Effects of Temperature on ADP-Ribosylation Factor Stimulation of Cholera Toxin Activity

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ABSTRACT: The effects of cholera toxin, a secretory product of *Vibrio cholerae*, result from ADP-ribosylation of the stimulatory guanine nucleotide-binding (G_s) protein of the adenylyl cyclase system. Cholera toxin A subunit (CTA) also uses agmatine, a simple guanidino compound, several proteins unrelated to G_s, and CTA itself as alternative ADP-ribose acceptors. The effects of toxin occur in the jejunum presumably at body core temperature. With agmatine as a model substrate, the optimal temperature for CTA-catalyzed ADP-ribosylation was 25–30 °C, and that for CTA-catalyzed auto-ADP-ribosylation was 20–25 °C. Both activities were significantly less at 37 °C, reflecting lower initial velocities, not heat-inactivation of the toxin. All the transferase activities of CTA are enhanced by ADP-ribosylation factors (ARFs), ~20-kDa guanine nucleotide-binding proteins that are ubiquitous in mammalian cells. Phospholipids and a soluble brain ARF, in a GTP-dependent manner, activated toxin NAD:agmatine ADP-ribosyltransferase activity; their simultaneous effect was maximal at physiological temperatures (~37 °C). At lower temperatures, the stimulation by ARF was much less. There were similar effects on other toxin-catalyzed reactions, notably, the ADP-ribosylation of $G_{s\alpha}$ and the hydrolysis of NAD. Thus, host factors, such as ARF and phospholipid, synergistically increase cholera toxin activity at 37 °C and may be important in toxin action in the mammalian gut.

Cholera toxin, a secretory product of *Vibrio cholerae* that is responsible for the devastating diarrheal syndrome characteristic of cholera, exerts its effect on intestinal cells in part through the ADP-ribosylation of the stimulatory GTP-binding protein (G_s)[1] of the adenylyl cyclase system (Field, 1979; Kelly, 1986). Cholera toxin is an oligomeric protein composed of A and B subunits. The A subunit (CTA) is an ADP-ribosyltransferase that catalyzes the ADP-ribosylation of the α subunit of G_s ($G_{s\alpha}$) (Gilman, 1984), simple guanidino compounds (e.g., arginine, agmatine) (Moss & Vaughan, 1977, 1988), and proteins unrelated to $G_{s\alpha}$ (Moss & Vaughan, 1978; Watkins et al., 1980) and auto-ADP-ribosylation of the A_1 fragment of CTA (Trepel et al., 1977; Moss et al., 1980), as well as the hydrolysis of NAD to ADP-ribose and nicotinamide (Moss et al., 1976).

A ~21-kDa protein that stimulates the ADP-ribosylation of $G_{s\alpha}$ (termed ADP-ribosylation factor or ARF) was purified from rat liver and bovine brain membranes (Kahn & Gilman, 1984, 1986). Like $G_{s\alpha}$, ARF is a guanine nucleotide-binding protein (Kahn & Gilman, 1986). In the presence of GTP or a nonhydrolyzable analogue, ARF enhances both the $G_{s\alpha}$-dependent and $G_{s\alpha}$-independent ADP-ribosyltransferase activities of cholera toxin, e.g., hydrolysis of NAD, ADP-ribosylation of simple guanidino compounds, and auto-ADP-ribosylation of CTA (Tsai et al., 1987), consistent with it serving as an allosteric activator of the toxin catalytic unit. ARFs are members of a multigene family; at least six different ARFs have been identified in human and bovine cDNA and genomic libraries (Price et al., 1988; Kahn et al., 1991; Tsuchiya et al., 1991). ARFs of three classes all synthesized in *Escherichia coli* as recombinant proteins stimulate the ADP-ribosyltransferase activities of CTA (Weiss et al., 1989; Price et al., 1992).

Although the toxin presumably functions in intestinal cells at body core temperature, we noted that the optimal temperature for CTA NAD:agmatine ADP-ribosyltransferase activity was apparently 25–30 °C. We, therefore, investigated the effects of certain assay conditions on toxin activity and report here evidence that phospholipids, but not detergents (e.g., SDS, cholate), modify the temperature optimum and allosteric properties of CTA in the presence of sARF II.

EXPERIMENTAL PROCEDURES

Materials. Agmatine, GTP, GDP, NAD, dimyristoylphosphatidylcholine (DMPC), bovine heart cardiolipin, cholate, bovine liver phosphatidylinositol (PI), bovine brain phosphatidylserine (PS), and dimyristoylphosphatidylethanolamine (DMPE) were purchased from Sigma Chemical Co., St. Louis, MO; sodium dodecyl sulfate (SDS) and AG 1-X2 anion-exchange resin were from Bio-Rad Laboratories, Richmond, CA; CTA was from List Biological Laboratories, Campbell, CA; nitrocellulose filters (25-mm diameter) were from Millipore Corp., Bedford, MA; [*adenine*-^{14}C]NAD (280 mCi/mmol) and [*carbonyl*-^{14}C]NAD (53 mCi/mmol) were from Amersham Corp., Arlington Heights, IL; [^{3}H]GTP (13 Ci/mmol), [^{32}P]NAD (20–40 Ci/mmol), and ^{35}S-labeled guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) (1260 Ci/mmol) were from New England Nuclear, Boston, MA; sources of other materials are published (Tsai et al., 1988; Noda et al., 1990; Bobak et al., 1990; Lee et al., 1991).

Purification of ADP-Ribosylation Factor. A soluble ADP-ribosylation factor (sARF II) was purified from the super-

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[1] Abbreviations: G_s, stimulatory guanine nucleotide-binding protein of the adenylyl cyclase system; $G_{s\alpha}$, α subunit of G_s; CTA, A subunit of cholera toxin; mARF and sARF, membrane-bound and soluble ADP-ribosylation factors, respectively; SDS, sodium dodecyl sulfate; DMPC, dimyristoylphosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; DMPE, dimyristoylphosphatidylethanolamine; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); NADase, NAD glycohydrolase.

natant fraction of bovine cerebral cortex by sequential chromatography on CM-Sepharose, hydroxylapatite, and Ultrogel AcA 54 as described (Tsai et al., 1988).

NAD:Agmatine ADP-Ribosyltransferase Assay. Activation of CTA by sARF II was evaluated using the NAD:agmatine ADP-ribosyltransferase assay (Moss & Stanley, 1981; Tsai et al., 1987). Assays contained 50 mM potassium phosphate (pH 7.5), 4 mM $MgCl_2$, 20 mM dithiothreitol (DTT), ovalbumin (0.3 mg/mL), 1 mM GTP, 2 mM (or 0.1 mM in Figures 4, 5, and 6) [^{14}C]NAD [(1.5–2) × 10^5 cpm/tube], 20 mM agmatine, CTA (0.5 μg), and sARF II (1 μg), and, where indicated, SDS, cholate, and/or phospholipids in a total volume of 120 μL. Phospholipid vesicles were prepared by sonification of the suspension for 30 min at a power output of 20 W under N_2 gas. After incubation for 1 h at 30 or 37 °C, unless otherwise indicated, assays were terminated by cooling at 4 °C, and [^{14}C]ADP-ribosylagmatine was isolated for radioassay (Moss & Stanley, 1981).

ADP-Ribosylation of $G_{s\alpha}$ by CTA. ADP-Ribosylation of $G_{s\alpha}$ by CTA was determined as described (Tsai et al., 1988; Bobak et al., 1990). Assays (total volume 100 μL) contained 50 mM potassium phosphate (pH 7.5), CTA (5 μg, previously activated with 30 mM DTT), 500 μM GTP, 5 mM $MgCl_2$, 20 mM thymidine, ovalbumin (0.3 mg/mL), G_s (0.5 μg in 0.3% cholate), and 10 μM [^{32}P]NAD (2 μCi) with or without sARF II (1 μg) and/or cardiolipin (1 mg/mL). G_s was purified from rabbit liver membranes essentially as described by Sternweis et al. (1981). After incubation for 30 min at the indicated temperature, 2 mL of 10% trichloroacetic acid and bovine serum albumin (5 μg) were added, and samples were kept at 4 °C for 1 h. Precipitated proteins were dissolved in 1% SDS/5% 2-mercaptoethanol (65 °C, 10 min) and subjected to electrophoresis in 16% polyacrylamide gels by the method of Laemmli (1970). Gels were exposed to Kodak X-Omat AR film.

NAD Glycohydrolase Assay. NAD glycohydrolase activity of CTA was measured by the method of Moss et al. (1976). Assays contained 50 mM potassium phosphate (pH 7.5), 4 mM $MgCl_2$, 20 mM DTT, 1 mM GTP, ovalbumin (0.3 mg/mL), 40 μM [*carbonyl*-^{14}C]NAD [(1–1.5) × 10^5 cpm/tube], CTA (2 μg), and, as indicated, sARF II (1 μg), SDS, cardiolipin, PI, and/or cholate in a total volume of 120 μL. After incubation for 1 h at the indicated temperature, assays were terminated by cooling at 4 °C, and [*carbonyl*-^{14}C]-nicotinamide was isolated using AG 1-X2 anion-exchange resin for radioassay.

[^{3}H]GTP and [^{35}S]GTPγS Binding by sARF II. Binding of [^{3}H]GTP and [^{35}S]GTPγS to sARF II was assayed by rapid filtration using nitrocellulose filters as described (Kahn & Gilman, 1986; Bobak et al., 1990) with modifications. sARF II (20 pmol, 0.4 μg) was incubated at 30 or 37 °C for 90 min in 25 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$, bovine serum albumin (0.4 mg/mL), and indicated concentrations of [^{3}H]GTP [(1–2) × 10^6 cpm/tube] or 20 nM [^{35}S]GTPγS [(2–3) × 10^6 cpm/tube] with or without cholate, DMPC, PI, or cardiolipin in a total volume of 100 μL. Samples were diluted with 2 mL of 25 mM Tris-HCl (pH 8.0)/5 mM $MgCl_2$/100 mM NaCl at 4 °C and applied to a filter, which was rapidly washed 5 times with 1-mL volumes of the same buffer. Background radioactivity (no sARF II) in the presence of phospholipids was <0.02–0.04% of the total radioactivity applied.

RESULTS

Modification of the Temperature Sensitivity of CTA by Phospholipids and sARF II. The optimal temperature for

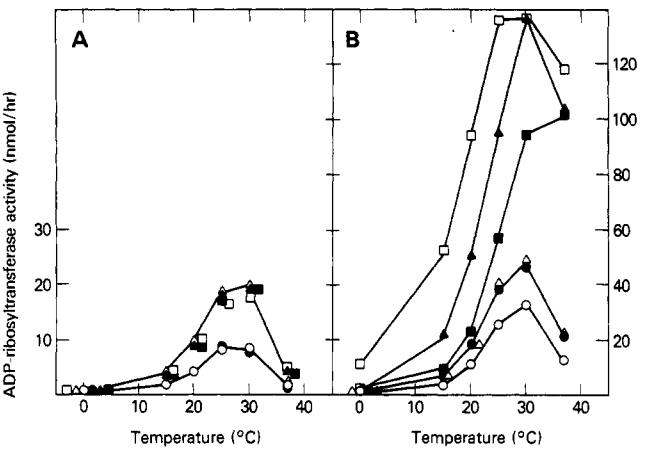

FIGURE 1: Effect of temperature on CTA ADP-ribosyltransferase activity with and without sARF II, phospholipids, and detergents. Assays containing CTA (1 μg) without (A) or with (B) sARF II (1 μg) were incubated for 1 h at the indicated temperature. Other additions were 0.003% SDS (●), 0.2% cholate (△), 2 mg/mL cardiolipin (□), 2 mg/mL PI and 0.2% cholate (■), 3 mM DMPC and 0.2% cholate (▲), or vehicle (○). Parts of this experiment were replicated 2 or 3 times.

ADP-ribosylation of agmatine catalyzed by CTA in the absence of sARF II or lipids was ~30 °C (Figure 1A). Addition of cholate, PI/cholate, DMPC/cholate, or cardiolipin, but not SDS, increased activity ~100% without significantly changing the optimal temperature. Activity was markedly increased by sARF II with or without lipids and detergents (Figure 1B). With DMPC/cholate, PI/cholate, or cardiolipin and sARF II, activity was further increased, and there was relatively little decline between 30 and 37 °C. The enhanced ADP-ribosyltransferase activity at 37 °C appeared not to result from stabilization of the toxin. With or without sARF II, phospholipid, and detergent, the activity of CTA was constant for at least 60 min at 30 or 37 °C (data not shown). CTA was not inactivated at 37 °C. A decrease in temperature from 37 to 25 °C resulted in a prompt increase in transferase activity (data not shown). With PI/cholate and sARF II, CTA activity at 37 °C was almost the same as that at 30 °C.

CTA catalyzes ADP-ribosylation of $G_{s\alpha}$ and also auto-ADP-ribosylation of the toxin A_1 subunit. Without phospholipids and sARF II, auto-ADP-ribosylation of CTA_1 was relatively low and ADP-ribosylation of $G_{s\alpha}$ was minimal (Figure 2A). The optimal temperature for auto-ADP-ribosylation was 20–25 °C. Addition of cardiolipin stimulated ADP-ribosylation of $G_{s\alpha}$ but not auto-ADP-ribosylation, and shifted the optimal temperature to ~30 °C (Figure 2B). sARF II enhanced ADP-ribosylation of $G_{s\alpha}$ and CTA_1, and was itself ADP-ribosylated as reported earlier (Tsai et al., 1987). The addition of sARF II also shifted the optimal temperature for all substrates to ~30 °C (Figure 2C). The labeled 36-kDa band is thought to be the β subunit of G_s; the β subunit of transducin is also ADP-ribosylated by CTA (Tsai et al., 1987). The addition of cardiolipin with sARF II further enhanced ADP-ribosylation of $G_{s\alpha}$ (Figure 2D). The combination of cardiolipin and sARF II had little effect on ADP-ribosylation of the β subunit of G_s or auto-ADP-ribosylation of CTA_1 between 15 and 30 °C but caused marked stimulation at 37 °C. The rate of ADP-ribosylation of each protein was constant at each temperature for 60 min.

CTA also catalyzes the hydrolysis of [*carbonyl*-^{14}C]NAD to [*carbonyl*-^{14}C]nicotinamide and ADP-ribose. The optimal temperature for NAD hydrolysis by CTA in the absence of sARF II or lipids was 25–30 °C (Figure 3A). Addition of

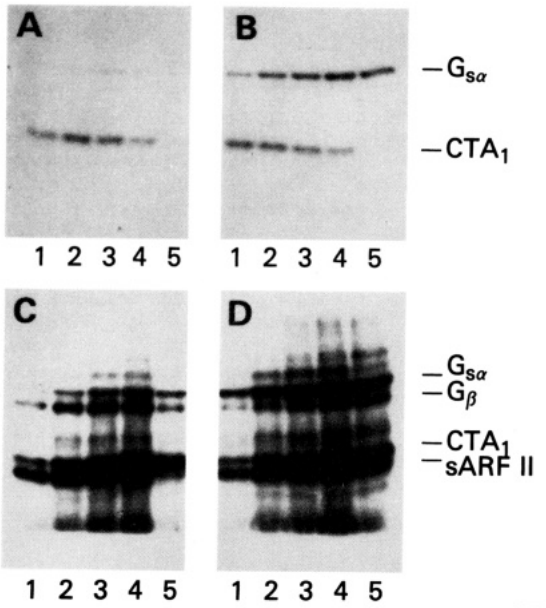

FIGURE 2: Effect of temperature on ADP-ribosylation of $G_{s\alpha}$ by CTA. ADP-ribosylation of $G_{s\alpha}$ and CTA was measured as described under Experimental Procedures. Other additions were none (A), cardiolipin (1 mg/mL) (B), sARF II (1 μg) (C), or cardiolipin plus sARF II (D). Samples were incubated for 30 min at 15 °C (lane 1), 20 °C (lane 2), 25 °C (lane 3), 30 °C (lane 4), or 37 °C (lane 5). The experiment was replicated twice.

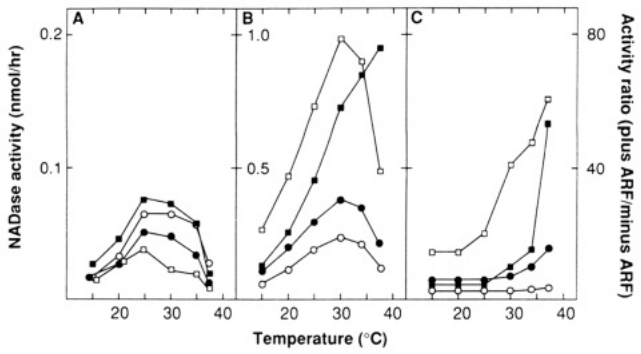

FIGURE 3: Effect of temperature on CTA NADase activity with and without sARF II and phospholipds. Assays containing CTA (2 μg) without (A) or with (B) sARF II (1 μg) were incubated for 1 h at the indicated temperature. Other additions were 0.003% SDS (●), PI (1 mg/mL) and 0.2% cholate (■), cardiolipin (1 mg/mL) (□), or vehicle (○). (C) The increase in NADase activity by sARF II at each temperature is shown as the ratio of activity with to that without ARF. All assays were run in duplicate. The experiment was replicated twice. The incorporation of [*adenine*-^{14}C]ADP-ribose into proteins in those assays was only 1–6% of [*carbonyl*-^{14}C]nicotinamide formation; i.e., the data substantially reflect NAD glycohydrolase, not ADP-ribosyltransferase, activity.

PI/cholate slightly stimulated the activity at low temperature, whereas SDS or cardiolipin inhibited between 25 and 37 °C, without significantly changing the optimal temperature. The activity was markedly increased by sARF II with or without lipids and SDS (Figure 3B). Addition of sARF II with PI/cholate also shifted the optimal temperature to 37 °C. Incorporation of [*adenine*-^{14}C]ADP-ribose into proteins in those assays was less than 3%, 6%, 5%, or 1% of [*carbonyl*-^{14}C]nicotinamide formation in control, SDS, cardiolipin, and PI/cholate, respectively. Thus, the data in Figure 3 substantially reflect NAD glycohydrolase activity, not the ADP-ribosyltransferase activity. Figure 3C shows the increase in NADase activity by sARF II as the ratio of activity with to that without ARF, at each temperature. PI/cholate and especially cardiolipin markedly enhanced the stimulatory effect of sARF II at higher temperatures. An alternative view is that in the presence of these phospholipids, as temperatures increased from 15 to 37 °C, toxin activity, relative to its potential, becomes very small in the absence of ARF, with increasing dependence on this activator protein.

Enhancement of sARF II-Stimulated CTA Activity by Phospholipids. To define the mechanism of lipid enhancement of sARF II-stimulated CTA ADP-ribosyltransferase, sARF II concentration in the NAD:agmatine ADP-ribosyltransferase assay was varied at 30 °C in the presence of various phospholipids and/or detergents. Without lipids or detergents, half-maximal activation by sARF II was observed at (2.15 ± 0.29) × 10^{-10} mol/assay (Table I). The addition of SDS or cardiolipin reduced the concentration at which half-maximal activation by sARF II was observed by ca. 10-fold. SDS or cardiolipin also increased the apparent affinity of CTA for sARF II at 37 °C. Cholate with or without other phospholipids had no effect.

Bobak et al. (1990) reported that high-affinity GTP binding by sARF II (apparent K_D, 70 nM) required DMPC/cholate and that sARF II in DMPC/cholate enhanced CTA activity with an apparent EC_{50} of 50 nM GTP. We measured the GTP dependency of the sARF II-stimulated CTA activity at 30 and 37 °C in the presence of phospholipids (Figures 4 and 5). In the presence of SDS or cholate, half-maximal activation of toxin ADP-ribosyltransferase activity by sARF II was observed at both temperatures with 10–100 μM GTP. At 30 °C, the EC_{50} for GTP in cardiolipin or PI/cholate (or PS/cholate, data not shown) was about 1 μM, and that in DMPC/cholate was about 0.1 μM. The EC_{50} values for GTP at 37 °C were slightly higher, except in the case of cardiolipin/cholate where a lower EC_{50} was observed. In these and the next experiments, 0.1 mM NAD was used to make data comparable to those of Bobak et al. (1990).

Effects of Cardiolipin and PI. PI enhanced sARF II-stimulated CTA activity with or without cholate with half-maximal activity at ~0.2 mg/mL (Figure 6). Cardiolipin markedly enhanced CTA activity in the presence of sARF II, and its effect was reduced by cholate. The effect of cardiolipin plus cholate was similar to that of PI/cholate, with a half-maximal effect at ~0.2 mg/mL in both cases. High-affinity [^{3}H]GTP binding to sARF II in cardiolipin was also reduced by the addition of cholate (data not shown).

The rate of ADP-ribosylation of agmatine by CTA at 30 °C was measured as a function of NAD concentration (Table II). The apparent K_m for NAD was ~6 mM in the presence of cardiolipin, DMPC/cholate, or PI/cholate. The addition of sARF II reduced the apparent K_m as reported by Noda et al. (1990). SDS, cardiolipin, PI/cholate, and PS/cholate, but not cholate or DMPC/cholate, further reduced the apparent K_m. Table III shows the V_{max} values estimated from the data in Tables I and II. The effectiveness at 30 °C was cardiolipin = PI/cholate ≥ DMPC/cholate = PS/cholate > cholate = SDS = no additions (Table III).

GTP Binding to sARF II in Phospholipids. [^{3}H]GTP binding at 30 °C was maximal in DMPC/cholate, with a K_D of ~0.1–0.2 μM (Figure 7) as reported by Bobak et al. (1990). [^{3}H]GTP binding (K_D ~1 μM) to sARF II was observed also in PI/cholate and cardiolipin, although maximal binding was less than that in DMPC/cholate. Apparent K_D values for [^{3}H]GTP binding at 30 °C were consistent with ED_{50} values at 30 °C in the sARF II-stimulated CTA ADP-ribosyltransferase assay. At 37 °C, maximal [^{3}H]GTP bound was less than at 30 °C, and the apparent K_D was higher with each phospholipid.

Table I: Effect of Phospholipids and/or Detergents on Interaction between sARF II and CTA[a]

phospholipid	detergent	sARF II–half-maximal activation (mol/assay) A	B
		$(2.15 \pm 0.29) \times 10^{-10}$ (10)	$(1.45 \pm 0.31) \times 10^{-10}$ (2)
	SDS (0.003%)	$(1.89 \pm 0.04) \times 10^{-11}$ (6)	$(2.41 \pm 0.21) \times 10^{-11}$ (2)
cardiolipin (2 mg/mL)		$(2.70 \pm 0.21) \times 10^{-11}$ (6)	$(3.74 \pm 0) \times 10^{-11}$ (2)
	cholate (0.2%)	$(1.83 \pm 0.27) \times 10^{-10}$ (6)	ND[b]
DMPC (3 mM)	cholate (0.2%)	$(2.09 \pm 0.26) \times 10^{-10}$ (4)	$(1.60 \pm 0.28) \times 10^{-10}$ (2)
PI (2 mg/mL)	cholate (0.2%)	$(2.13 \pm 0.39) \times 10^{-10}$ (5)	$(1.26 \pm 0.07) \times 10^{-10}$ (2)
PS (2 mg/mL)	cholate (0.2%)	$(1.94 \pm 0.55) \times 10^{-10}$ (3)	ND

[a] The ADP-ribosyltransferase activity of CTA (0.5 μg/assay, 1.85×10^{-11} mol) was measured for 1 h at 30 °C (A) or 37 °C (B) with varying amounts of sARF II and the indicated concentrations of phospholipids and/or detergents. The data are means ± SEM of values from 3–10 experiments (*n* in parentheses) in A and means ± half the range of values from 2 experiments in B. [b] ND, not determined.

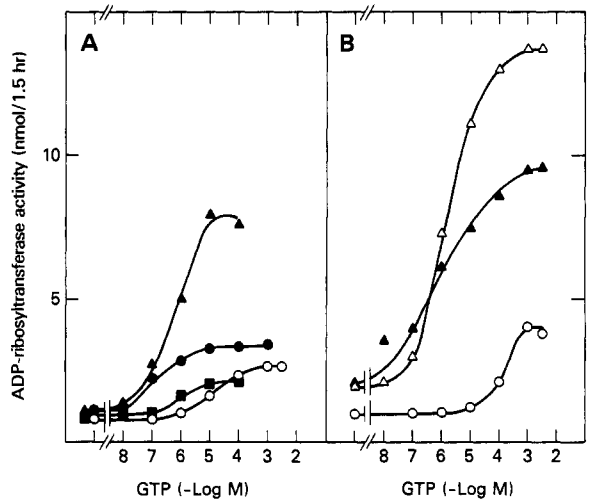

FIGURE 4: Effect of GTP on sARF II enhancement of CTA ADP-ribosyltransferase activity with phospholipids and/or detergents at 30 °C. Assays containing 0.1 mM NAD [as used by Bobak et al. (1990)], 10 mM agmatine, 0.5 μg of CTA, 0.5 μg of sARF II, and the indicated concentrations of GTP were incubated at 30 °C for 1.5 h. Other additions were (in A) 0.2% cholate (○), 3 mM DMPC and 0.2% cholate (●), 3 mM DMPE and 0.2% cholate (■), and PI (2 mg/mL) and 0.2% cholate (▲) and (in B) 0.003% SDS (○), cardiolipin (2 mg/mL) (△), and cardiolipin plus 0.2% cholate (▲). All assays were run in duplicate. The experiment was replicated twice.

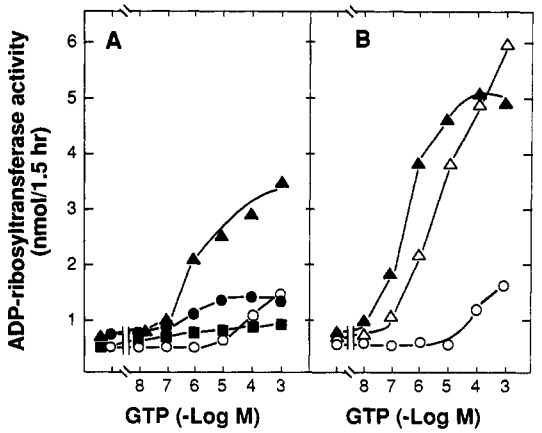

FIGURE 5: Effects of GTP on sARF II enhancement of CTA ADP-ribosyltransferase activity with phospholipids and/or detergents at 37 °C. Measurement of ADP-ribosyltransferase activity and additions to (A) and (B) are described in the legend to Figure 4. The experiment was replicated twice.

Apparent K_D values were also assessed by competition of guanine nucleotides for [^{35}S]GTPγS binding to sARF II at 30 °C (Table IV). The K_D for [^{35}S]GTPγS binding by ARF was reported to be 20 nM (Kahn & Gilman, 1986). The calculated K_D is approximately half of the apparent K_D because of the presence of 20 nM [^{35}S]GTPγS in our experiments.

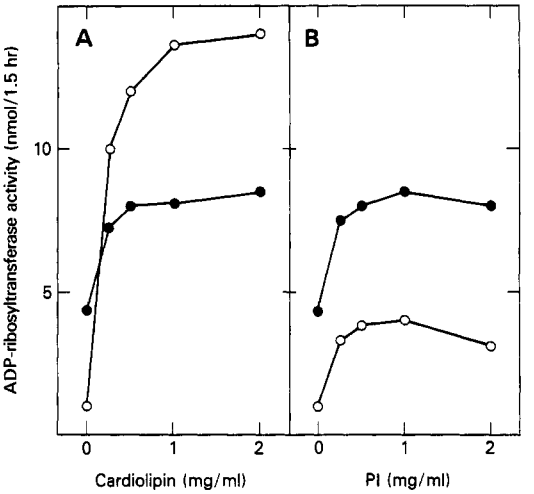

FIGURE 6: Effects of cardiolipin and PI on ADP-ribosyltransferase activity. Assays as described in the legend to Figure 4 were supplemented with 1 mM GTP and the indicated concentrations of cardiolipin (A) or PI (B) without (○) or with 0.2% cholate (●). The experiment was replicated twice.

Table II: Effect of Phospholipids, Detergents, and sARF II on Apparent K_m Values for NAD[a]

phospholipid	detergent	apparent K_m for NAD (mM) –sARF II	+sARF II
		6.53 ± 0.72 (6)	2.77 ± 0.14 (13)
	SDS	ND[b]	1.26 ± 0.10 (5)
cardiolipin		7.47 ± 0.73 (4)	1.25 ± 0.13 (6)
	cholate	ND[b]	2.41 ± 0.12 (6)
DMPC	cholate	7.38 ± 0.49 (3)	2.19 ± 0.20 (5)
PI	cholate	6.30 ± 1.03 (3)	1.29 ± 0.05 (3)
PS	cholate	ND[b]	1.22 ● 0.04 (4)

[a] Assays containing 20 mM agmatine, 1 mM GTP, 0.5 μg/tube of CTA, 1 μg/tube of sARF II, and various concentrations of NAD were incubated for 1 h. Other additions were 0.003% SDS, 0.2% cholate, 2 mg/mL cardiolipin, PI, or PS, and 3 mM DMPC. Data are means ± SEM of values from 3–13 experiments (*n* in parentheses). [b] ND, not determined.

The K_D for GTP in DMPC/cholate was 0.14 ± 0.10 μM, which was in the same range as the K_D for [^{3}H]GTP binding and the ED_{50} for GTP in sARF II-stimulated CTA activity. K_D values with cardiolipin, PI/cholate, and PS/cholate were 0.5–0.8 μM. Under these conditions, no significant difference was observed in the binding of GTP and GDP to sARF II.

DISCUSSION

Cholera toxin, which exerts its effect on intestinal cells in part through the ADP-ribosylation of $G_{s\alpha}$ in the adenylyl cyclase system, can use agmatine as an alternative ADP-ribose acceptor. Although the toxin presumably functions in

Table III: Effect of Phospholipids, Detergents, and sARF II on the V_{max} of ADP-Ribosyltransferase[a]

phospholipid	detergent	V_{max} of ADP-ribosyltransferase activity (nmol/h)		
		-sARF II	+sARF II	c
		20.2 ± 4.4	54.4 ± 3.0	(58.8 ± 3.8)[c]
	SDS	ND[b]	68.4 ± 7.8	(67.8 ± 4.3)
cardiolipin		33.5 ± 7.9	165 ± 8	(189 ± 9)
	cholate	ND	76.1 ± 6.3	(56.1 ± 5.7)
DMPC	cholate	52.2 ± 3.4	124 ± 9	(217 ± 29)
PI	cholate	44.9 ± 5.8	141 ± 5	(192 ± 42)
PS	cholate	ND	114 ± 7	(95.4 ± 2.6)

[a] CTA activity was measured as described in Table II. Data are means ± SEM of values from those experiments. [b] ND, not determined. [c] In parentheses, V_{max} values estimated from data from experiments described in Table I.

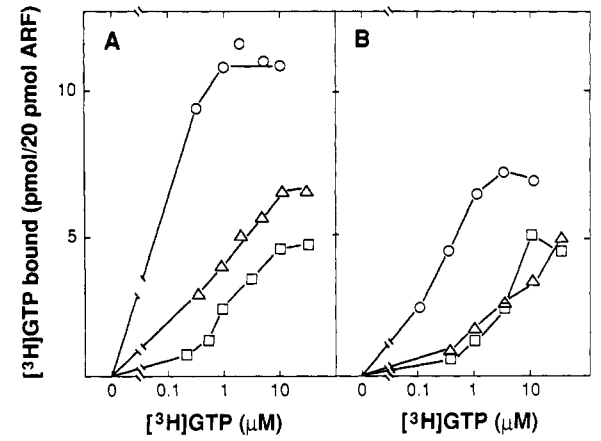

FIGURE 7: GTP binding by sARF II in the presence of cholate and lipids. sARF II (0.4 μg, 20 pmol) was incubated with the indicated concentration of [^{3}H]GTP at 30 °C (A) or 37 °C (B) as described under Experimental Procedures. Other additions were 3 mM DMPC and 0.2% cholate (○), PI (2 mg/mL) and 0.2% cholate (△), or cardiolipin (1 mg/mL) (□). Each point is the mean of values from duplicate assays in a typical experiment, which was replicated 3 times.

Table IV: Competition by GTP and GDP for [^{35}S]GTPγS Binding by sARF II[a]

lipid	% cholate	apparent K_D for nucleotides (μM)	
		GTP	GDP
cardiolipin, 2 mg/mL		1.56 ± 0.42 (4)	1.08 ± 0.28 (4)
DMPC, 3 mM	0.2	0.28 ± 0.19 (4)	0.29 ± 0.14 (4)
PI, 2 mg/mL	0.2	0.88 ± 0.12 (3)	0.77 ± 0.04 (3)
PS, 2 mg/mL	0.2	1.27 ± 0.09 (3)	0.94 ± 0.09 (3)

[a] sARF II (0.4 μg) was incubated with 20 nM [^{35}S]GTPγS [(2–3) × 10^6 cpm] and several concentrations of GTP or GDP, with lipid and cholate as indicated. Data are means ± SEM of values from three or four experiments (n in parentheses). Specific binding without GTP or GDP was 890 000 cpm in DMPC/cholate, 93 000 cpm in PI/cholate, 87 000 cpm in cardiolipin, and 49 000 cpm in PS/cholate, in a typical experiment.

intestinal cells at body core temperature, we found that the optimal temperature for CTA NAD:agmatine ADP-ribosyltransferase activity was in the range of 25–30 °C and that for CTA-catalyzed auto-ADP-ribosylation was in the range of 20–25 °C. The optimal temperature for NADase activity of CTA was also 25–30 °C. Several bacterial enzymes are apparently inhibited at temperatures above 20–25 °C (Neidhardt et al., 1990). For instance, Rohrer et al. (1975) purified an enzyme from bacteriophage T4-infected cells that was capable of ADP-ribosylating *E. coli* RNA polymerase as well as other arginine-containing polypeptides, with an optimal temperature of 15–20 °C. The enzyme was irreversibly inhibited at 37 °C. Noninfected *E. coli* also contain an activity that ADP-ribosylates several bacterial and nonbacterial proteins and is maximal at 20 °C (Skorko & Kur, 1981).

The addition of certain phospholipids and sARF II had two effects on the NAD:agmatine ADP-ribosyltransferase activity of CTA. They enhanced reaction velocity and shifted the optimal temperature so that activity remained high at ~37 °C. Either phospholipids or sARF II alone stimulated CTA activity, but activities at 37 °C were still relatively low. The lower activity at higher temperatures reflects a lower initial velocity, not inactivation of the toxin. CTA-catalyzed ADP-ribosylation of $G_{s\alpha}$, which is a component of host cells, was increased by addition of a phospholipid like cardiolipin or sARF II, as reported by Kahn and Gilman (1986) and Tsai et al. (1988), and in the presence of both, the reaction rate was essentially maximal at 37 °C. These data are consistent with the conclusion that interactions between CT and host cell components such as ARF, phospholipids, and G_s modify the effects of temperature on enzyme activity. Since at temperatures well below 37 °C, i.e., 15–20 °C, the stimulation by ARF is much reduced, in vitro studies of cholera toxin activity by ARF would best be done at physiological temperatures.

Activation of CTA by sARF II in 0.003% SDS required micromolar concentrations of GTP (Bobak et al., 1990). Noda et al. (1990) reported that interaction of sARF II with the toxin lowered the apparent K_m values for both substrates, NAD and agmatine, and activation by SDS was associated with a further decrease in K_m values for both substrates. In DMPC/cholate, sARF II enhanced CTA activity in the presence of nanomolar concentrations of GTP, and increased GTP binding by sARF II was observed (Bobak et al., 1990). We found several other phospholipids (cardiolipin, PI/cholate, PS/cholate) that also stimulated sARF II-enhanced CTA activity in the presence of GTP. Cardiolipin and SDS, but not other phospholipids, increased the apparent affinity of CTA for sARF II. Cardiolipin, PI/cholate, or PS/cholate lowered the apparent EC_{50} for GTP (to about 1 μM), whereas with SDS or cholate, 10–100 μM GTP was needed. Cardiolipin, PI/cholate, or PS/cholate, but not DMPC/cholate, in the presence of sARF II, further reduced the apparent K_m for NAD. With cardiolipin, PI/cholate, or PS/cholate, sARF II exhibited high-affinity (~1 μM) nucleotide binding. We conclude that, although all lipids did not have exactly the same effects, phospholipids can enhance interactions among CTA, sARF II, guanine nucleotide, and NAD at body core temperature. The reasons for differences in effects of phospholipids on the transferase and glycohydrolase activities of the toxin are unclear.

DMPC/cholate increased binding of GTP to sARF II and sARF II-stimulated CTA activity. GTP binding to sARF II was not observed in DMPC without cholate or in DMPC with SDS (Bobak et al., 1990). In our experiments, cholate partially inhibited the stimulatory effect of cardiolipin on CTA activity. Only cardiolipin promoted effective interaction between sARF II and CTA. Cardiolipin (diphosphatidylglycerol) differs in basic structure from phospholipids like PI or PC, which may contribute to differences in the effects of cholate with it and other phospholipids.

There are reports that DMPC/cholate stimulates ADP-ribosylation of $G_{s\alpha}$ and inhibits auto-ADP-ribosylation of CTA_1 in the presence of ARF (Tsai et al., 1988; Bobak et al., 1990). In our experiments, sARF II without phospholipids stimulated ADP-ribosylation of α and β subunits of G_s, and CTA_1 was itself ADP-ribosylated, whereas cardiolipin with

or without sARF II stimulated ADP-ribosylation of $G_{s\alpha}$, but not the other proteins. Tsai et al. (1991) recently reported that in DMPC/cholate, a fraction of sARF II formed a GTPγS-dependent aggregate with CTA that exhibited a substrate specificity different from that of monomeric CTA and sARF II. These results are consonant with the possibility that phospholipids can influence not only temperature sensitivity but also substrate specificity of the toxin. Whether or not ARF plays a role in cholera toxin action on cells, our observations emphasize again that the behavior of an enzyme in vitro may be very different from that in its natural milieu.

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