement of CT-A ADP-Ribosyltransferase Activity. Because sARF II activity was greater in SDS than in cholate with or without DMPC, experiments were performed to investigate the effect of assay conditions on the rate of sARF II activation of CT-A. In the presence of SDS, stimulation of toxin activity by sARF II occurred with no detectable delay and was much greater with 100 μM GTP than with 400 nM (Figure 6A). Toxin activity, however, was constant for only ~120 min. It declined rapidly thereafter and by 3 h was nil, whether or not sARF II was present (Figure 6B). On the other hand, with DMPC and cholate, achievement of maximal sARF II activation was delayed (Figure 6A) although basal toxin activity was constant from zero time (Figure 6B). After maximal activation was achieved (60–90 min), the sARF II stimulated toxin activity remained constant throughout (Figure 6A), likely due to the fact that the activity of toxin was stabilized (Figure 6B). Under these conditions, 400 nM was as effective as 100 μM GTP in supporting sARF II activation (consistent with data in Figure 4B).

Incubation of sARF II with GTP Allows Attainment of a Maximal Rate of sARF II Stimulation of CT-A Activity without Delay. When sARF II, in DMPC/cholate, was incubated at 30 °C with GTP with or without CT-A before initiation of the transferase assay, no delay in establishing a maximal rate of sARF-stimulated CT-A activity was observed (Figure 7). Prior incubation of sARF II without GTP and with or without CT-A did not diminish the delay (Figure 7), nor did incubation of sARF II with GTP at 0 °C (Figure 8),

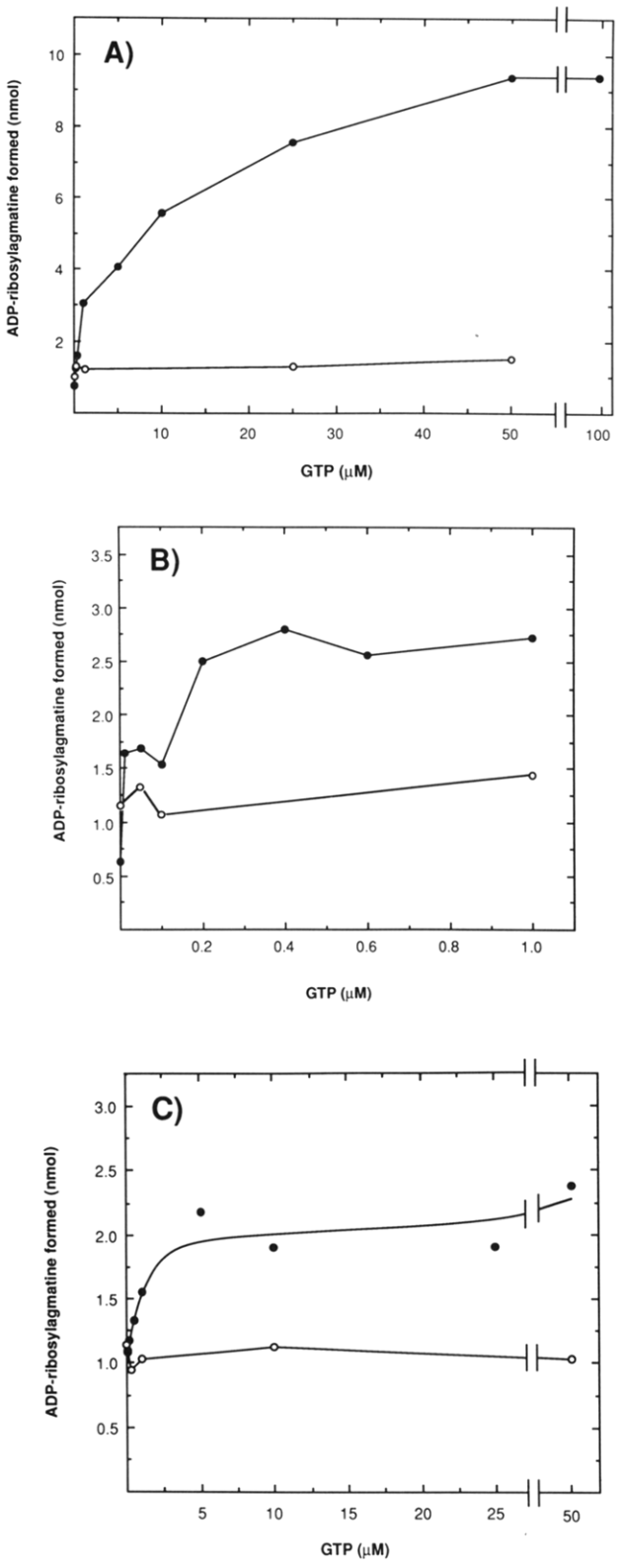

FIGURE 4: Effect of GTP on sARF II enhancement of CT-A ADP-ribosyltransferase in phospholipid and detergents. Assays (total volume 300 μL) containing CT-A (1 μg) with (●) or without (○) sARF II (1 μg) along with 100 μM [*adenine*-^{14}C]NAD (60 000 cpm), 10 mM agmatine, 20 mM DTT, and the indicated concentration of GTP were incubated for 2 h at 30 °C. Other additions were (A) 50 mM potassium phosphate (pH 7.5)/5 mM $MgCl_2$/ovalbumin (0.1 mg/mL)/0.003% SDS, (B) 20 mM HEPES (pH 8)/1 mM EDTA/6 mM $MgCl_2$/3 mM DMPC/0.2% sodium cholate, or (C) 20 mM HEPES (pH 8)/1 mM EDTA/6 mM $MgCl_2$/0.2% sodium cholate. Samples (50 μL) were assayed for ADP-ribosylagmatine formation as described under Experimental Procedures. Note that the *y*-axis scales are different in each panel.

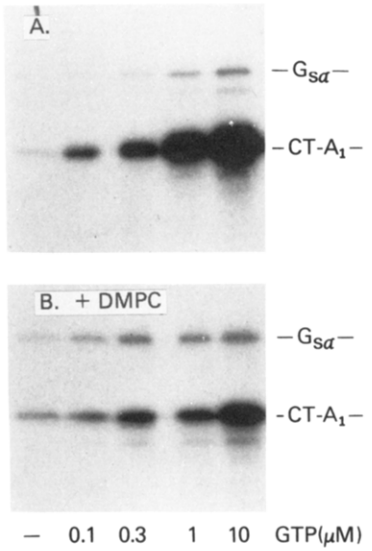

FIGURE 5: Effect of GTP on sARF II enhancement of ADP-ribosylation of $G_{s\alpha}$ by CT. Assays (total volume 100 μL) containing 25 mM potassium phosphate, pH 7.4, 25 μg of CT previously activated with 30 mM DTT, GTP at the indicated concentration, 5 mM $MgCl_2$, 20 mM thymidine, $G_{s\alpha}$ (0.2 μg, in 0.3% cholate), 10 μM [^{32}P]NAD (2 μCi), sARF II (1.3 μg), and the absence (A) or presence (B) of 1 mM DMPC were incubated for 60 min at 30 °C before precipitation with trichloroacetic acid, SDS–PAGE, and autoradiography.

a temperature at which no high-affinity binding of GTP by sARF II was detected (data not shown).

sARF II Stimulation of CT-A Activity in DMPC/Cholate Is Not Due to the Effect of Cholate Alone. The data presented up to this point are consistent with the hypothesis that a CT-A-independent high-affinity interaction of GTP with sARF II is a relatively slow step that could account, at least in part, for the delay in attaining maximal sARF II activation of CT-A in DMPC/cholate. In the presence of cholate alone, sARF II activation is less but occurs with no delay (Figure 9). In the presence of DMPC/cholate, the sARF II–GTP interaction necessary for optimal activation occurs relatively slowly, although the final level of activation is essentially equal to that seen with SDS (Figure 9).

Discussion

ARF has been purified from rabbit liver membranes (Kahn & Gilman, 1984), bovine brain membranes (Kahn & Gilman, 1986; Tsai et al., 1988b), and bovine brain cytosol (Tsai et al., 1988b), although the majority of activity is present in cytosol (Tsai et al., 1988b; Kahn et al., 1988). It is not known whether mARF and sARF(s) are identical in primary structure or are very closely related, but distinct, members of a family of proteins. Deduced amino acid sequences of putative ARF cDNA clones from bovine adrenal chromaffin cells (bovine ARF1) and yeast (Sewell & Kahn, 1988), bovine retina (bovine ARF2) (Price et al., 1988), and human cerebellum (human ARF1 and ARF3) (Bobak et al., 1989) show a high degree of relatedness, and all contain the proposed consensus sequences for GTP binding and GTP hydrolysis. On the basis of sequences of CNBr peptides from mARF and sARF II and deduced amino acid sequences from the ARF cDNAs, sARF II protein corresponds to either ARF1 or ARF3 cDNAs whereas mARF may represent an ARF gene product which has not been cloned (Kahn et al., 1988; Tsai, 1988b; Sewell & Kahn, 1988; Price et al., 1988; Bobak et al., 1989).

Enhancement of cholera toxin catalyzed ADP-ribosyltransferase activity by sARF II requires GTP or a non-

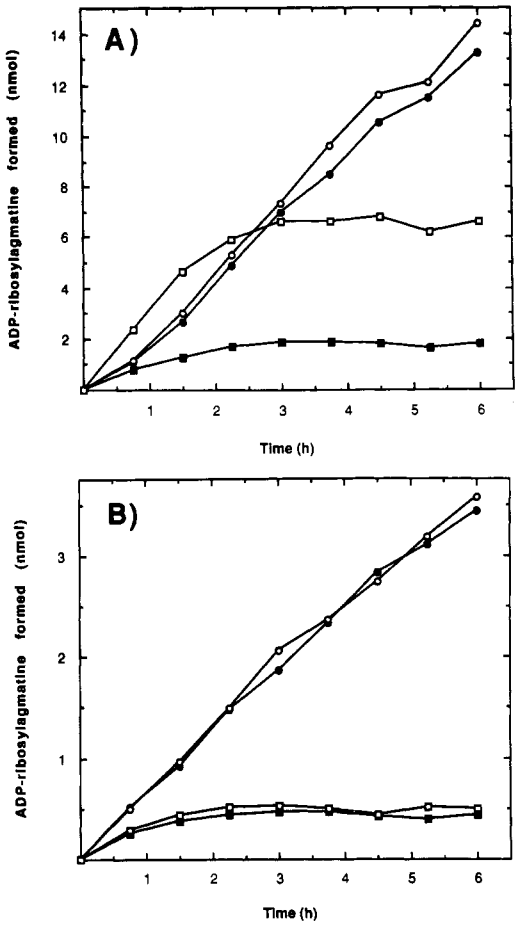

FIGURE 6: Effect of phospholipid and detergent on sARF II enhancement of CT-A ADP-ribosyltransferase activity. Assays (total volume 600 μL) containing (A) sARF II and CT-A or (B) CT-A with 100 μM [*adenine*-^{14}C]NAD (60 000 cpm), 10 mM agmatine, and 20 mM DTT were incubated at 30 °C; samples (50 μL) were removed at the indicated time for determination of ADP-ribosylagmatine formation. Other additions included 20 mM HEPES (pH 8), 1 mM EDTA, 6 mM $MgCl_2$, 3 mM DMPC, and 0.2% sodium cholate with 400 nM (●) or 100 μM (○) GTP and 50 mM potassium phosphate (pH 7.5), 5 mM $MgCl_2$, ovalbumin (0.1 mg/mL), and 0.003% SDS with 400 nM (■) or 100 μM (□) GTP. The final concentrations of sARF II and CT-A were 1 μg/300 μL.

hydrolyzable analogue (Tsai et al., 1987, 1988b). In the present study, sARF II, in DMPC/cholate, enhanced CT-A ADP-ribosyltransferase activity with an apparent EC_{50} for GTP of ~50 nM; these conditions also lowered the apparent EC_{50} of GTP for sARF II stimulation of the ADP-ribosylation of $G_{s\alpha}$ by cholera toxin. There was a delay of an achievement of a maximal rate of sARF-stimulated toxin activity in DMPC/cholate, which was abolished by incubation of sARF II with GTP, with or without CT-A, at 30 °C before initiation of ADP-ribosyltransferase assays. Maximal activation in DMPC/cholate exceeded that in cholate or buffer alone and was approximately equal to that in the presence of SDS, which occurred without delay. High-affinity binding of GTP by sARF II was observed in the presence of Mg^{2+}, DMPC, and cholate, was not detected when assays contained cholate without DMPC, and did not require NaCl as reported necessary for binding of GTPγS by mARF (Kahn & Gilman, 1986). In that study, a cholate requirement for guanine nucleotide binding by mARF was not described, although 0.1% cholate was present in the binding assay (Kahn & Gilman, 1986). The results of the present study, then, indicate that in DMPC/cholate, a functional high-affinity interaction of GTP with a species of sARF II occurs prior to direct activation of CT-A and resultant ADP-ribosylaton of substrate.

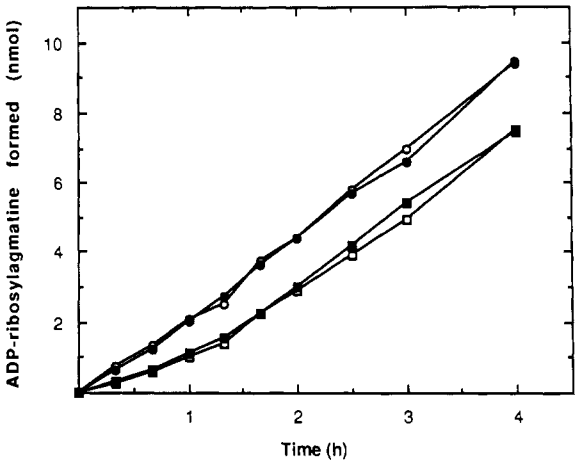

FIGURE 7: Effect of incubation of sARF II with or without GTP and/or CT-A before assay. sARF II (□), sARF plus activated CT-A (■), sARF plus 400 nM GTP (○), and sARF with GTP plus activated CT-A (●) were incubated for 2 h at 30 °C in 20 mM HEPES (pH 8)/1 mM EDTA/6 mM $MgCl_2$/3 mM DMPC/0.2% sodium cholate before initiation of transferase assays by the addition of [*adenine*-^{14}C]NAD and agmatine with GTP and/or activated CT-A as needed to render each transferase reaction mixture complete (total volume 625 μL). CT-A was activated immediately prior to use in the assay as noted under Experimental Procedures. Final concentrations of sARF II and CT-A were 1 μg/300 μL. After the indicated time at 30 °C, samples (50 μL) were removed for assay of ADP-ribosylagmatine formation.

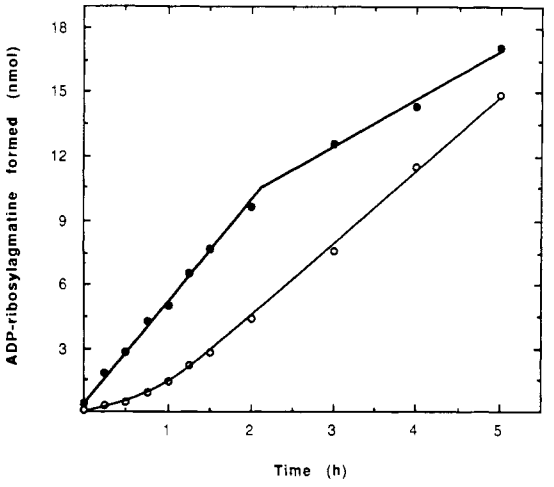

FIGURE 8: Effect of incubation temperature on activation of sARF II by GTP. sARF II was incubated at 30 °C (●) or 0 °C (○) for 2 h in 20 mM HEPES (pH 8)/1 mM EDTA/6 mM $MgCl_2$/1 mM DTT/3 mM DMPC/0.2% sodium cholate/400 nM GTP before addition of [*adenine*-^{14}C]NAD, agmatine, and activated CT-A to initiate transferase assays (total volume 1.9 mL). Final concentrations of sARF II and CT-A were 1 μg/300 μL. CT-A was activated immediately prior to use in the assay as noted under Experimental Procedures. After incubation at 30 °C for the indicated time, samples (50 μL) were assayed for ADP-ribosylagmatine formation.

It was originally reported that, in the presence of rabbit liver mARF, 5–15 μM GTP was required to achieve the half-maximal initial rate of activation of G_s by cholera toxin; however, no high-affinity binding of guanine nucleotides to ARF could be detected (Kahn & Gilman, 1984). A brief delay in achieving the maximal rate of cyclase activation by mARF-stimulated cholera toxin was observed. In contrast to the present study, this delay was eliminated by incubation of mARF with $G_{s\alpha}$, with or without GTP, before initiation of assays (Kahn & Gilman, 1984). It is unclear whether the elimination of the delay was the result of the detergent present with the $G_{s\alpha}$, or of the $G_{s\alpha}$ itself. More recently, these investigators reported that membrane ARF (purified by using

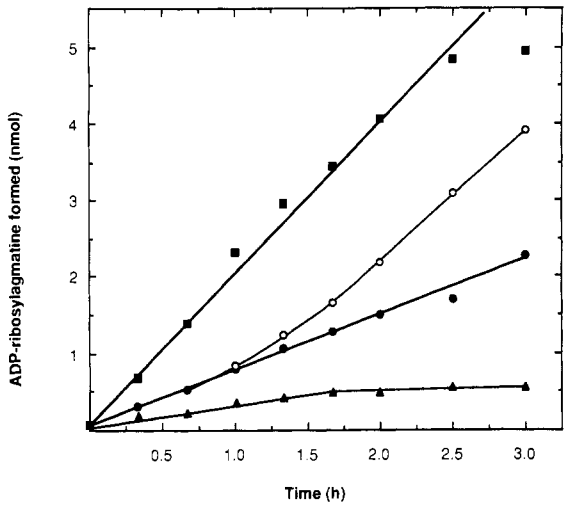

FIGURE 9: Effects of phospholipid and detergent on sARF II enhancement of CT-A ADP-ribosyltransferase activity. Assays (total volume 750 μL) containing sARF II, activated CT-A, 100 μM [*adenine*-^{14}C]NAD (60 000 cpm), and 10 mM agmatine with 20 mM HEPES (pH 8)/1 mM EDTA/6 mM $MgCl_2$/1 mM DTT/400 nM GTP/0.2% sodium cholate (●), 20 mM HEPES (pH 8)/1 mM EDTA/6 mM $MgCl_2$/1 mM DTT/400 nM GTP/3 mM DMPC/0.2% sodium cholate (○), 50 mM potassium phosphate (pH 7.5)/5 mM $MgCl_2$/20 mM DTT/100 μM GTP/ovalbumin (0.1 mg/mL)/0.003% SDS (■), or 20 mM HEPES (pH 8)/1 mM EDTA/6 mM $MgCl_2$/1 mM DTT/400 nM GTP (▲) were incubated at 30 °C; samples (50 μL) were removed at the indicated time for determination of ADP-ribosylagmatine formation. CT-A was activated immediately prior to use in the assay as noted under Experimental Procedures. Final concentrations of sARF II and CT-A were 1 μg/300 μL.

a different procedure and source) did bind GTPγS, GTP, and GDP with apparent K_D's (in competition with GTPγS) of 20, 90, and 40 nM, respectively (Kahn & Gilman, 1986). We interpret these discordant observations to indicate that there may be a difference between the conditions optimal for high-affinity GTP binding and those sufficient for ARF activity, albeit GTP-dependent.

As shown here, activation of cholera toxin by sARF in the presence of 0.003% SDS and 400 nM GTP (a concentration that would require high-affinity GTP interaction with sARF II) was very low. In SDS, the apparent EC_{50} for GTP was ~5 μM, indicating that a relatively low-affinity GTP binding site is active under these conditions. High-affinity GTP binding by sARF II was not demonstrable in SDS (without DMPC/cholate). Tsai et al. have reported that an active ARF–CT-A complex was observed under certain conditions, but only a fraction of ARF was capable of complex formation (Tsai et al., 1988a). The results of that study, taken in context with the present report, support the hypothesis that in the presence of GTP and either SDS or DMPC/cholate, ARF and CT-A can exist as complexed or uncomplexed species which exhibit a different substrate specificity and time course of activation.

It is not known how intracellular conditions modulate GTP activation of ARF. DMPC and cholate are required for the optimal stimulation of CT-catalyzed ADP-ribosylation of $G_{s\alpha}$ by sARF I or sARF II (Tsai et al., 1988b; Figure 5). Ionic detergents, such as cholate, are speculated to produce packing defects in DMPC bilayers, which in turn facilitate oriented insertion of certain proteins into the bilayers (Jain & Zakim, 1987). Our finding that a species of sARF II, in the presence of DMPC/cholate, can exhibit a functional high-affinity interaction with GTP suggests that sARF could also stimulate cholera toxin mediated ADP-ribosylation of $G_{s\alpha}$ after association within a membrane environment.

Gill (1976) reported that the ability of cholera holotoxin to activate adenylyl cyclase was enhanced by SDS and suggested that "more A1 can be generated by reduction of denatured toxin than of native toxin". Others have subsequently reported that detergents, used in assays without purified components, can stimulate or inhibit activation of adenylyl cyclase by cholera toxin (Gill, 1976; Moss et al., 1979; Schleifer et al., 1982; Neer et al., 1987). The present study used purified CT-A_1 subunit (activated by DTT) in a cell-free system with purified components. Our data indicate that the SDS effect is on the ARF–CT-A interaction and not on CT-A directly. In systems using holotoxin, SDS may also enhance the release and activation of CT-A subunit.

Many enzymes are inhibited or inactivated by detergents. It is highly unusual, however, for even nondenaturing concentrations of anionic detergents, such as SDS, to activate or enhance enzymatic activity. Examples include catalase from *Aspergillus niger* (Jones et al., 1987), pyruvate oxidase (Walsh & Bell, 1986), and glycerol kinase (Blake et al., 1978; Mather & Gennis, 1985) from *Escherichia coli*, protein kinase C (Murakami et al., 1986), phospholipase A_2 (Volwerk et al., 1986), and certain phospholipase neurotoxins (Radvanyi et al., 1987). SDS, also at very low concentrations, is reported to stimulate NADPH-dependent superoxide production in postnuclear cell-free extracts obtained from neutrophils or macrophages (Bromberg & Pick, 1984; Curnutte et al., 1987; Pick et al., 1987). Because arachidonic acid, as well as certain other unsaturated fatty acids, can also stimulate the NADPH oxidase (Bromberg & Pick, 1984; McPhail et al., 1985), it has been suggested that these stimulants (SDS and arachidonate) act as anionic amphiphiles to activate this enzyme (Bromberg et al., 1986). The physiochemical properties of these agents alone, however, may not be sufficient to explain the differences in their relative potency of stimulation (Seifert & Schultz, 1987). sARF II stimulation of CT-A NAD:agmatine ADP-ribosyltransferase activity is also enhanced by arachidonic acid (EC_{50} ~50 μM) to an extent similar to that produced by 0.003% SDS (data not shown). This EC_{50} for arachidonic acid is within the concentration range reported effective for stimulation of the NADPH oxidase system in cell-free extracts. CT-A ADP-ribosyltransferase activity is slightly inhibited by arachidonic acid in the absence of sARF II.

We recently reported (Bobak et al., 1989) that the deduced amino acid sequences of the ARF cDNAs exhibit a modest, but significant, percentage of identity with the deduced amino acid sequence of bovine phospholipase C II (PLC II) (Stahl et al., 1988). The extent of overlap involves 82% of the ARF amino acid sequences and a domain of the PLC II believed to be involved in catalytic activity. In view of the unusual detergent/phospholipid requirements for ARF activity and high-affinity GTP binding shown in the present paper, it is tempting to speculate that the possible area of homology between the deduced amino acid sequences of the ARFs and PLC II may reflect domains of the proteins involved in phospholipid recognition/interaction or membrane association. Evaluation of the significance of the apparent homology between the ARFs, the phospholipase C family of enzymes, and other enzymes with unusual detergent/phospholipid requirements awaits characterization of the functional protein domains of these molecules.

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Registry No. GTP, 86-01-1; DMPC, 18194-24-6; Mg, 7439-95-4;

cholic acid, 81-25-4; ADP-ribosyltransferase, 58319-92-9.

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