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ADP-ribosylation of a 21–24 kDa eukaryotic protein(s) by C3, a novel botulinum ADP-ribosyltransferase, is regulated by guanine nucleotide

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Besides botulinum C2 toxin, *Clostridium botulinum* type C produces another ADP-ribosyltransferase, which we termed 'C3'. ADP-ribosyltransferase C3 has a molecular mass of 25 kDa and modifies 21–24 kDa protein(s) in platelet and brain membranes. C3 was about 1000 times more potent than botulinum C1 toxin in ADP-ribosylation of membrane proteins. C3-catalysed ADP-ribosylation of the 21–24 kDa protein(s) was decreased by stable guanosine triphosphates, with the potency order GTP[S] ≫ p[NH]ppG > p[CH_2]ppG. GTP[S] inhibited the ADP-ribosylation caused by C3 by maximally 70–80%, with half-maximal and maximal effects occurring at 0.3 and 10 μM-GTP[S] respectively. The concomitant addition of GTP decreased the inhibitory effect of GTP[S]. GTP[S]-induced inhibition of ADP-ribosylation was resistant to washing of pretreated platelet membranes. The data suggest that the novel botulinum ADP-ribosyltransferase C3 modifies eukaryotic 21–24 kDa guanine nucleotide-binding protein(s).

INTRODUCTION

Various microbial toxins affect the eukaryotic cell by ADP-ribosylation of regulatory proteins (Foster & Kinney, 1985). Well-known examples of this category of toxins are diphtheria, pertussis and cholera toxins. Whereas diphtheria toxin ADP-ribosylates elongation factor II, which modification inhibits protein synthesis (Collier, 1982), pertussis and cholera toxins affect the regulation of the adenylate cyclase system. Cholera toxin ADP-ribosylates the stimulatory G_s-protein of adenylate cyclase (Gill, 1982), thereby persistently activating the production of cyclic AMP. On the other hand, pertussis toxin modifies the G_i-protein (Ui, 1984) and impairs the inhibitory regulation of adenylate cyclase. Furthermore, recent studies have shown that cholera and pertussis toxin not only ADP-ribosylate G-proteins involved in adenylate cyclase regulation, but also modify various proteins belonging to a family of homologous G-proteins (transducin, G_o and G_n), which are involved in different transmembrane signal-transduction processes (Stryer & Bourne, 1986).

Botulinum C2 toxin, which is produced by certain strains of *Clostridium botulinum*, is another microbial ADP-ribosyltransferase (Simpson, 1984). Botulinum C2 toxin ADP-ribosylates isolated actin (Aktories *et al.*, 1986*a*,*b*) and actin in intact cells (Reuner *et al.*, 1987), thereby reducing the ability of the microfilament protein to polymerize (Aktories *et al.*, 1986*a*). It has been shown that, in addition to botulinum C2 toxin, certain strains of *Clostridium botulinum* type C produce a second ADP-ribosyltransferase, named 'C3', which is clearly distinct from the C2 toxin (Aktories *et al.*, 1987). The substrate of the novel ADP-ribosyltransferase appears to be 21–24 kDa protein(s) found in various cell types, including platelets, neuroblastoma × glioma hybrid cells, leucocytes and sperm (Aktories *et al.*, 1987). The nature of the eukaryotic substrate is not known at present. As pertussis, cholera and diphtheria toxins modify GTP-binding proteins (Foster & Kinney, 1985; Collier, 1982; Gill, 1982; Ui, 1984), we were prompted to investigate whether this was also true for the substrate of the novel ADP-ribosyltransferase C3. It is known that guanine nucleotides modify the properties of G-proteins to serve as substrates of ADP-ribosylating toxins (Kahn & Gilman, 1984; Ui *et al.*, 1985). Therefore we studied the influence of various guanine nucleotides on the ADP-ribosylation of the 21–24 kDa protein(s) by C3.

Recently it has been reported that botulinum neurotoxin D (Ohashi & Narumiya, 1987) and C1 (Ohashi *et al.*, 1987) can also ADP-ribosylate an eukaryotic 21 kDa protein. Therefore a further object of the present study was to compare the ADP-ribosyltransferase activities of C3 with those of botulinum neurotoxins.

MATERIALS AND METHODS

Materials

Botulinum ADP-ribosyltransferase C3 was purified from the culture medium of *Clostridium botulinum* type C strain 486 kindly donated by Dr. M. Sebald, Pasteur Institute, Paris, France, as described by Aktories *et al.* (1987). Botulinum C2 toxin was purified as described by Ohishi *et al.* (1980). Botulinum C1 toxin was purified from *Clostridium botulinum* Type C strain C 250, kindly

Abbreviations used: C3, novel botulinum ADP-ribosyltransferase (not to be confused with 'complement component C3', which is frequently abbreviated to 'C3' also); GTP[S](ATP[S]), guanosine (adenosine) 5'-[γ-thio]triphosphate; p[NH]ppG, guanosine 5'-[$\beta\gamma$-imido]triphosphate; p[CH_2]ppG, guanosine 5'-[$\beta\gamma$-methylene]triphosphate; G_i, the inhibitory guanine nucleotide-binding regulatory protein of adenylate cyclase; G_s, the stimulatory guanine nucleotide-binding regulatory protein of adenylate cyclase; G_o, a GTP-binding protein from bovine brain; G_n, a GTP-binding protein from neutrophil leucocytes; DTT, dithiothreitol; PAGE, polyacrylamide-gel electrophoresis.

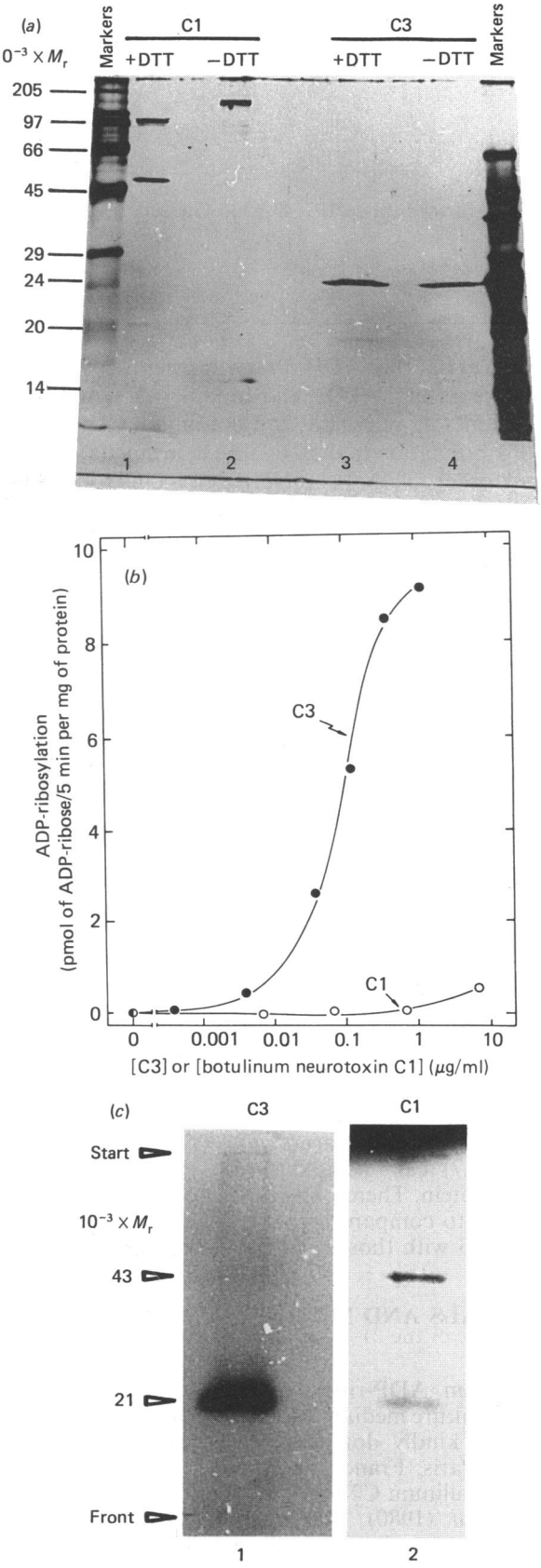

Fig. 1. Comparison of ADP-ribosylation of platelet membrane proteins by C3 and botulinum neurotoxin C1

(*a*) SDS/PAGE of purified C3 and botulinum neurotoxin C1 preparations. Neurotoxin C1 (1.4 μg; lanes 1 and 2) and C3 (2 μg; lanes 3 and 4) were heated without DTT (lanes 2 and 4) or with 100 mM-DTT (lanes 1 and 3) for 10 min at 95 °C and thereafter subjected to SDS/PAGE. The M_r markers were myosin (M_r 205000), phosphorylase *b* (97000), bovine serum albumin (66000), ovalbumin (45000), carbonic anhydrase (29000), trypsinogen (24000), trypsin inhibitor (20000) and α-lactalbumin (14000). (*b*) ADP-ribosylation of platelet membrane proteins by C3 and botulinum neurotoxin C1. Human platelet membranes were incubated in the presence of 0.5 μM-[^{32}P]NAD with increasing concentrations of C3 (●) or neurotoxin C1 (○) for 30 min. The radioactivity of labelled proteins was determined as described in the Materials and methods section. (*c*) Autoradiogram of SDS/PAGE of human platelet proteins labelled by C3 or neurotoxin C1. Human platelet membranes were ADP-ribosylated by C3 (1.4 μg/ml; lane 1) or neurotoxin C1 (7 μg/ml; lane 2) as described in the Materials and methods section, with the exception that 10 mM-DTT was present during the incubation. The incubation time was 30 min. Lane 1, exposed for 1 day; lane 2, exposed for 3 days.

donated by Dr. G. Sakaguchi, Osaka, Japan, as described by Kurazono *et al.* (1985). *Clostridium botulinum* were cultured essentially as described by Aktories *et al.* (1987). All nucleotides used were obtained from Boehringer (Mannheim, Germany). [^{32}P]NAD was prepared as described by Cassel & Pfeuffer (1978).

Preparation and pretreatment of human platelet membranes

Human platelet membranes were prepared as described by Aktories & Jakobs (1984). For pretreatment, 250 μl of a suspension of human platelet membranes (1–4 mg of protein/ml) were incubated with 30 μl of GTP[S] (100 μM) and 20 μl of triethanolamine/HCl (50 mM, pH 7.5) for 5 min at 37 °C. Thereafter treatment was stopped by the addition of 1 ml of ice-cold triethanolamine/HCl (50 mM, pH 7.5) and centrifugation of the mixture for 4 min at 10000 *g*. The pellet was twice washed with 1 ml of triethanolamine/HCl buffer and finally resuspended in the appropriate volume of 10 mM-triethanolamine/HCl, pH 7.5, for use in the ADP-ribosylation assay.

ADP-ribosylation assay

C3-induced ADP-ribosylation of the 21–24 kDa protein(s) in human platelet membranes was performed essentially as described by Aktories *et al.* (1987). Briefly, human platelet membranes (50–250 μg of protein/tube) were preincubated in a medium containing 10 mM-thymidine, 1 mM-$MgCl_2$, 0.5 mM-ATP, 0.1–1 μM-[^{32}P]-NAD (about 0.3–1 μCi/tube) and 50 mM-triethanolamine/HCl, pH 7.5, for 5 min at 37 °C in a total volume of 100 μl. The reaction was initiated by the addition of C3 ADP-ribosyltransferase and the incubation was continued for further 5 min or as indicated. Determination of the incorporated ADP-ribose was performed by stopping the reaction with 400 μl of a solution containing SDS (2%, w/v) and bovine serum albumin (1 mg/ml) and precipitation of the proteins with 500 μl of trichloroacetic acid (30%, w/v). Proteins were collected on to nitrocellulose filters. The filters were washed ten times with 2 ml of 6% trichloroacetic acid and placed in scintillation fluid for analysis of retained radioactivity. The filter blank in the absence of botulinum

Table 1. Influence of nucleotides on C3-induced ADP-ribosylation

Human platelet membranes were pretreated with GTP, GDP, GTP[S] and ATP[S] (all at 10 μM) for 5 min in the medium described in the Materials and methods section, except that $MgCl_2$ was 5 mM. Thereafter, the ADP-ribosylation was initiated by the addition of C3 (0.1 μg/ml) and the reaction allowed to continue for 10 min. The [^{32}P]NAD concentration was 0.5 μM. The radioactivity of the labelled proteins was determined as described in the text. Data are means ± S.E.M.

Nucleotide	ADP-ribosylation (pmol of ADP-ribose/10 min per mg of protein)
None	2.12±0.08 (n = 8)
GTP (10 μM)	2.14±0.06 (n = 4)
GDP (10 μM)	1.90±0.1 (n = 4)
GTP[S] (10 μM)	0.34±0.04 (n = 4)
ATP[S] (10 μM)	2.05±0.12 (n = 4)

ADP-ribosyltransferase was 0.1–0.2% of added [^{32}P]-NAD and was subtracted from retained radioactivity.

SDS/polyacrylamide-gel electrophoresis

After the indicated incubation time the ADP-ribosylation reaction was stopped by the addition of 1 ml of trichloroacetic acid (20%). The resulted pellet was washed with diethyl ether and dissolved in 50 μl of electrophoresis buffer and analysed by SDS/PAGE as described by Laemmli (1970). Gels were stained, destained and subjected to autoradiography. A similar procedure was used for SDS/PAGE analysis of the purified C1 toxin and C3 ADP-ribosyltransferase. Silver staining was performed as described by Wray *et al.* (1981).

Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

RESULTS

Recently we reported the purification of a novel ADP-ribosyltransferase from *Clostridium botulinum* culture medium (Aktories *et al.*, 1987). Fig. 1(*a*) shows the silver-stained SDS/PAGE gel of the ADP-ribosyltransferase C3 purified from *Clostridium botulinum* type C as described recently (Aktories *et al.*, 1987). The M_r of C3 was about 25000 in both the presence and the absence of DTT. The preparation of C3 was apparently free of any higher-M_r contaminants. In particular, there was no indication of contamination by C1. For comparison, the gel for botulinum neurotoxin C1 is shown on the left of Fig. 1(*a*). The M_r of the botulinum neurotoxin C1 was about 150000 in the absence of DTT. Under reducing conditions (100 mM-DTT), C1 was cleaved into a light and a heavy chain with M_r 50000 and 100000 respectively. The ADP-ribosylation of 21–24 kDa protein(s) in human platelet membranes by C3 and C1 is shown in Fig. 1(*b*). A half-maximal and maximal ADP-ribosylation of the 21–24 kDa protein(s) by C3 was observed at 0.1 and 1–3 μg respectively. This effect was not increased with DTT (result not shown). In contrast with the extensive ADP-ribosylation caused by C3, botulinum neurotoxin C1 caused only marginal labelling, measured by means of the filtration assay. Surprisingly, SDS/PAGE analysis of the proteins labelled by neurotoxin C1 revealed labelling of 43 and 21–24 kDa proteins (Fig. 1*c*). ADP-ribosylation of the ~ 21 kDa protein in platelet membranes by C1 was not substantially increased by DTT or trypsin treatment.

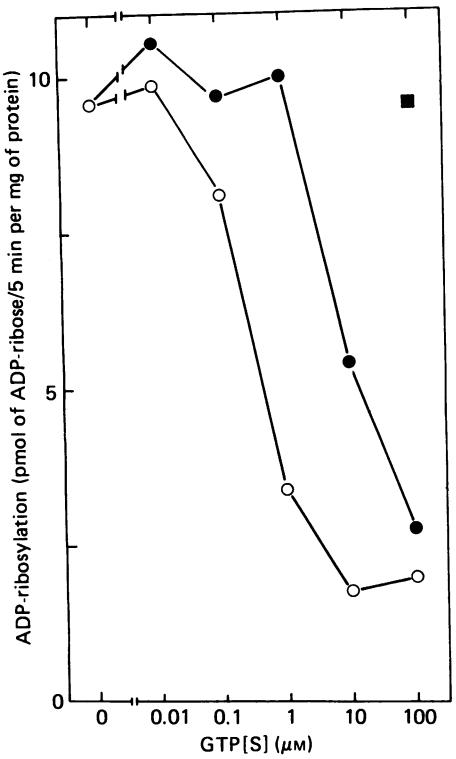

Fig. 2. Influence of GTP[S] on the ADP-ribosylation by C3

Human platelet membranes were pretreated with increasing concentrations of GTP[S] without GTP (○) and with GTP (100 μM; ●) or with GTP alone (■) for 5 min. Thereafter the ADP-ribosylation was initiated by the addition of C3 (9 μg/ml) and [^{32}P]NAD (0.1 μM) and the reaction allowed to continue for a further 5 min. The radioactivity of the labelled proteins was determined as described in the text.

Influence of guanine nucleotides on the ADP-ribosylation of the 21–24 kDa substrate protein(s) by botulinum ADP-ribosyltransferase C3

Next we studied the effects of nucleotides on the ADP-ribosylation of the 21–24 kDa substrate by C3. As shown in Table 1, GTP[S], but not GTP, GDP or ATP[S], inhibited the ADP-ribosylation by C3. Similar effects of the nucleotides were obtained in the absence or presence of 0.5 mM-ATP and without and with creatine kinase and phosphocreatine (0.4 mg/ml and 5 mM respectively) as a GTP/ATP-regenerating system (results not shown). Fig. 2 shows that GTP[S] decreased the ADP-ribosylation in a concentration-dependent manner, with half-maximal and maximal inhibition occurring at about 0.3 and 10 μM-GTP[S] respectively. GTP (100 μM), which by itself was without effect under these conditions, caused a shift to the right of the concentration–response curve of GTP[S]. Fig. 3 shows that not only GTP[S], but also p[NH]ppG and p[CH_2]ppG, decreased the labelling

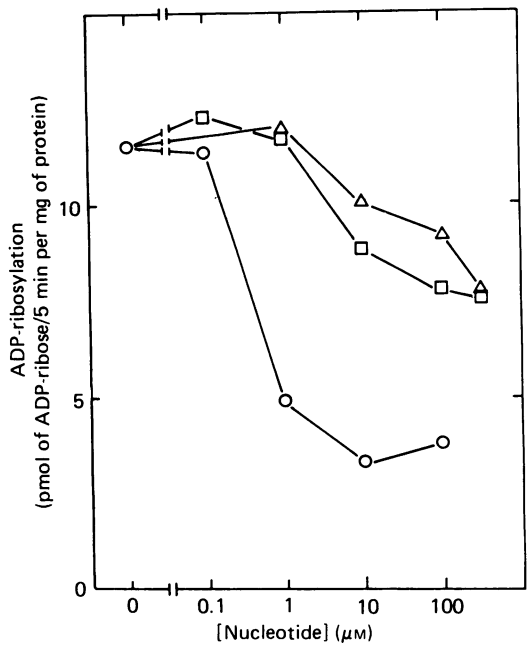

Fig. 3. Influence of GTP[S], p[NH]ppG and p[CH_2]ppG on C3-catalysed ADP-ribosylation

Human platelet membranes were pretreated with the indicated concentrations of GTP[S] (○), p[NH]ppG (□) and p[CH_2]ppG (△) for 5 min. Thereafter the ADP-ribosylation was initiated by the addition of C3 (1.4 μg/ml) and [^{32}P]NAD (0.1 μM). The radioactivity of the labelled proteins was determined as described in the text.

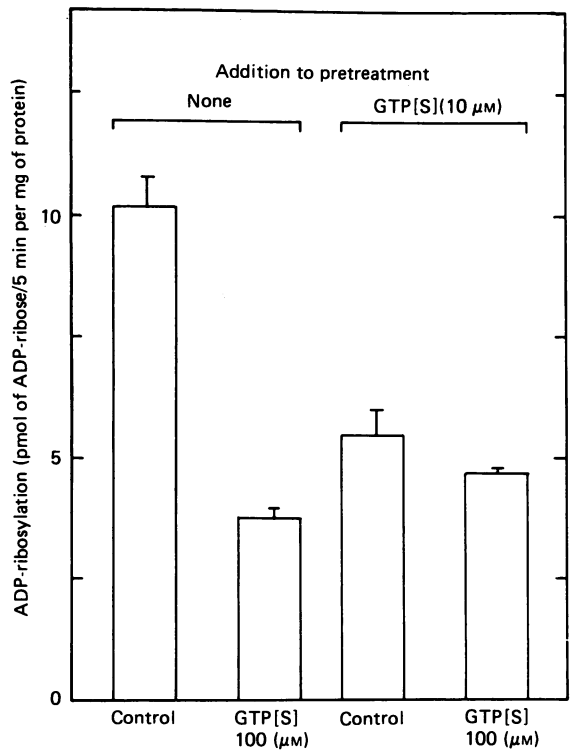

Fig. 4. Influence of pretreatment of human platelet membranes with GTP[S] on C3-catalysed ADP-ribosylation

Platelet membranes were pretreated without and with GTP[S] (10 μM) and washed as described in the text. Thereafter the treated membranes were ADP-ribosylated by C3 (1.4 μg/ml) without (Control) and with GTP[S] (100 μM). Results are given as means ± S.E.M. (n = 3).

caused by C3. However, GTP[S] was much more potent and more efficient than the other stable GTP analogues.

In order to study whether GTP[S] acts on C3 or on the protein substrate, platelet membranes were pretreated with 10 μM-GTP[S] for 5 min at 37 °C. Pretreatment was stopped by diluting the incubation mixture with ice-cold buffer, followed by the washing off of unbound GTP[S]. Thereafter the membranes were used for the ADP-ribosylation assay. As shown in Fig. 4, in control membranes the stable GTP analogue decreased C3-catalysed protein labelling. In GTP[S]-pretreated platelet membranes the ADP-ribosylation was greatly decreased, even in the absence of GTP[S] during the ADP-ribosylation assay. The addition of GTP[S] had only a marginal further inhibitory effect. In order to clarify further the specificity of the GTP[S] action, we studied the effects of the stable guanine nucleotide on ADP-ribosylation catalysed by C3 in comparison with botulinum C2 toxin in the same crude preparation of platelet membranes. As shown in Fig. 5, the ADP-ribosylation of the 21–24 kDa protein by C3 was clearly decreased by GTP[S]. In contrast, the labelling by botulinum C2 toxin was not significantly affected by GTP[S].

DISCUSSION

Recently, we have shown that a 21 kDa protein in platelet membranes is ADP-ribosylated by a novel ADP-ribosyltransferase C3 found in the culture medium of *Clostridium botulinum* (Aktories *et al.*, 1987). The pathophysiological role of this microbial enzyme is not known at present. Interestingly, it has been reported that botulinum neurotoxins C1 and D also possess ADP-ribosyltransferase activity (Ohashi & Narumiya, 1987; Ohashi *et al.*, 1987), suggesting that the enzymic activity is involved in the neurotoxic effects of these agents. Furthermore, substrates of C1 and D-induced ADP-ribosylation were also 21 kDa proteins (Ohashi & Narumiya, 1987; Ohashi *et al.*, 1987). As shown in the present paper, we tried to reproduce these findings with highly purified C1 toxin and compared the ADP-ribosylation caused by C3 with the labelling of proteins caused by the neurotoxin. However, under our experimental conditions, C1 toxin caused only a weak ADP-ribosylation of 21–24 kDa protein(s). In addition, we found labelling of a 43 kDa protein by C1, which is probably due to contamination with botulinum C2 toxin. Labelling of the 21–24 kDa substrate by C1 was not substantially increased either by DTT or by pretreatment of the toxin with trypsin. Also, in rat brain membranes, C3 was much more potent in ADP-ribosylating the 21–24 kDa protein(s) than was C1 (results not shown). Two explanations are feasible for the discrepancies between our results and those published by others (Ohashi & Narumiya, 1987; Ohashi *et al.*, 1987). First, C3 is structurally and functionally unrelated to C1, and the ADP-ribosylation of the ~21 kDa protein(s) by C1 is due merely to contamination of the C1 preparation used with C3. Second, C3 may be an active subunit of C1 that has to be released to elicit the ADP-ribosylating activity, a process that might not have

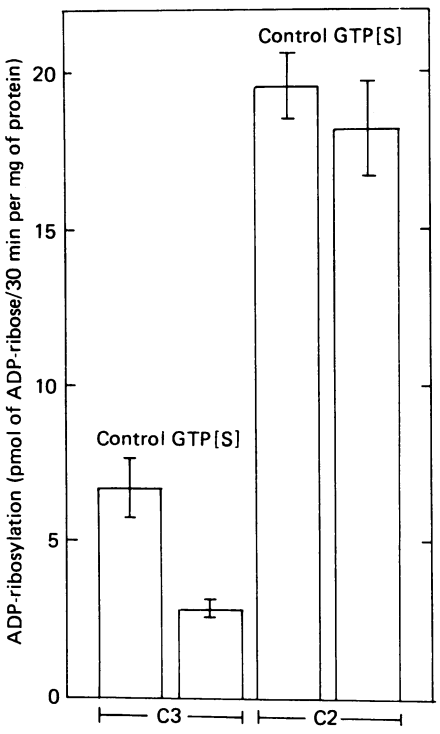

Fig. 5. Influence of GTP[S] on the ADP-ribosylation by C3 and by botulinum C2 toxin

Human platelet membranes were pretreated for 5 min without and with GTP[S] (100 μM). Thereafter the ADP-ribosylation was initiated by the addition of C3 (1.4 μg/ml) or of botulinum C2 toxin (1 μg/ml) and the incubation was continued for a further 5 min. The labelled proteins were determined as described in the text. The [^{32}P]NAD concentration was 1 μM. Data are given as means $\pm$ S.E.M. ($n = 3$).

occurred under our experimental conditions. Although it is not possible to decide at present which explanation is correct, one should be cautious in assuming that botulinum neurotoxins are ADP-ribosyltransferases until the purity of the neurotoxin preparations used has been checked.

Ohashi and colleagues (Ohashi & Narumiya, 1987; Ohashi *et al.*, 1987) reported that the ADP-ribosylation of the 21 kDa protein in brain membranes by botulinum neurotoxins C1 and D was affected by guanine nucleotides. Here we report that guanine nucleotides also influenced the ADP-ribosylation of the 21–24 kDa substrate(s) caused by C3. GTP[S], but not ATP[S], inhibited the labelling of the 21–24 kDa protein(s) in a concentration-dependent manner, with a maximal inhibitory effect at about 10 μM. This effect was most probably due to a specific reaction of GTP[S] at a GTP-binding protein, since GTP effectively shifted the inhibition curve of GTP[S] to higher nucleotide concentrations. The reason why GTP itself did not inhibit ADP-ribosylation by C3 is not known at present. A non-specific degradation of GTP is unlikely. Even in the presence of ATP and with a GTP/ATP-regenerating system, which effectively block unspecific GTPase activity (Cassel & Selinger, 1976; Aktories & Jakobs, 1981), GTP was without any inhibitory effect.

The site of GTP[S] action could be at one or more of at least three different molecular levels. First, GTP[S] may act directly on the ADP-ribosyltransferase C3; secondly, the 21–24 kDa protein substrate(s) may be the target; and thirdly, NAD metabolism may be affected by the stable GTP analogue, leading finally to decreased ADP-ribosylation. Our finding that pretreatment of platelet membranes followed by extensive washing decreased the C3-induced ADP-ribosylation argues against a direct interaction of GTP[S] with C3. The finding that GTP[S] impaired C3-catalysed ADP-ribosylation but not the botulinum-C2-toxin-induced reaction suggests that NAD metabolism, but not the C3 substrate, was modified by GTP[S].

GTP-binding proteins (G-proteins) are substrates of ADP-ribosylating bacterial toxins like pertussis, cholera and diphtheria toxins (Collier, 1982; Gill, 1982; Ui, 1984; Foster & Kinney, 1985). These G-proteins are functionally and structurally modified by guanine nucleotides (Stryer & Bourne, 1986). On the other hand, guanine nucleotides modify the properties of these G-proteins to serve as substrates for ADP-ribosylating toxins. For instance ADP-ribosylation of G_s depends on the presence of GTP (Gill, 1982), whereas the stable GTP analogue, GTP[S], inhibits the ADP-ribosylation of G_s and G_i by cholera toxin (Kahn & Gilman, 1984) and pertussis toxin (Ui *et al.*, 1985) respectively. Considering these data, one can speculate also that the substrate of C3 is a GTP-binding protein. Recently several GTP-binding proteins with M_r values of about 21000 have been described. Among these are the various *ras*-oncogene products (Weinberg, 1983; Hagag *et al.*, 1986), the ADP-ribosylation factor (Kahn & Gilman, 1986) and the GTP-binding protein isolated from placenta (Evans *et al.*, 1986). These proteins, which may be involved in transmembrane signalling, remain to be tested as substrates of C3.

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