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Activation of *Clostridium botulinum* C3 Exoenzyme-Catalyzed ADP-Ribosylation of RhoA by K^+ in a Mg^{2+}-Dependent Manner[1]

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The effect of KCl on ADP-ribosylation of the recombinant RhoA protein catalyzed by the *Clostridium botulinum* C3 enzyme was studied. When the recombinant glutathione S-transferase-RhoA fusion protein (GST-RhoA) was incubated with C3 and [adenylate-^{32}P]NAD, incorporation of radioactivity into the recombinant RhoA increased in the presence of KCl. The increase in ADP-ribose incorporation into RhoA due to KCl appeared in the presence of $MgCl_2$ and was abolished by EDTA. C3 was stabilized by KCl, but the stabilization was also seen with BSA. The KCl-induced increase in the ADP-ribosylation was observed even in the presence of BSA during the modification reaction, thus the effect of KCl was not due to the stabilization of C3. While the initial rate of the reaction was increased by KCl, maximum incorporation of ADP-ribose per GST-RhoA molecule did not increase in the presence of KCl. Kinetic analysis revealed that KCl increased V_{max} but did not alter K_m for either NAD or RhoA. The NAD glycohydrolase activity of C3 was also increased by KCl. These results indicate that KCl directly activates the C3 enzyme.

Key words: activation, ADP-ribosylation, *Clostridium botulinum* C3 exoenzyme, potassium ion, recombinant GST-RhoA fusion protein.

The Rho/Rac protein family, a member of the ras-GTP-binding protein superfamily, consists of Rho A, B, and C, and Rac1 and 2. The Rho proteins are thought to be involved in the regulation of actin stress fiber formation (*1, 2*), cell motility (*3, 4*), smooth muscle contraction (*5*), and malignant transformation (*6, 7*).

The C3 exoenzyme, produced by strains C and D of *Clostridium botulinum*, catalyzes ADP-ribose transfer from NAD to Rho/Rac proteins (*8-14*), and the modified amino acid residue was determined to be asparagine-41 in a Rho protein purified from the cytosol of bovine adrenal glands (*15*). ADP-ribosylation of the Rho proteins by the C3 exoenzyme closes off the pathway of signal transduction through the small GTP-binding protein (*16, 17*). The notion that Rho proteins regulate the organization of actin in the cytoskeleton is partly derived from observations that C3 treatment of Swiss 3T3 cells led to the disappearance of stress fibers and an altered cell shape (*1*).

Since the discovery of ADP-ribosyltransferase activity of the C3 enzyme, several factors, including cytosolic proteins and detergents, have been reported to affect the ADP-ribosylation of Rho proteins by the enzyme (*18-21*). However, analysis of the mechanism which modulates C3-catalyzed ADP-ribosylation of Rho is difficult especially in systems involving cell lysates or partially purified proteins as the substrate source, since in these crude preparations, the substrate may be a mixture of different Rho/Rac proteins, in addition to the possible coexistence of factors affecting the modulation, such as Rho-GDI, which was reported to bind tightly to Rho proteins and to inhibit C3-catalyzed ADP-ribosylation of Rho (*22*). The activity of several eucaryotic ADP-ribosyltransferases was shown to be modulated by salts; salt-activated and salt-inhibited transferases were found in turkey erythrocytes (*23, 24*) and chicken bone marrow cells (*25*). On the other hand, data on the effect of salt on C3 activity are limited. We studied the effect of KCl on C3 activity using a recombinant RhoA protein which is free from other C3 substrates or eucaryotic proteins which may influence the C3-catalyzed ADP-ribosylation of Rho proteins. We report here that the ADP-ribosyltransferase activity of C3 was enhanced by KCl, in a Mg^{2+}-dependent manner while the NAD glycohydrolase activity was also enhanced by the salt, but Mg^{2+}-independently.

MATERIALS AND METHODS

Materials—[adenylate-^{32}P]NAD (29.6 TBq/mmol) and

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Abbreviations: C3, M_r=25,000 protein produced by *Clostridium botulinum* type C displaying ADP-ribosyltransferase activity; DTT, dithiothreitol; G-protein, guanine nucleotide-binding regulatory protein; GST-RhoA, recombinant glutathione S-transferase-RhoA fusion protein; MSH, 2-mercaptoethanol; TCA, trichloroacetic acid; TFA, trifluoroacetic acid.

Enzymes: ADP-ribosyltransferase [EC 2.4.2.31]; NAD glycohydrolase [EC 3.2.2.5]; glutathione S-transferase [EC 1.2.1.12].

[carbonyl-^{14}C]NAD (1.96 GBq/mmol) were purchased from New England Nuclear. Botulinum C3 exoenzyme (C3) was from Wako, Osaka. pGEX5G (*26*) was kindly donated by Dr. Randy S. Haun of NHLBI, NIH, Bethesda, USA.

Preparation of Recombinant RhoA Proteins—The RhoA protein was expressed in *Escherichia coli* as described previously (*1, 27*) and purified as described for the Rac1 protein (*28*). Briefly, the complete RhoA coding sequence was amplified from poly(A)$^+$ RNA of a human leukocyte cell line, HL-60, by the RT-PCR technique, using human RhoA specific primers 1 (GGCCTGGTTCCGCGGGCAGC-CATCCGGAAGAA) and 2 (CTGCGCCTCGCTCCTCACA-AGACAAGGCAACCA). The PCR product was cloned into the pGEX5G expression plasmid and expressed in *E. coli* DH5α cells (*26*). The correct reading frame was confirmed by nucleotide sequencing of both strands of the recombinant plasmid and microsequencing of N-terminal amino acids of the recombinant RhoA protein after thrombin cleavage. The 49 kDa GST-RhoA fusion protein was purified by glutathione Sepharose 4B affinity chromatography (Pharmacia). The purified GST-RhoA protein was digested with bovine thrombin (Sigma), and then subjected to heparin Sepharose 6B affinity chromatography (Pharmacia) to remove thrombin. The purified preparation was dialyzed against salt-free buffer (20 mM Tris-HCl, pH 7.5, 1 mM MSH) just before use. Recombinant RhoA proteins, both fusion and post-digested, were shown to bind GTP and were ADP-ribosylated by C3.

Partial Purification of Brain Cytosolic Proteins Serving as Substrates for C3-Catalyzed ADP-Ribosylation—Botulinum C3 substrates were partially purified from bovine brain cytosol according to the method described previously (*18*) with modifications. Briefly, bovine brains were homogenized in 5 volumes of 0.25 M sucrose containing 20 mM Tris-HCl (pH 7.5), aprotinin (25 kallikrein inhibitory units/ml), 2 mM MSH, and 1 mM EDTA (TSAME buffer), and then centrifuged at 100,000$\times g$ for 1 h. The supernatant fraction thus obtained was subjected to DE52 column chromatography. The substrate for C3-catalyzed ADP-ribosylation was eluted from the column at about 50 mM NaCl. The peak fractions containing the substrate were pooled, concentrated with ammonium sulfate (90% saturation), and then fractionated on a gel filtration column of Sephadex G-100 pre-equilibrated in TSAME buffer containing 100 mM NaCl. The peak fractions (25 kDa) containing the substrate for C3-catalyzed ADP-ribosylation were pooled and used as endogenous Rho proteins of bovine brain.

ADP-Ribosylation Assay—For the standard assay, recombinant GST-RhoA (60 pmol) was incubated with 1 μM [^{32}P]NAD (200 Bq/pmol) and 0.6 ng of C3 in a reaction mixture consisting of 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 10 mM DTT, and 6 μg of BSA (total volume, 50 μl) at 37°C for 30 min. The reaction was terminated by the addition of 150 μl of 0.4% SDS containing 0.2 M Tris-HCl (pH 7.5) and 10 μg/ml of BSA, followed by further addition of 150 μl of 30% TCA. The acid-insoluble proteins were collected on a glass fiber filter (Whatman GF/A), and radioactivity counted with a liquid scintillation spectrometer. In some experiments, the reaction was terminated by boiling of the mixture with 25 μl of Laemmli sample buffer (*29*) containing 2% SDS, and then a sample (50 μl) was subjected to SDS/PAGE (15% gel), followed by Coomassie Blue staining and autoradiography. For quantification of radioactivity in the gel, a bio-imaging analyzer, BAS 2000 (Fuji), was used. The assays were repeated four times.

NAD Glycohydrolysis—The activity of NAD glycohydrolysis by C3 was determined by measuring nicotinamide release. [carbonyl-^{14}C]NAD (10 μM, 1.6 kBq/nmol) was incubated with 10 ng of C3 enzyme for 2 h at 37°C in a reaction mixture comprising 50 mM Tris-HCl, pH 7.5, 10 μg of BSA, 10 mM DTT, and 1 mM EDTA, in a total volume of 0.1 ml. The reaction was terminated by adding 0.4 ml of 0.1% TFA and then the mixture was passed through a filter. The filtrate (0.4 ml) was applied to a COSMOCIL 5C18-MS column (4.6$\times$150 mm) and developed with 0.1% TFA under isocratic conditions (*30*) at the flow rate of 0.3 ml/min. Fractions of 0.6 ml were collected and the radioactivity in each fraction was counted. Under these conditions, the retention times of authentic nicotinamide and NAD were 12 and 50 min, respectively. The amount of radioactivity in fractions corresponding to nicotinamide was estimated as the activity of NAD glycohydrolase. When the reaction was carried out without the C3 exoenzyme, the count in the nicotinamide fraction was about 0.02% of the total count. This value was subtracted from the count for nicotinamide fraction in each assay.

RESULTS

ADP-Ribosylation of the GST-RhoA Protein by C3—The human RhoA protein was expressed as a fusion protein with glutathione S-transferase in *E. coli*, and affinity-purified with glutathione-Sepharose. The obtained preparation showed a single band (49 kDa) on SDS-PAGE, followed by Coomassie Blue staining (data not shown). The C3 enzyme purified according to Morii *et al.* (*31*) was obtained from a commercial source. The enzyme preparation also exhibited electrophoretically a single band (25 kDa) (data not shown). When this enzyme was incubated with bovine brain cytosol in the presence of [^{32}P]NAD, only a single band corresponding to a 22 kDa protein was detected on SDS-PAGE/autoradiography, but no incorporation was observed at the position corresponding to actin (data not shown), a substrate of the botulinum C2 toxin (*32*).

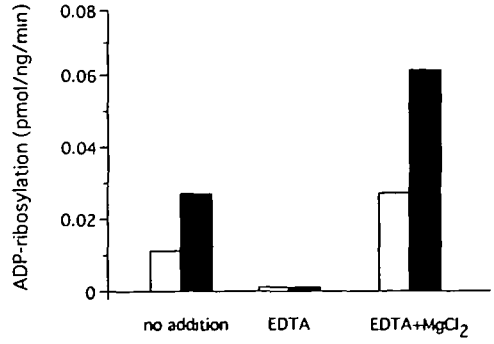

Fig. 1. **Effect of KCl on C3-catalyzed ADP-ribosylation with or without $MgCl_2$.** ADP-ribosylation of the GST-RhoA protein (60 pmol) by C3 (0.6 ng) was determined as described under "MATERIALS AND METHODS" in the absence (open bars) or presence (closed bars) of 100 mM KCl, with the addition of EDTA or $MgCl_2$, or their combination. The final concentrations of EDTA and $MgCl_2$ were 1 and 5 mM, respectively.

The fusion protein was incubated with C3 ADP-ribosyltransferase and [^{32}P]NAD, and then analyzed by SDS-PAGE, followed by Coomassie Blue staining and autoradiography. Radioactivity was observed only in a band (49 kDa) corresponding to the GST-RhoA protein, no other radioactive bands being observed. Since in this reconstitutional system the incorporation of radioactivity was observed only into the GST-RhoA protein, we measured the modification by counting the radioactivity of acid-insoluble materials in the reaction mixture collected on a glass filter. The result showed good correlation with that obtained by counting of the radioactivity associated with GST-RhoA on SDS-PAGE, using a bio-imaging analyzer, BAS 2000. Thus, determination of C3-catalyzed ADP-ribosylation of GST-RhoA was essentially carried out by means of this assay and the results obtained with the filter assay were confirmed by SDS-PAGE/BAS 2000. Mg^{2+}-dependency of the C3-catalyzed modification was noted when the purified native Rho protein was used as the C3-substrate protein (*33-35*). We examined this point with GST-RhoA and observed the labeling of GST-RhoA, without the addition of $MgCl_2$, but in the presence of EDTA the modification was completely inhibited (Fig. 1). This result may reflect the presence of a trace amount of Mg^{2+} in the preparation. To abolish the effect of contaminating divalent cations, further experiments were carried out in the presence of 1 mM EDTA, unless otherwise stated. BSA was also included in all reaction mixtures to prevent protein-destabilization during incubation for ADP-ribosylation.

KCl-Induced Increase in C3-Catalyzed ADP-Ribosylation—The effect of KCl on C3-catalyzed ADP-ribosylation of the GST-RhoA protein was then examined (Fig. 1). In the presence of 5 mM $MgCl_2$, the addition of KCl increased the modification up to 2.2-fold, while in the absence of $MgCl_2$, the effect of the salt was not observed. NaCl and LiCl showed similar effects to KCl, albeit to lesser extents, 75 and 72% of the KCl-induced increase, respectively (data not shown). When $MgCl_2$ was replaced by $CaCl_2$, the addition of KCl also increased the incorporation to 66% of the ADP-ribosylation with $MgCl_2$ (data not shown). These results indicate that the KCl-induced increase in ADP-ribosylation depends on the presence of divalent cations and that $MgCl_2$ can be replaced by $CaCl_2$. In the presence of $MgCl_2$, the most effective concentration of KCl was 100 mM, and higher concentrations of the salt inhibited the incorporation (Fig. 2A). Though inhibitory effects of higher concentrations (0.5-1.0 M) of NaCl on C3-catalyzed modification were reported (*18, 34*), the activating effect of KCl, NaCl, or LiCl has apparently not heretofore been described.

To exclude the possibility that the effect of the salt is only observed when the GST-RhoA fusion protein is used as a substrate for C3, similar experiments were carried out with the recombinant RhoA peptide or partially purified C3-substrate protein from bovine brain. As shown in Fig. 2B, KCl enhanced the modification of both the recombinant RhoA peptide and the bovine brain C3 substrate, and in each case, the maximal effect of KCl was observed at the concentration of 100 mM. When the ADP-ribosylated GST-RhoA labeled in the presence or absence of KCl was cut into GST and RhoA by thrombin, the incorporation was observed only into the RhoA portion in each case (data not shown). From these results, we concluded that the KCl-induced increase in C3-catalyzed ADP-ribosylation of the Rho protein is a commonly observed phenomenon among different preparations of Rho.

Effect of KCl on the pH Optimum—The effect of KCl on the pH optimum of C3-catalyzed ADP-ribosylation of GST-RhoA was also examined. As shown in Fig. 3, an increase in the modification by KCl was observed in the pH range of 6.5-9.5. The addition of KCl did not alter the pH

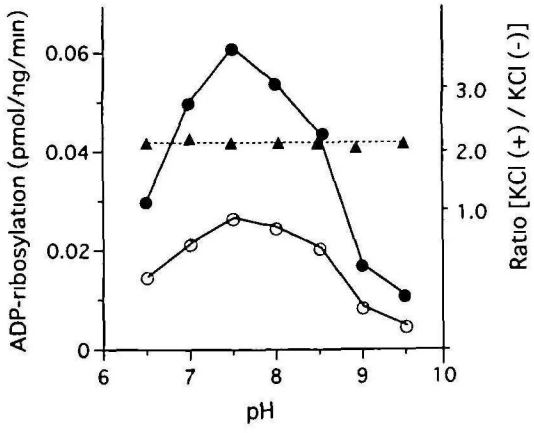

Fig. 3. **pH optimum of C3-catalyzed ADP-ribosylation of recombinant GST-RhoA in the presence and absence of KCl.** Recombinant GST-RhoA (60 pmol) was ADP-ribosylated with 1 μM [^{32}P]NAD and C3 (0.6 ng) in the presence (closed circles) or absence (open circles) of KCl (100 mM) at the indicated pH for 30 min Thereafter, the amount of [^{32}P]ADP-ribose incorporated into the protein was determined by the filter assay. The ratio of the activity in the presence of KCl to that in the absence of the salt [KCl (+)/KCl (−)] over the pH range of 6 5-9.5 is expressed as triangles.

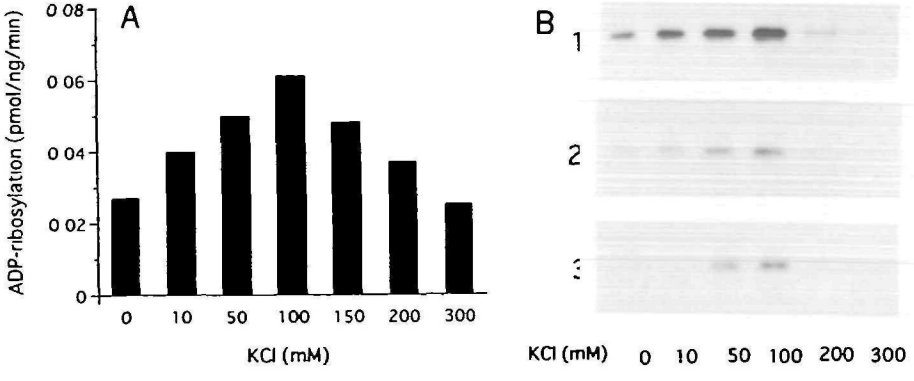

Fig. 2. **Activation of C3-catalyzed ADP-ribosylation by KCl.** (A) Recombinant GST-RhoA (60 pmol) was ADP-ribosylated by C3 (0.6 ng) in the presence of increasing concentrations of KCl. (B) Recombinant GST-RhoA (60 pmol) (lane 1), recombinant RhoA fragment (10 pmol) (lane 2) or partially purified Rho from bovine brain cytosol (0 5 μg) (lane 3) was ADP-ribosylated with 1 μM [^{32}P]NAD and C3 (0.6 ng) in the presence of increasing concentrations of KCl Thereafter, the labeled proteins were analyzed by SDS-PAGE and autoradiography.

optimum (pH 7.5) of the modification of the Rho protein. In addition, the ratio of the activity in the presence of KCl to that in its absence [KCl(+)/KCl(−)] was unchanged over the pH range of 6.5-9.5.

Effect of KCl on the Stability of GST-RhoA and C3—In an attempt to define the mechanism of the KCl effect on the acceptor activity of GST-RhoA, GST-RhoA was preincubated with 50 mM Tris-HCl (pH 7.5) containing 10 mM DTT at 37°C prior to the ADP-ribosylation assay. The incorporation markedly decreased with an increase in preincubation time. Preincubation of the recombinant protein for 10 min caused a decrease to 50% of the value obtained without preincubation and the reduction reached 75% on preincubation for 80 min. The addition of KCl during the preincubation of RhoA did not prevent this decrease of ADP-ribose incorporation. With the addition of BSA, $MgCl_2$, GTP, or GDP during the preincubation the initial level of the incorporation was maintained even after 80-min preincubation (Fig. 4A). Next, we tested the effect of KCl on the stability of the C3 enzyme. Though C3 was also destabilized by preincubation in the presence of 50 mM Tris-HCl (pH 7.5) containing 10 mM DTT, the addition of KCl as well as BSA maintained the modification at over 90% of the initial level, while guanine nucleotides or $MgCl_2$ were less effective in preventing the destabilization of C3 (Fig. 4B). These results indicate that KCl can stabilize C3 during the preincubation and that BSA has a stabilizing effect similar to that of KCl. Because BSA was included in the standard ADP-ribosylation assay mixture, it is unlikely that the KCl-induced increase in the modification is due to its ability to stabilize C3.

KCl-Induced Increase in the Initial Rate of C3-Cata-

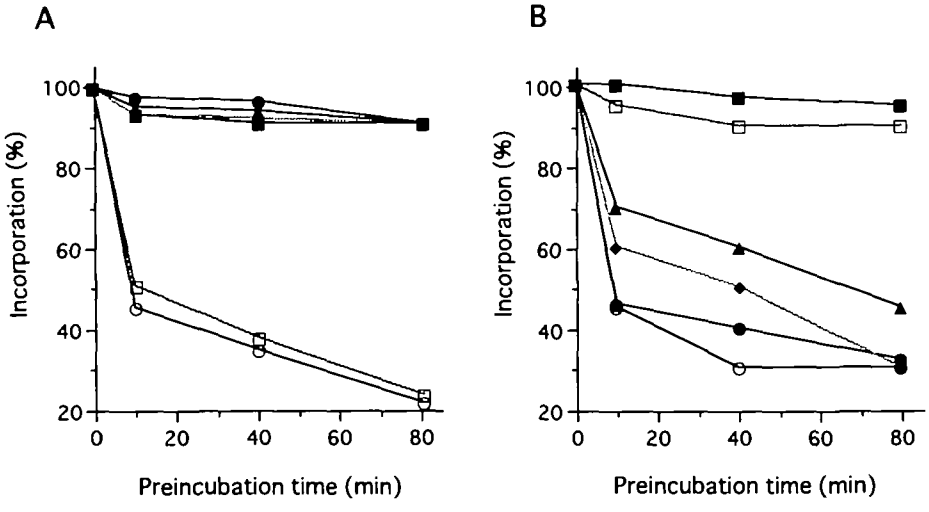

Fig. 4. **Effects of KCl, $MgCl_2$, guanine nucleotides, and BSA on the stability of GST-RhoA or C3 during preincubation.** Recombinant GST-RhoA (60 pmol) (A) or C3 (0.6 ng) (B) was preincubated in 50 mM Tris-HCl (pH 7.5) containing 1 mM EDTA and 10 mM DTT for the indicated periods with no addition (open circles), or the addition of one of the following five reagents, 6 μg BSA (closed squares), 5 mM $MgCl_2$ (closed circles), 0.5 mM GTP (closed triangles), 0.5 mM GDP (closed diamonds), or 100 mM KCl (open squares). Thereafter, each preincubated solution was supplemented with the rest of the reagents in addition to C3 (0.6 ng) (A) or GST-RhoA (60 pmol) (B). The modification was started by the addition of 1 μM [^{32}P]NAD. After 30 min incubation, the radioactive incorporation into the acid-insoluble fraction was determined. ADP-ribosylation without preincubation (0.061 pmol/ng/min) was taken as 100%.

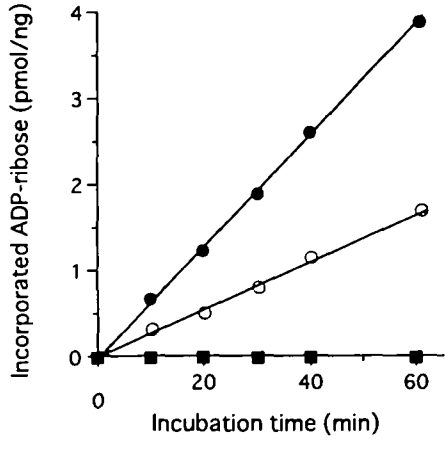

Fig. 5. **Time course of C3-catalyzed ADP-ribosylation of recombinant GST-RhoA in the presence and absence of KCl.** Recombinant GST-RhoA (60 pmol) was ADP-ribosylated with 1 μM [^{32}P]NAD and C3 (0.6 ng) in the presence of 1 mM EDTA for the indicated periods. The additions were none (squares), $MgCl_2$ (5 mM) (open circles), or $MgCl_2$ (5 mM) plus KCl (100 mM) (closed circles). Thereafter, the amount of [^{32}P]ADP-ribose incorporated into the protein was determined by the filter assay.

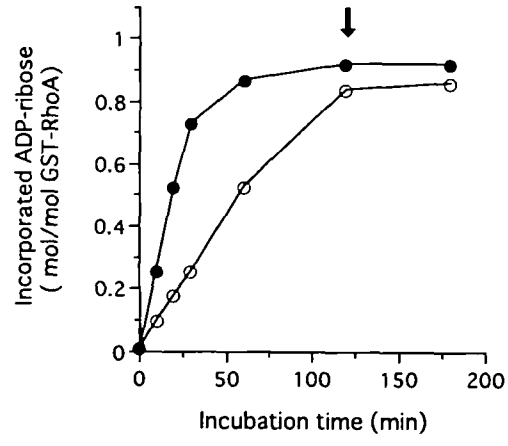

Fig. 6. **Stoichiometry of ADP-ribose incorporation into recombinant GST-RhoA with and without KCl.** Recombinant GST-RhoA (2.0 pmol) was incubated with 5 μM [^{32}P]NAD and C3 (10 ng) in the presence (closed circles) or absence (open circles) of KCl (100 mM) for the indicated periods. The amount of [^{32}P]ADP-ribose incorporated into the protein was determined by the filter assay. An additional 10 ng of C3 was added at the time point indicated by the arrow.

lyzed ADP-Ribosylation—To confirm that KCl indeed enhances the modification of RhoA in the presence of BSA and Mg^{2+}, we examined the time course of the reaction, with or without KCl, in the presence of those compounds. As shown in Fig. 5, the time course of the ADP-ribosylation of GST-RhoA by C3 was linear in both the presence and absence of KCl, thus the C3 activity remained constant, at least within 60 min of incubation, even in the absence of KCl, and the possibility that the KCl-induced increase in the modification is due to stabilization of C3 can be ruled out. Thus, we concluded that KCl increased the initial rate of C3-catalyzed ADP-ribosylation of RhoA. The increase in the initial rate of C3-catalyzed ADP-ribosylation on the addition of KCl might reflect an increase in sites of the modification in a molecule of GST-RhoA protein. However, this possibility was ruled out by the finding that the maximal incorporation of ADP-ribose/mol of GST-RhoA was 0.9 in both the presence and absence of KCl. Further addition of the C3 enzyme at 120 min of incubation did not affect the maximal incorporation of ADP-ribose (Fig. 6). To examine the mechanism of the KCl-induced increase in the initial velocity, the effect of KCl on the kinetics of ADP-ribosylation of GST-RhoA by C3 was examined. As shown in Fig. 7, A and B, KCl did not alter the K_m values for GST-RhoA and NAD, but did increase V_{max} 2.1-2.2 fold.

Effect of KCl on NAD Glycohydrolase Activity—The effect of KCl on the NAD glycohydrolase activity of the C3 exoenzyme was then examined. [carbonyl-^{14}C]NAD was incubated in the presence of 1 mM EDTA with the C3

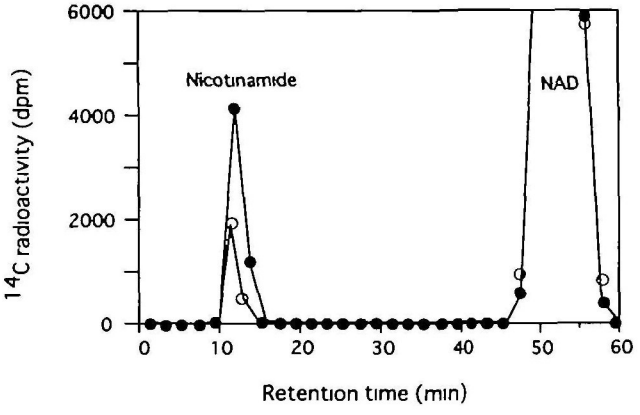

Fig. 8 **HPLC analysis of reaction products of C3 exoenzyme-catalyzed NAD glycohydrolysis.** The reaction was carried out at 37°C for 2 h and the products were analyzed by HPLC, as described under "MATERIALS AND METHODS." HPLC analysis of the reaction products in the absence (open circles) or presence (closed circles) of KCl (100 mM) is shown

TABLE I. **Effect of KCl on the NAD glycohydrolase activity of C3 in the presence and absence of Mg^{2+}.** The NAD glycohydrolase activity of C3 was measured as described under "MATERIALS AND METHODS" in the absence or presence of 100 mM KCl with the indicated additions.

Additions	NAD glycohydrolase activity of C3 (pmol/ng/min)	
	KCl (−)	KCl (+)
None	0.021	0.043
EDTA (1 mM)	0 020	0 042
EDTA (1 mM)+$MgCl_2$ (5 mM)	0.020	0.042

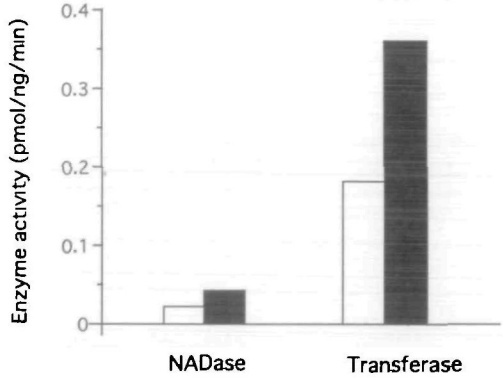

Fig 9. **Comparison of the C3 activities of ADP-ribosylation and NAD glycohydrolysis.** Both the ADP-ribosylation (Transferase) and NAD glycohydrolysis (NADase) assays were performed with 10 μM NAD in the absence (open bars) or presence (closed bars) of 100 mM KCl. For ADP-ribosylation, GST-RhoA (225 pmol) was incubated with 10 μM [^{32}P]NAD (70 Bq/pmol) and 0 6 ng of C3 in the reaction mixture described under "MATERIALS AND METHODS." NAD glycohydrolysis assay was also carried out as described under "MATERIALS AND METHODS"

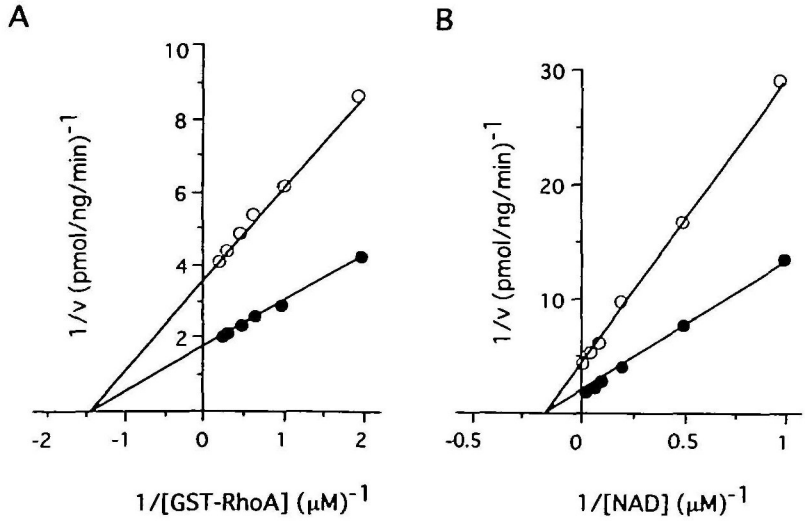

Fig 7 **Effect of KCl on the kinetics of C3-catalyzed ADP-ribosylation of recombinant GST-RhoA.** C3-induced ADP-ribosylation of recombinant GST-RhoA was studied with increasing concentrations of recombinant GST-RhoA (A) or NAD (B) in the presence (closed circles) or absence (open circles) of KCl (100 mM). The assays were performed with 50 μM NAD (A) or 4 5 μM GST-RhoA (B), respectively. The incorporation of [^{32}P]ADP-ribose was determined using the filter assay The data are shown as Lineweaver-Burk plots. The values of kinetic parameters obtained from the plots are: (A) K_m=0.71 μM, V_{max} [KCl(−)]=0.27 pmol/ng/min, and V_{max} [KCl(+)]=0.56 pmol/ng/min, (B) K_m=8.0 μM, V_{max} [KCl(−)]=0.25 pmol/ng/min, and V_{max} [KCl(+)]=0.55 pmol/ng/min.

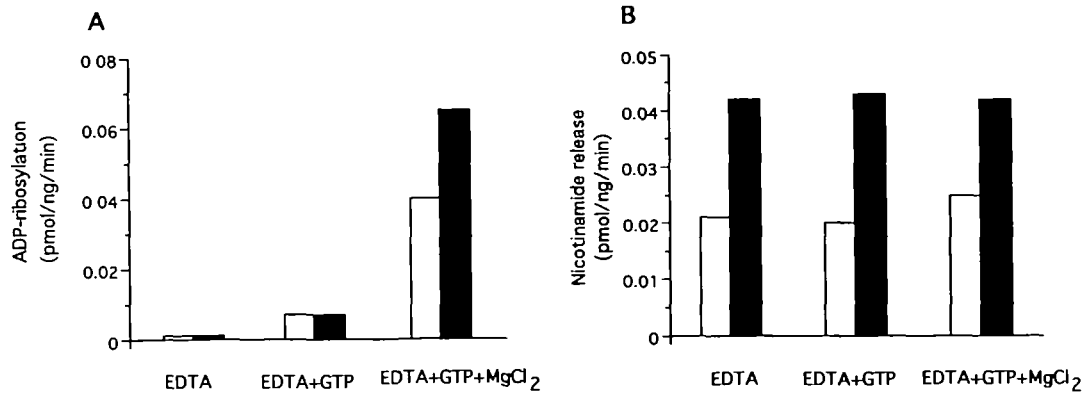

Fig. 10. **Effects of KCl, $MgCl_2$, and GTP, and their combinations on C3 exoenzyme-catalyzed ADP-ribosylation or NAD glycohydrolysis.** The effects of various agents on ADP-ribosylation (A) or NAD glycohydrolysis (B) by C3 exoenzyme were measured as described under "MATERIALS AND METHODS" in the absence (open bars) or presence (closed bars) of 100 mM KCl. The final concentrations of EDTA, $MgCl_2$, and GTP were 1, 5, and 0.5 mM, respectively.

enzyme, and the released nicotinamide was isolated by reversed phase HPLC. This release of nicotinamide increased in C3-dose- and incubation time-dependent manners (data not shown). As shown in Fig. 8, the addition of KCl to the assay system increased the amount of nicotinamide ~2.1 fold, while the addition of $MgCl_2$ did not affect the amount of released nicotinamide, in both the absence and presence of KCl (Table I). When the NAD glycohydrolase activity of C3 was compared with its ADP-ribosyltransferase activity with the same concentration of NAD (10 μM), the activity of NAD hydrolysis was about one-ninth that of the transferase, in both the presence and absence of KCl (Fig. 9). GTP with or without Mg^{2+} did not change either the basal or KCl-stimulated NAD glycohydrolase activity, whereas the guanine nucleotide restored the ADP-ribosylation to some extent in the absence of $MgCl_2$ (Fig. 10) in a KCl-independent manner. GTP plus $MgCl_2$ further increased the C3 transferase activity (Fig. 10), as noted by other workers (*33-35*).

DISCUSSION

We have described here the enhancement of botulinum C3 exoenzyme-catalyzed ADP-ribosylation of the recombinant RhoA protein by KCl, in a Mg^{2+}-dependent manner. The effects of salt on its ADP-ribosyltransferase activity have been reported (*23-25*). Moss *et al.* found that NaCl decreased K_m for NAD, in studies on ADP-ribosyltransferase from turkey erythrocytes (*24*). We previously reported that one type of ADP-ribosyltransferase from chicken bone marrow cells was activated by 200 mM NaCl (*25*). We also reported that NaCl stabilized the endogenous ADP-ribosyltransferase from chicken heterophils against mechanical treatment (*36*). Since there has been no documentation on the increasing effect of KCl on C3-catalyzed ADP-ribosylation, though higher concentrations of NaCl was found to inhibit the modification (*34*), it is interesting that C3-catalyzed reactions are also affected by these salts.

To elucidate the mechanism of the KCl-induced increase in the modification, we examined the effects of KCl on the pH optimum, kinetics and stoichiometry of the reaction, and found that KCl increased V_{max} of C3-catalyzed ADP-ribosylation but did not alter K_m for either RhoA or NAD, indicating that KCl does not affect the affinities of the C3 enzyme for these substrates. RhoA is a guanine nucleotide binding protein and the effect of Mg^{2+} on the nucleotide binding of G proteins has been widely recognized (*37, 38*). Our finding that Mg^{2+} and GTP stabilize RhoA but not C3 also support the idea that RhoA directly interacts with Mg^{2+} and guanine nucleotides. In contrast, KCl prevented destabilization of C3 but had no effect on the stabilization of RhoA. Since BSA, which can also protect C3 from destabilization, was present in all the ADP-ribosylation reactions, the KCl-induced increase in the ADP-ribosylation of RhoA cannot be explained by a stabilizing effect of KCl. The protective effect of KCl on destabilization of C3 indicates that KCl directly interacts with C3 and enhances its catalytic ability to ADP-ribosylate RhoA. If the target of KCl is C3 but not RhoA, NAD glycohydrolase activity, the other enzymatic activity of C3, would also be increased by KCl. Indeed, we found that the NAD glycohydrolase activity of C3 was activated 2-fold on the addition of KCl. Based on these results, we conclude that KCl increases the modification by activating the C3 enzyme. We observed that the NAD glycohydrolase activity does not require Mg^{2+} or guanine nucleotides. The NAD glycohydrolase activity of C3 has also been shown but only in the presence of Mg^{2+} (*11*). This seems to be the first report of the Mg^{2+}-independence of C3-glycohydrolase activity, in addition to the activating effect of KCl. The cholera and pertussis toxins were also reported to exhibit NAD glycohydrolase activity, though at low levels in the absence of appropriate acceptors (*39, 40*). The ratio of the C3 transferase activity to that of NAD glycohydrolase of C3 measured with same concentration (10 μM) of NAD was about 9.

Mg^{2+} did not affect the ability of KCl to increase NAD hydrolysis, while KCl required a divalent cation such as Mg^{2+} to increase the ADP-ribosyltransferase activity of C3. Based on the findings that the Rho protein requires Mg^{2+} to bind guanine nucleotides and that Mg^{2+} stabilizes RhoA but not C3, we presume that Mg^{2+} binds to RhoA directly. It is feasible that only Mg^{2+}-bound RhoA can serve as an acceptor for C3-catalyzing ADP-ribose transfer. The discrepancy in the Mg^{2+}-dependence of KCl-induced activation between ADP-ribosylation and NAD glycohydrolysis, catalyzed by C3, may reflect a difference in the Mg^{2+}

requirements of ADP-ribosylation and NAD glycohydrolysis. Why KCl did not enhance GTP-dependent ADP-ribosylation occurring in the absence of Mg^{2+} is not clear. Since GTP restores only part of the ADP-ribosylation achieved by Mg^{2+}, the role of GTP in the ADP-ribosylation of RhoA is not identical to that of Mg^{2+}. The mechanism by which GTP partly supports ADP-ribosylation of RhoA in the absence of free Mg^{2+} is the subject of ongoing studies.

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