Inhibition of cytochalasin D-stimulated G-actin ATPase by ADP-ribosylation with *Clostridium perfringens* iota toxin

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Clostridium perfringens iota toxin belongs to a novel family of actin-ADP-ribosylating toxins. The effects of ADP-ribosylation of skeletal muscle actin by *Clostridium perfringens* iota toxin on cytochalasin D-stimulated actin ATPase activity was studied. Cytochalasin D stimulated actin-catalysed ATP hydrolysis maximally by about 30-fold. ADP-ribosylation of actin completely inhibited cytochalasin D-stimulated ATP hydrolysis. Inhibition of ATPase activity occurred at actin concentrations below the critical concentration (0.1 μM), at low concentrations of Mg^{2+} (50 μM) and even in the actin-DNAase I complex, indicating that ADP-ribosylation of actin blocks the ATPase activity of monomeric actin and that the inhibitory effect is not due to inhibition of the polymerization of actin.

INTRODUCTION

Various bacterial ADP-ribosylating toxins modify regulatory GTP-binding proteins, thereby affecting eukaryotic cell function. Pertussis toxin and cholera toxin ADP-ribosylate GTP-binding proteins involved in transmembrane signal transduction (for a review, see Pfeuffer & Helmreich, 1988). Diphtheria toxin and *Pseudomonas* exotoxin A modify elongation factor II (Collier, 1967; Iglewski & Kabat, 1975). In all of these cases, the eukaryotic protein substrates of these toxins possess inherent GTPase activities, which are apparently crucial for the regulatory functions of the GTP-binding proteins. *Clostridium perfringens* iota toxin belongs to a novel class of ADP-ribosylating toxins which modify actin (Simpson *et al.*, 1987; Schering *et al.*, 1988). ADP-ribosylation inhibits the ability of actin to polymerize (Schering *et al.*, 1988). Additionally, ADP-ribosylated actin acts like a capping protein to inhibit polymerization of non-modified actin at the fast-growing end of actin filaments (Wegner & Aktories, 1988). Actin is an ATP-binding protein and possesses inherent ATPase activity (for a review, see Pollard & Cooper, 1986). It appears that ATP hydrolysis plays an important role in the dynamic transition between monomeric and polymeric actin. Polymerization of actin largely increases ATP hydrolysis. Since ADP-ribosylation of actin inhibits polymerization, it is important to clarify whether ADP-ribosylation affects the ATPase activity of actin directly or indirectly. Basal ATPase activity of non-polymerized G-actin is very low. The mycotoxin cytochalasin is known to stimulate G-actin ATPase activity severalfold (Brenner & Korn, 1980; for a review, see Cooper, 1987); we therefore studied the effects of ADP-ribosylation on cytochalasin-stimulated actin ATPase activity.

MATERIALS AND METHODS

Materials

The ADP-ribosylating component of *C. perfringens* iota toxin was purified from the culture medium of *C.*

perfringens type E strain CN5063, which was kindly donated by Dr. S. Thorley (Wellcome Biotech, Beckenham, Kent, U.K.) essentially according to the procedure described (Stiles & Wilkens, 1986). Cytochalasin D was obtained from Sigma (Deisenhofen, Germany). DNAase I was a gift from Dr. H. G. Mannherz (Marburg, Germany). [α - ^{32}P]ATP, [γ - ^{32}P]ATP and [^{32}P]NAD were prepared according to Walseth & Johnson (1979) and Cassel & Pfeuffer (1978) respectively, or were obtained from NEN (Dreieich, Germany). All other reagents were of analytical grade and were purchased from commercial sources.

Purification of skeletal muscle actin

Rabbit skeletal muscle actin was purified as described (Spudich & Watt, 1971).

ADP-ribosylation of actin

Skeletal muscle actin was ADP-ribosylated as described (Aktories *et al.*, 1986a) in a medium containing 40 μM - CaCl_2 , 40 μM -ATP, 1 mM-dithiothreitol, 50 μM -NAD 10 mM-triethanolamine/HCl, pH 7.5, and the indicated concentrations of iota toxin (ADP-ribosylating component) in a total volume of 100 μl for 1 h.

Filter assay for determination of actin ADP-ribosylation

This was performed as described (Schering *et al.*, 1988).

ATPase activity

ATPase activity was determined essentially as described for GTPase activity (Aktories *et al.*, 1982). After ADP-ribosylation of actin (in a volume of 100 μl), free nucleotides were removed by the addition of 20 μl of Dowex 1 $\times$ 8 (50 %). The samples were vortex-mixed and centrifuged for 1 min. The supernatant was removed. MgCl_2 (50 μM or 1 mM), cytochalasin D (1 μM) and [γ - ^{32}P]ATP (50 μM , about 200000 c.p.m., all final concentrations) were added to the supernatant. ATP hydrolysis was terminated after incubation for 2 h or as indicated at 37 °C by the addition of 450 μl of ice-cold sodium phosphate buffer (20 mM, pH 2) containing 5 %

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(w/v) activated charcoal to 50 μ l aliquots of the reaction mixture. After centrifugation for 15 min at 15000 g, 300 μ l of the supernatant was counted for radioactivity.

Binding of cytochalasin D to actin

Actin (15 μ M) was incubated in G-buffer [2 mM-Tris/HCl (pH 8.0)/0.2 mM- CaCl_2 /0.005% NaN_3 /0.2 mM-ATP/0.5 mM- β -mercaptoethanol] containing 110 μ M-NAD without or with iota toxin (3 μ g) for 45 min in a total volume of 180 μ l. Thereafter, MgCl_2 (50 μ M) and cytochalasin D (1 μ M) were added to give a final volume of 200 μ l. The mixture was incubated for 1 h at room temperature. Then the mixture was centrifuged in a Centricon filter membrane holder (Amicon, Witten, Germany) for 10 min at 4000 g. Thereafter, an aliquot of the filtrate (5 μ l) was used in the ATPase assay.

Polyacrylamide-gel electrophoresis

This was performed under non-denaturing conditions according to Laemmli (1970), with the exception that the 11% polyacrylamide gel contained no SDS but did contain 0.5 mM-ATP.

Actin concentration

This was determined spectrophotometrically by using a specific absorption coefficient at 290 nm of $0.62 \text{ ml}^{-1} \cdot \text{mg} \cdot \text{cm}^{-1}$.

RESULTS

Fig. 1 illustrates the influence of increasing concentrations of iota toxin on the ADP-ribosylation of actin and on ATP hydrolysis stimulated by cytochalasin D (1 μ M). ATP hydrolysis was determined in the presence of 50 μ M- MgCl_2 , a concentration which does not induce polymerization. ADP-ribosylation of actin inhibited actin-catalysed ATP hydrolysis in a concentration-dependent manner. Half-maximal and maximal inhibition of ATPase activity was obtained with 0.4 μ g and 6.0 μ g of iota toxin/ml respectively. Half-maximal and maximal ADP-ribosylation of actin was observed at 1 μ g and

Fig. 1. Influence of increasing concentrations of iota toxin on ADP-ribosylation of actin (●) and inhibition of actin ATPase (○)

Skeletal muscle actin (7 μ M) was incubated for 2 h with the indicated concentrations of iota toxin. Thereafter, the ATPase activity of actin (5 μ M) was determined in the presence of 1 μ M-cytochalasin D, 50 μ M- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and 50 μ M- MgCl_2 .

Fig. 2. Inhibition of cytochalasin D-stimulated actin ATPase by ADP-ribosylation with iota toxin

Skeletal muscle actin was ADP-ribosylated in the presence of iota toxin (5 μ g) and 50 μ M-NAD (○) or was not ADP-ribosylated (●). Thereafter, the actin concentration was made up to 0.1 μ M. Actin ATPase was stimulated by the indicated concentrations of cytochalasin D in the presence of 1 mM- MgCl_2 and 50 μ M- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$.

6.0 μ g of iota toxin/ml respectively. Fig. 2 shows that cytochalasin D increased ATP hydrolysis by skeletal muscle actin in a concentration-dependent manner. A maximal 30-fold increase in ATP hydrolysis occurred at 1 μ M-cytochalasin D. ADP-ribosylation of actin by iota toxin almost completely inhibited the cytochalasin-stimulated ATPase activity. In order to exclude further the possibility that inhibition of ATPase activity was caused by ADP-ribosylation-induced inhibition of actin polymerization, cytochalasin-stimulated ATPase activity was studied at actin concentrations (0.1 μ M) below its critical concentration. The MgCl_2 concn. was 1 mM, a concentration which causes an increase in G-actin ATPase activity. Fig. 3(a) illustrates the time course of ATP hydrolysis by actin. Actin ATPase activity was constant for at least 150 min. In the absence of iota toxin, NAD did not affect ATP hydrolysis; subsequent addition of iota toxin almost completely inhibited ATP hydrolysis. The time course of the ADP-ribosylation of actin shows that 2 min after addition of iota toxin, about 50% of the actin was ADP-ribosylated (Fig. 3b). The high rate of ADP-ribosylation correlates with the almost immediate inhibition of ATPase. Inhibition of actin ATPase activity by ADP-ribosylation was revealed by direct determination of the nucleotide bound to actin. For this purpose $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ -loaded actin was treated by Dowex to remove unbound nucleotides. Then, actin was ADP-ribosylated by iota toxin in the presence of cytochalasin D for a further 60 min and the nucleotides bound were determined by t.l.c. Fig. 4 shows that unmodified actin bound almost only ADP. In contrast, the ADP-ribosylated actin bound significantly more ATP. Inhibition of stimulation of actin ATPase could be due to the inability of cytochalasin to interact with ADP-

Fig. 3. Time course of ATP hydrolysis (a) and ADP-ribosylation (b) of actin

(a) ATP hydrolysis by actin was determined in the presence of 50 μM -[γ - ^{32}P]ATP, 50 μM -NAD, 50 μM -MgCl₂ and 1 μM -cytochalasin D as described (○). After 60 min, 5 μg of iota toxin was added (●). The P_i released was determined at the indicated time points. (b) ADP-ribosylation of actin was determined under identical conditions as in (a) with the exception that 50 μM -[^{32}P]NAD and unlabelled ATP were present. The actin concentration was 6 μM .

ribosylated actin. Studies on the binding of cytochalasin to actin are hampered by the fact that the affinity between G-actin and cytochalasin D is very low (about 18 and 3 μM with Ca²⁺ and Mg²⁺ respectively; Goddette & Frieden, 1986). We addressed this issue by using a filtration assay. Control and ADP-ribosylated actin were incubated with cytochalasin for 1 h at room temperature. The incubation mixture was then filtered through Centricon filter membranes (exclusion M_r of 10000). Thereafter, an aliquot of the Centricon filtrate was used to stimulate actin ATPase. When actin was absent during the preincubation period, ATP hydrolysis was stimulated by cytochalasin present in the Centricon filtrate [43 ± 1 mmol of ATP hydrolysed/min per mol of actin (mean $\pm$ S.E.M., $n = 3$)]. Stimulation was less with the Centricon filtrates of the cytochalasin D solutions preincubated with unmodified or ADP-ribosylated actin (31 ± 0.2 and 33 ± 0.5 mmol of ATP hydrolysed/min per mol of actin respectively), indicating that cytochalasin D binds to either form of actin.

It has been shown by Polzar *et al.* (1989) that cytochalasin stimulates actin ATPase even in an actin-DNAase I complex, which is not able to polymerize. As ADP-ribosylated actin still binds to

Fig. 4. Actin-bound nucleotides after ADP-ribosylation of actin with iota toxin

Actin (9 μM), loaded with [α - ^{32}P]ATP, was ADP-ribosylated with iota toxin (5 $\mu\text{g}/\text{ml}$) (b) or was not ADP-ribosylated (a) in the presence of 50 μM -NAD and 1 μM -cytochalasin D for 1 h. Thereafter, the actin-bound nucleotides were determined by t.l.c. (polyethyleneimine-cellulose; 1 M-LiCl). Scan analysis of the radioactive nucleotides is shown.

Fig. 5. Polyacrylamide gel showing the actin-DNAase I complex

Actin (1 μM) was [^{32}P]ADP-ribosylated as described. Thereafter, ADP-ribosylated actin (1 μM) was incubated with 2 μM -DNAase I for 17 h and the complex formed was analysed by non-denaturing polyacrylamide-gel electrophoresis (lane a) and autoradiography (lane b).

DNAase I (Aktories *et al.*, 1986b), we studied the effect of ADP-ribosylation on cytochalasin D-stimulated ATPase under this quasi-monomeric state of actin. For this purpose, actin (0.1 μM) was ADP-ribosylated by iota toxin and then incubated for 17 h with 0.2 μM -DNAase I. Fig. 5 shows a native gel and autoradiogram of the actin-DNAase I complex indicating that all ADP-ribosylated actin was complexed under the conditions used.

Table 1. Effects of ADP-ribosylation on the ATPase activity of the actin-DNAase I complex

Actin (1 μ M) was ADP-ribosylated without or with 50 μ M-NAD and iota toxin (5 μ g/ml) as described. The actin-DNAase I complex was then formed by incubation of the ADP-ribosylated actin (0.1 μ M) with DNAase I for 17 h at 4 °C. Thereafter, the ATPase activity of the actin-DNAase I complex was determined in the presence or the absence of cytochalasin D (1 μ M) in the presence of [³²P]ATP and 1 mM-MgCl₂. Data are means $\pm$ S.E.M. ($n = 3$).

Additions	ATPase activity (mmol of ATP/min per mol of actin)	
	Actin	Actin-DNAase I complex
Without cytochalasin D		
Iota toxin	6.3 $\pm$ 0.2	5.9 $\pm$ 0.2
NAD	5.7 $\pm$ 0.2	4.6 $\pm$ 0.2
Iota toxin + NAD	1.7 $\pm$ 0.1	1.6 $\pm$ 0.1
With cytochalasin D (1 μ M)		
Iota toxin	222 $\pm$ 4	117 $\pm$ 4
NAD	199 $\pm$ 5	103 $\pm$ 2
Iota toxin + NAD	47 $\pm$ 1	28 $\pm$ 1

Non-complexed actin migrated as DNAase I (results not shown). As shown in Table 1, stimulation of ATP hydrolysis in the actin-DNAase complex was inhibited by prior ADP-ribosylation of actin. Cytochalasin stimulated ATP hydrolysis of the actin-DNAase I complex to a lesser extent than with uncomplexed actin. However, under both conditions ADP-ribosylation inhibited actin ATPase.

DISCUSSION

It has recently been shown that ADP-ribosylation of actin inhibits polymerization (Aktories *et al.*, 1986a; Schering *et al.*, 1988) and blocks actin-associated ATPase activity (Geipel *et al.*, 1989). ATP hydrolysis by actin occurs concomitantly with polymerization (Carlier, 1987). Therefore the question arises as to whether inhibition of ATP hydrolysis is secondary to a blockade of actin polymerization. The data presented in this report show that the effects of the toxin on ATPase activity are not due to inhibition of actin polymerization. ADP-ribosylation of actin inhibited ATP hydrolysis stimulated by cytochalasin D, which has been shown to increase G-actin ATPase activity (Brenner & Korn, 1980). In agreement with this view, stimulation of ATPase by cytochalasin was observed at concentrations below the critical concentration of actin and/or at very low concentrations of Mg²⁺ (50 μ M). Inhibition of actin ATPase was observed by direct determination of actin-bound nucleotide. These studies showed that the amount of total nucleotide bound (ADP and ATP) to actin was not changed after ADP-ribosylation, and indicated that ADP-ribosylated actin had bound ATP rather than ADP. Studies on the concentration dependency of the effects of iota toxin on ADP-ribosylation of actin and inhibition of ATPase activity revealed that ADP-ribosylation of not more than about 20 % of actin caused inhibition of more

than 50 % of actin ATPase. The reason for this discrepancy is not known, but it was also observed with actin which had been ADP-ribosylated by botulinum C2 toxin (Geipel *et al.*, 1989). It is noteworthy that cytochalasin supposedly stimulates ATPase activity by formation of actin dimers (Goddette & Frieden, 1986). One could argue that ADP-ribosylation inhibits formation of dimers, thereby indirectly blocking cytochalasin D-stimulated ATP hydrolysis. However, recent findings that cytochalasin stimulates actin ATPase in the actin-DNAase I complex question this view (Polzar *et al.*, 1989). Also in the actin-DNAase I complex, ADP-ribosylation of actin inhibits ATP hydrolysis stimulated by cytochalasin D. Thus all of these findings indicate that ADP-ribosylation of actin blocks the ATPase activity of monomeric actin.

The mechanism responsible for inhibition of cytochalasin-stimulated ATPase by ADP-ribosylation activity is not known. The ADP-ribosylation site in actin has been determined to be Arg-177 (Vandekerckhove *et al.*, 1988). So far the binding site of cytochalasin to actin has not been analysed. Our binding data suggest that ADP-ribosylated actin is still capable of binding cytochalasin. ADP-ribosylation of actin did not prevent binding of nucleotides, which supposedly bind to actin amino acid residues 114-118 and/or 336 and 356 (Hegyi *et al.*, 1986; Barden & Kemp, 1987). Furthermore, the data shown herein and reported recently (Geipel *et al.*, 1989), indicate that ADP-ribosylation also inhibits basal G-actin ATPase activity in the absence of cytochalasin. Thus it is suggested that ADP-ribosylation of actin, which inhibits the ability of actin to polymerize and turns actin into a capping protein, blocks ATP hydrolysis by affecting the catalytic region of the actin ATPase.

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