Botulinum ADP-ribosyltransferase activity as affected by detergents and phospholipids

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GTP-binding proteins with M_r values of 22000 and 25000 in bovine brain cytosol were ADP-ribosylated by an exoenzyme (termed C3) purified from *Clostridium botulinum* type C. The rate of C3-catalyzed ADP-ribosylation of the partially purified substrates was extremely low by itself, but was increased enormously when a protein factor(s) obtained from the cytosol was simultaneously added. The rate of the C3-catalyzed reaction was also stimulated by the addition of certain types of detergents or phospholipids even in the absence of the protein factors. The ADP-ribosylation appeared to be enhanced to an extent more than the additive effect of either the protein factors or the detergents (and phospholipids). Thus, ADP-ribosylation catalyzed by botulinum C3 enzyme was affected not only by cytoplasmic protein factors but also by detergents or phospholipids in manners different from each other.

ADP-ribosylation; GTP-binding protein; *Clostridium botulinum*; Phospholipid; Detergent

1. INTRODUCTION

A family of structurally homologous membrane-associated GTP-binding proteins (G proteins) is present in a variety of cells [1]. They act as a signal transducer between membrane receptors and effectors such as adenylate cyclase, phospholipase C (or A_2), K^+ (or Ca^{2+}) channels and cGMP-specific phosphodiesterase. Some bacterial toxins exert their influences on eukaryotic cells by catalyzing ADP-ribosylation of the α-subunits (M_r = 39000–45000) of G proteins. Cholera toxin ADP-ribosylates the α-subunit of G_s and activates the G protein. A protein cofactor, termed ARF, which has also a GTP-binding activity, is required for the ADP-ribosylation [2]. On the other hand, pertussis toxin (islet-activating protein; IAP) ADP-ribosylates the α-subunits of G_i and G_o. This reaction requires $\beta\gamma$-subunits of G proteins [3], and is enhanced by ATP and Chaps [4].

There is another family of structurally homologous GTP-binding proteins with M_r values of about 20000 in mammalian tissues. This family includes the GTP-binding proteins of *ras*- and *rho*-gene products and other novel proteins with similar molecular weight. Recently, Narumiya and coworkers [5] reported that certain types of botulinum toxins (or enzymes) displayed an ADP-ribosyltransferase activity whose substrate was a protein with an M_r value of 21000. Aktories et al. [6,7] also reported that another ADP-ribosyltransferase, termed C3, was produced by *Clostridium botulinum* type C. These toxin (or enzyme) substrates have been copurified with a GTP-binding activity [8,9], and some of them have recently been identified as GTP-binding proteins identical with putative products of *rho* and *rac* genes [9–12].

In the previous paper [13], we have reported that the rate of C3-catalyzed ADP-ribosylation of substrate proteins partially purified from bovine brain cytosol was extremely low by itself, and that this reaction was markedly enhanced by cytoplasmic protein factor(s) obtained from the brain cytosol. In this paper, we report that C3-catalyzed ADP-ribosylation occurred very rapidly even in the absence of the cytoplasmic factor(s), when membrane-bound proteins extracted with cholate and partially purified from bovine brain were used as the substrates. Both activities as ADP-ribosyltransferase and NAD glycohydrolase of C3 enzyme were markedly stimulated by certain types of detergents and phospholipids. Some characteristics of the stimulation are described with relation to the protein factor(s) that have been identified in the previous paper [13].

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Abbreviations: IAP, islet-activating protein or pertussis toxin; ARF, ADP-ribosylation factor; GTPγS, guanosine 5′-(3-*O*-thio)triphosphate; C3, the M_r = 25000 protein produced by *Clostridium botulinum* type C displaying ADP-ribosyltransferase activity; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfate

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2. MATERIALS AND METHODS

2.1. *Purifications of C3 enzyme and bovine brain cytoplasmic substrate and activator proteins for C3-catalyzed ADP-ribosylation*

Botulinum ADP-ribosyltransferase C3 was purified from the culture medium of *Clostridium botulinum* type C strain 903 as described previously [13]. Cytoplasmic substrate and activator proteins for C3-catalyzed ADP-ribosylation were partially purified from bovine brain as described by Ohtsuka et al. [13].

2.2. *Partial purification of substrate proteins for C3-catalyzed ADP-ribosylation from bovine brain membranes*

The initial steps used for purification of C3 substrate proteins from bovine brain membranes were similar to those described previously for purification of IAP substrates [14,15]. Briefly, cholate extract (550 ml) was prepared from the membrane and applied to a column (4.4 × 40 cm) of DEAE-Sephacel (Pharmacia LKB Biotechnology Inc.) which had been equilibrated with 1.8 l of TED (20 mM Tris-HCl, pH 8.0, 1 mM EDTA and 1 mM dithiothreitol) containing 25 mM NaCl, aprotinin and 1% sodium cholate. The column was then eluted with a linear gradient of NaCl (1.2 l; 0–200 mM) in TED containing 1% sodium cholate and aprotinin and followed by further elution with 500 ml of 250 mM NaCl in the same solution. The activity of C3 substrates was eluted with NaCl at 250 mM, while IAP substrates were recovered from the column at about 125 mM NaCl [24]. The C3 substrate-rich fractions (240 ml) from the DEAE-Sephacel were concentrated and then fractionated on a column (3.2 × 90 cm) of Sephacryl S-300HR (Pharmacia LKB) in TMDG (20 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, 1 mM dithiothreitol and 1 μM GDP) containing 100 mM NaCl, aprotinin and 1% sodium cholate. The activity of C3 substrates was eluted from the column in a single peak along with a GTPγS-binding activity. The fractions (140 ml), after being concentrated to approximately 20 ml, were diluted with 5 vols of TMDG containing 300 mM NaCl and 5% ethyleneglycol, and then applied to a column (2.2 × 10 cm) of phenyl-Sepharose CL-4B (Pharmacia LKB) which had been equilibrated with 250 ml of TMDG containing 250 mM NaCl, 5% ethyleneglycol and 0.25% sodium cholate. The column was washed with 50 ml of the equilibration buffer and eluted with a linear gradient of 0.25% sodium cholate/250 mM NaCl to 2% sodium cholate/0 mM NaCl (200 ml) in TMDG containing 5% ethyleneglycol. C3 substrates were eluted from the column with NaCl at about 155 mM and with sodium cholate at about 0.9% as a single peak. The fractions that also contained GTPγS-binding activity with a specific activity similar to the C3 substrate were used for the following experiments.

2.3. *Assays of C3-catalyzed ADP-ribosylation and NAD glycohydrolase*

Assay of C3-catalyzed ADP-ribosylation of protein substrates was described in [13]. The [^{32}P]ADP-ribosylated proteins were also electrophoresed through SDS-polyacrylamide gels (12.5%) as described in [3,16], and an autoradiogram was made with a Kodak X-Omat AR film using an intensifying screen at −85°C. NAD glycohydrolase activity was assayed by a method described in [17,18]. The reaction mixture contained 20 mM sodium Hepes (pH 7.4), 1 mM $MgCl_2$, 20 μM [4-^{3}H-*nicotinamide*]NAD (approximately 1000 cpm/pmol) and other additions as indicated in Table I. Reaction was initiated with the addition of C3 enzyme (2.7 μg/ml), and continued at 37°C for 3 h. [4-^{3}H]Nicotinamide formed during the incubation was separated by a column (0.8 × 1 cm) of AG1-X2 (Bio-Rad) as described in [18].

2.4. *Miscellaneous*

[^{35}S]GTPγS-binding activity was determined with the use of a rapid filtration method as described in [3]. Protein was quantitated by staining with Amide black with bovine serum albumin as a standard protein [19]. [α-^{32}P]NAD was obtained from DuPont New England Nuclear; [4-^{3}H-*nicotinamide*]NAD from Amersham. Phospholipids were obtained from Serdary Research Laboratories. Other materials and chemicals were obtained from the same sources as described in [13–15,20–22].

3. RESULTS

In a previous paper [13], we have partially purified GTP-binding proteins with M_r values of 22000 and 25000 from bovine brain cytosol as the substrate for a botulinum ADP-ribosyltransferase (termed C3). As shown in Fig. 1A, the actual rate of the ADP-ribosylation was extremely low when the cytoplasmic substrates by themselves were incubated with C3 enzyme in the presence of [^{32}P]NAD. However, the reaction was markedly enhanced by the addition of protein factor(s) obtained from the cytosol fraction in accordance with our previous results [13]. There were also C3-substrate proteins in the membrane fractions of which the M_r values were the same as the cytosol proteins (data not shown). The membrane-bound substrates were partially purified from the membrane cholate extracts and subjected to C3-catalyzed ADP-ribosylation under the same assay conditions. The ADP-ribosylation occurred very rapidly even in the absence of the cytoplasmic activator protein; there was no further stimulation by the activator protein (Fig. 1B). When the membrane substrates were reconstituted into phospholipid vesicels free from cholate, there was a marked reduction in the ADP-ribosylation of the membrane substrates (data not shown); under this condition, the activator protein effectively stimulated the C3-catalyzed ADP-ribosylation of the membrane substrates.

Since the above data showed that C3-catalyzed ADP-ribosylation of membrane substrates as well as its susceptibility to the activator protein was markedly affected by cholate, we studied the effect of the detergent on the same C3-induced modification of cytoplasmic substrates that were in solution without detergent or phospholipids. As expected, the ADP-ribosylation of cytoplasmic substrates was stimulated significantly, though slightly, by the addition of cholate to the reaction mixture (Fig. 2). The degree of the stimulation by cholate was far less than that by the cytoplasmic activator protein. The combined addition of cholate with the activator protein enhanced the C3-catalyzed reaction to an extent more than the additive of the effect of either alone, suggesting that these activators acted through mechanisms different from each other.

The effects of various detergents on C3-catalyzed ADP-ribosylation of the cytoplasmic substrates were examined in the presence or the absence of the activator protein, and the results are summarized in Fig. 3. The rate of the ADP-ribosylation was stimulated upon the addition of cholate (Fig. 3A) or deoxycholate (Fig. 3B) regardless of whether the reaction mixture was supplemented with the activator protein or not. The max-

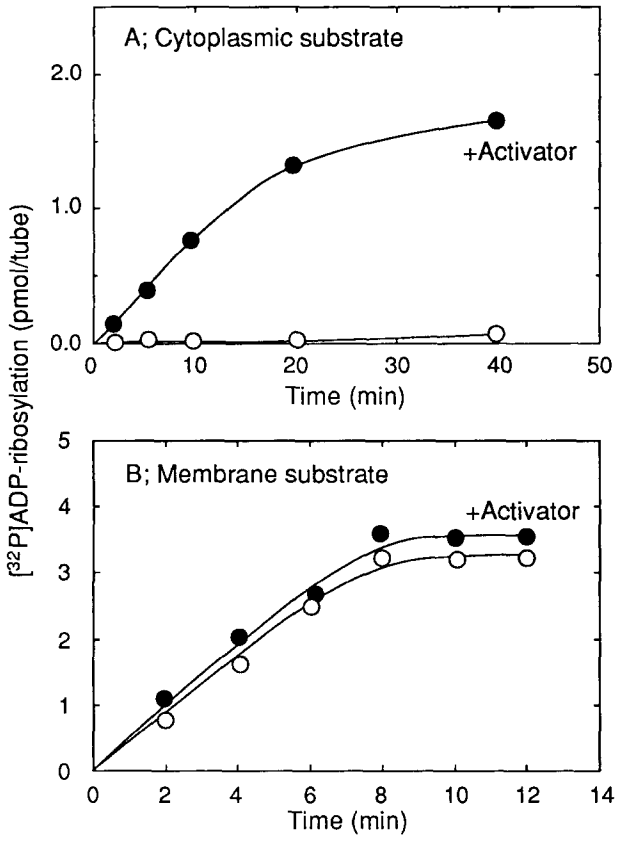

Fig. 1. C3-catalyzed ADP-ribosylation of the cytoplasmic and membrane substrates partially purified from bovine brain. Cytoplasmic (A) or membrane (B) proteins that had been partially purified from bovine brain as substrates for C3-catalyzed ADP-ribosylation were incubated at 37°C in 175 μl of a reaction mixture containing C3 enzyme (70 ng) and 2 μM [^{32}P]NAD in the presence (●) or the absence (○) of a protein factor (70 μg) partially purified as the activator for the C3-induced reaction. At the indicated times, aliquots (25 μl) were withdrawn and analyzed for [^{32}P]ADP-ribosylated proteins as described in section 2.3.

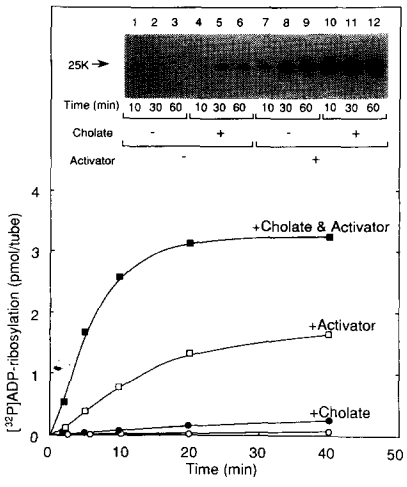

Fig. 2. Time course of C3-catalyzed ADP-ribosylation of the cytoplasmic substrates in the presence of cholate and/or the activator protein. The partially purified cytoplasmic substrates (24 μg) were incubated at 37°C in 150 μl of a reaction mixture containing C3 enzyme (60 ng) and [^{32}P]NAD in the presence (●, ■; lanes 4–6 and 10–12) or the absence (○, □; lanes 1–3 and 7–9) of 0.3% sodium cholate. The incubation mixture was further supplemented with 60 μg of the partially purified activator protein (□,○; lanes 7–12) or not (■,●; lanes 1–6). At the indicated times, aliquots (25 μl) were withdrawn and analyzed for [^{32}P]ADP-ribosylated proteins as described in section 2.3. Inset: Other aliquots were subjected to SDS-polyacrylamide gel electrophoresis and autoradiography.

imum stimulations were obtained with cholate and deoxycholate at 0.2% and 0.1%, respectively, in the presence of the activator protein. Further increases in concentration beyond these points caused marked inhibition of the ADP-ribosylation in either case. A similar biphasic effect was observed for deoxycholate even in the absence of the activator with a peak value at the concentration of 0.1%. On the other hand, Chaps (Fig. 3C) or Lubrol-PX (Fig. 3D) inhibited the C3-catalyzed ADP-ribosylation in a concentration-dependent manner. The concentration dependence of the inhibition caused by Chaps or Lubrol was affected by the presence of the activator protein; essentially the total inhibition occurred at a lower concentration in the absence of the activator than in its presence.

Possible interactions of various detergents with C3 enzyme was studied (Table I) in which NAD glycohydrolase activity of C3 enzyme was determined in the absence of protein serving as the substrate of ADP-ribosylation. When [4-^{3}H-*nicotinamide*]NAD was incubated with C3 enzyme at 37°C, NAD was hydrolyzed to radioactive nicotinamide in accordance with previous results [23]. The hydrolysis was significantly stimulated by the addition of cholate or deoxycholate, an activator of the C3-catalyzed ADP-ribosylation of the cytoplasmic substrate proteins. Chaps or Lubrol-PX was also effective in stimulating the glycohydrolase reaction, although the two detergents inhibited the ADP-ribosylation of the substrate proteins (see Fig. 3C and D). Thus, NAD glycohydrolysis catalyzed by C3 enzyme was stimulated by all detergents tested in the present study.

Table I also shows that C3-catalyzed NAD glycohydrolase was activated by crude phospholipids, azolectin. Therefore, the effects of various phospholipids on the C3-catalyzed ADP-ribosylation of the cytoplasmic substrates were further studied with individual phospholipids (Fig. 4). Anionic phospholipids, such as phosphatidylinositol (lanes 11 and 12), phosphatidylserine (lanes 13 and 14), phosphatidic acid (lanes 19 and 20) and cardiolipin (lanes 21 and 22), markedly enhanced C3-catalyzed ADP-ribosylation of cytoplasmic substrates, while lysophosphatidylcholine (lanes 5 and 6), lysophosphatidylethanolamine (lanes 7 and 8), phosphatidylcholine (lanes 15 and 16) and phosphatidylethanolamine (lanes 17 and 18) appeared to inhibit the ADP-ribosylation. There was no apparent stimulation or inhibition of the

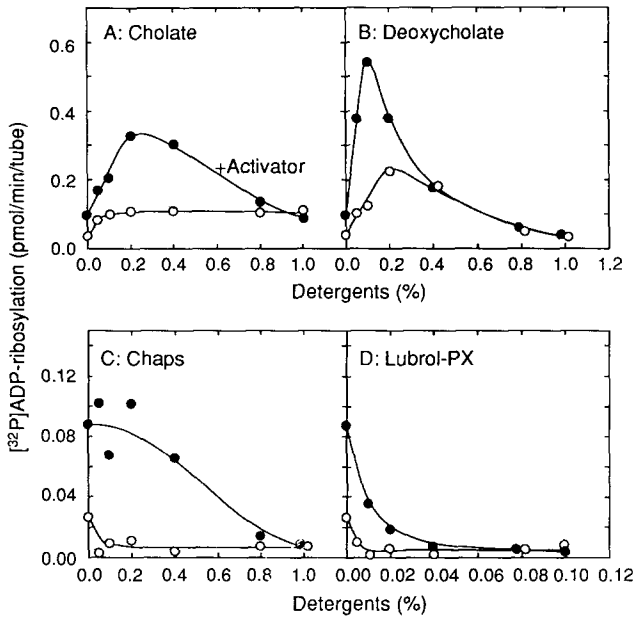

Fig. 3. Effects of various detergents on C3-catalyzed ADP-ribosylation of the cytoplasmic substrates in the presence or the absence of the activator protein. The partially purified cytoplasmic substrates (4 μg; approximately 3 pmol of the substrate activity) were incubated at 37°C for 1 min in 25 μl of a reaction mixture containing C3 enzyme (10 ng) and [^{32}P]NAD in the presence of various concentrations of cholate (A), deoxycholate (B), Chaps (C) or Lubrol-PX (D). The incubation mixture was further supplemented with the activator protein (•) or not (○). The initial rate of ADP-ribosylation (pmol of ADP-ribose incorporated per min/tube) were plotted against the concentrations of detergents added to the reaction mixture.

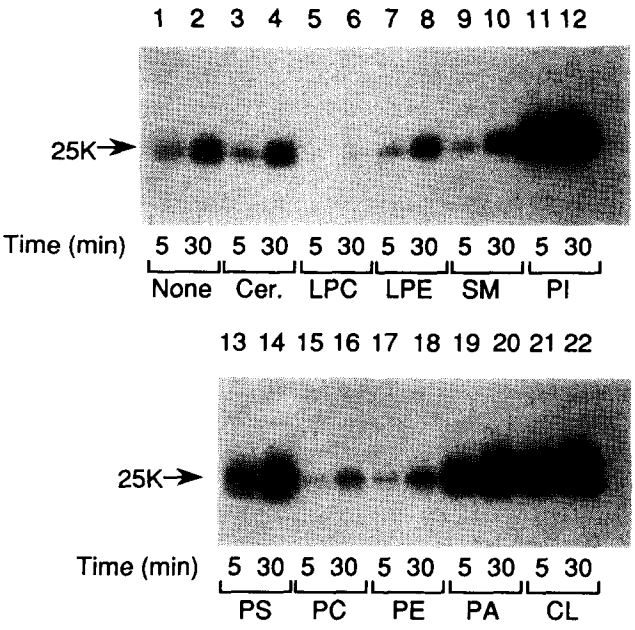

Fig. 4. Effects of various phospholipids on C3-catalyzed ADP-ribosylation of the cytoplasmic substrates. The partially purified cytoplasmic substrates (4 μg) were incubated at 37°C in 25 μl of a reaction mixture containing C3 enzyme (10 ng), [^{32}P]NAD and the activator proteins (10 μg) in the presence of various phospholipids at the concentration of 0.4 mg/ml or their absence (lanes 1 and 2). The phospholipids used were cerebroside (3 and 4), lysophosphatidylcholine (5 and 6), lysophosphatidylethanolamine (7 and 8), sphingomyelin (9 and 10), phosphatidylinositol (11 and 12), phosphatidylserine (13 and 14), phosphatidylcholine (15 and 16), phosphatidylethanolamine (17 and 18), phosphatidic acid (19 and 20) and cardiolipin (21 and 22). After the incubation for the indicated periods, [^{32}P]ADP-ribosylated proteins were subjected to SDS-polyacrylamide gel electrophoresis and autoradiography.

ADP-ribosylation when cerebroside (lanes 3 and 4) or sphingomyelin (lanes 9 and 10) was added. Although these results were obtained with the cytoplasmic activator protein, essentially the same effects of various phospholipids were observed in the absence of the activator protein (data not shown).

Table I

Effects of detergents and phospholipids on C3-catalyzed NAD glycohydrolysis

Additions (concentrations)	Nicotinamide formed (nmol/min per mg protein)
None	0.10 ± 0.009
Cholate (0.2%)	0.20 ± 0.006
Deoxycholate (0.07%)	0.19 ± 0.008
Chaps (0.3%)	0.24 ± 0.012
Lubrol-PX (0.02%)	0.24 ± 0.012
Azolectin (0.5 mg/ml)	0.16 ± 0.013

[4-^{3}H-*nicotinamide*]NAD (20 μM) was incubated at 37°C for 3 h with C3 enzyme in the presence of the additions indicated as described in section 2.3, and the activities are expressed as nmol of nicotinamide formed per min/mg of C3 enzyme. The values are means ± SE from four observations

4. DISCUSSION

The enzymatic activity of bacterial toxins dependent on ADP-ribosylation for their action appears to be latent; upon mild denaturation by detergents, protease digestion, or the disulfide bond reduction of native protein, there is a large increase in the enzymatic activity. For example, activation of cholera toxin by thiol results from reduction of a single disulfide bond between the two peptides in the A subunit [24]. Action of ATP to stimulate IAP-catalyzed ADP-ribosylation appears to be due to the dissociation of the A protomer, enzymatically active subunit (S_1), from the binding component of B oligomer [4]. The activities of the two toxins are also stimulated by a respectively specific type of detergents, such as SDS [25] and Chaps [4], as a result of their direct interactions with the toxin molecules rather than their substrates, G proteins.

In the previous study [13], we have shown that ADP-ribosylation of cytoplasmic substrates catalyzed by botulinum C3 enzyme was markedly stimulated by protein factors contained in the brain cytosol, and the factor-induced stimulation was profoundly affected by

the ionic composition of the reaction mixture. The present paper indicates that the C3-catalyzed ADP-ribosylation was activated not only by the protein factor(s) but also by certain types of detergents or phospholipids. The major findings obtained here are summarized as follows. (i) C3-catalyzed ADP-ribosylation of cytoplasmic substrates was stimulated by anionic detergents, such as cholate or deoxycholate, at their optimum concentrations, but inhibited by zwitterionic (Chaps) or nonionic (Lubrol-PX) detergents (Fig. 3); the stimulation or inhibition occurred in either the presence or the absence of the protein factor(s) that appeared to be a potent activator by a different mechanism. (ii) NAD glycohydrolase activity of C3 enzyme observable in the absence of substrate proteins, was stimulated by any of the detergents tested (Table I). (iii) Certain types of phospholipids stimulated, but other phospholipids did not (or inhibited), C3-catalyzed ADP-ribosylation of substrate proteins (Fig. 4). (iv) C3-catalyzed ADP-ribosylation appeared to be enhanced synergistically by the protein factor(s) and detergents or phospholipids (Figs 2 and 4).

Thus, it is very likely that detergents act not only on the C3 enzyme but also on the substrate proteins, probably dependent on their concentrations, since Chaps and Lubrol-PX exerted their stimulatory effect only on the NAD glycohydrolase activity of C3 despite their inhibitory action on the ADP-ribosylation of the substrate protein added. The action of the cytoplasmic protein activator also appeared to result from its interaction with both C3 enzyme and the substrate proteins [13] as had been also the case with ARF, which activated cholera toxin-catalyzed ADP-ribosylation of G_s [2,26,27]. However, the mechanism whereby the protein factor activated the C3-catalyzed ADP-ribosylation seems to be different from the mechanism for the detergent-induced activation; the maximum activity of ADP-ribosylation induced by optimal concentration of cholate or deoxycholate was further increased by combined addition of the protein factor (Fig. 3).

Effects of phospholipids on bacterial toxin-catalyzed ADP-ribosylations have also been reported previously [4,27]. Not only ARF but also phospholipids were essentially required for cholera toxin-catalyzed ADP-ribosylation of purified α-subunit of G_s [27]. Lysophosphatidylcholine and phosphatidylcholine stimulated thiol-dependent IAP-catalyzed NAD glycohydrolase activity [4]. Although the effects of other phospholipids have not been studied in the above papers, the specificity of phospholipids observed with botulinum C3 enzyme seemed to be somehow different from that observed with cholera toxin or IAP; the C3-catalyzed ADP-ribosylation was markedly enhanced by negatively charged phospholipids such as phosphatidylinositol, phosphatidylserine, phosphatidic acid and cardiolipin (Fig. 4). Conceivably, these phospholipids may share the same mechanism as anionic detergents for the activation of C3 enzyme.

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