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ADP-ribosylation of Rho proteins inhibits sperm motility

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The highly homologous Rho proteins RhoA, RhoB and RhoC are low-molecular-mass GTP-binding proteins. They are selectively ADP-ribosylated by *Clostridium botulinum* ADP-ribosyltransferase C3 (C3 exoenzyme). The biological function of the Rho proteins is still unclear; there is evidence that they are involved in the regulation of the filamental network of cells. Here we report that C3 exoenzyme-like toxins ADP-ribosylate small GTP-binding proteins in bovine spermatozoa and inhibit sperm motility. These findings indicate that Rho proteins which reportedly regulate the microfilament system are basically involved in sperm motility.

GTP-binding protein; Rho protein; ADP-ribosylation; sperm motility

1. INTRODUCTION

Rho proteins are low-molecular-mass GTP-binding proteins with apparent molecular masses of about 21–24 kDa. They belong to the large family of Ras proteins. RhoA, RhoB and RhoC which are about 40% homologous to Ras proteins are suggested to be involved in the regulation of the microfilamental cytoskeleton [1]. Rho proteins are selective substrates for *Clostridium botulinum* ADP-ribosyltransferase C3 (C3 exoenzyme) [2–5] and other C3 exoenzyme-like bacterial ADP-ribosyltransferases [6]. ADP-ribosylation of Rho proteins renders the small GTP-binding proteins functionally inactive [7]. Rho proteins appear to control the formation of actin filaments but not of microtubules in various types of cells [3,7]. Ridley and Hall [1] reported that this family of low-molecular-mass GTP-binding proteins regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Recently it has been shown that Rho proteins are apparently involved in the migration of neutrophil leukocytes and swiss 3T3 fibroblasts [8,9]. It is generally accepted that actin is essential for cell movement [10]. Actin has been detected immunologically also in spermatozoa from various species, including bovine spermatozoa [11]. It has been proposed that actin could play a role in the stabilization of the acrosome structure [12] but no direct involvement of actin in sperm motility has been offered.

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This paper is dedicated to the memory of Eycke Böhme.

Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

To assess the occurrance of Rho proteins in bovine spermatozoa, spermaozoal membrane proteins and cytosolic proteins were ADP-ribosylated in the presence of [^{32}P]NAD, and Rho proteins were visualized by autoradiography after SDS-PAGE. We also investigated the effects of the ADP-ribosylation of Rho proteins upon sperm motility by incubating live bovine spermatozoa with C3 exoenzyme.

2. MATERIALS AND METHODS

2.1. *[α-^{32}P]GTP-binding to spermatozoal proteins*

For membrane and cytosol preparation, freshly ejaculated spermatozoa were separated from contaminating cells on a two step gradient of Percoll (45% and 90%, v/v, of Percoll in Biggers Whitten's Willingham's medium [13]). The pellets were washed, and enriched bovine spermatozoal tail membranes and cytosol were prepared as described previously [14]. Proteins were solubilized in Laemmli sample buffer [15], heated at 95°C in the presence of 10 mM dithiothreitol, separated on a 12.5% SDS slab gel and subsequently electroblotted onto nitrocellulose (Amersham Buchler, Braunschweig, Germany) [16]. After electroblotting, [α-^{32}P]GTP-binding was performed [17]. The blots were washed in binding buffer (1 mM EGTA, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4, supplemented with 0.3% (v/v) Tween 20) and subsequently incubated with 0.5 μCi [α-^{32}P]GTP (DuPont-New England Nuclear, Dreieich, Germany), for 1 h at room temperature. After thoroughly washing with binding buffer, the dried blots were subjected to autoradiography.

2.2. *[^{32}P]ADP-ribosylation assay*

C3 exoenzyme was prepared as described previously [18]. For [^{32}P]ADP-ribosylation of Rho proteins in bovine spermatozoa, enriched bovine sperm tail membrane proteins (40 μg) and cytosolic proteins (40 μg) were incubated with 0.4 μg of C3 exoenzyme in a medium containing 1 mM dithiothreitol, 1 mM EDTA, 2 mM $MgCl_2$, 1 mM ATP, 10 mM thymidine, 100 μM GDP, 0.01% (w/v) SDS, 1.5 μCi/tube [^{32}P]NAD (Amersham Buchler, Braunschweig, Germany) and 50 mM triethanolamine-HCl (pH 7.5) in a total volume of 200 μl for 30 min at 37°C. Incubation was terminated by addition of sample

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buffer. Subsequently, proteins were heated at 95°C in the presence of 10 mM dithiothreitol and analyzed on a 12.5% SDS-polyacrylamide gel. Gels were stained with Coomassie blue followed by drying and subsequent autoradiography. For 2D-gel electrophoresis, spermatozoal tail membranes (40 μg/rod gel) and human platelet membranes (5 μg/rod gel) were ADP-ribosylated by C3 exoenzyme. Human platelet membranes were prepared from outdated platelet concentrates as previously described [19]. Proteins were dissolved in sample buffer (4.9 M urea, 1% (w/v), Nonidet NP 40, 1% ampholine, pH gradient 5–7 and 50 mM dithiotreitol) and loaded onto rod gels for isoelectric focussing. The second dimension was run on 12.5% SDS-polyacrylamide slab gels. Gels were dried and subjected to autoradiography for 24 h.

2.3. *Analysis of sperm motility*

Freshly ejuculated bovine spermatozoa were separated from contaminating cells. About 70–90% of motile spermatozoa were collected, washed in Biggers Whitten's Willingham's medium, supplemented with 5 mg/ml bovine serum albumine (BWW/BSA medium), and adjusted to 3×10^7 cells/ml by dilution with BWW/BSA medium. Samples without or with the addition of 1 μg/ml, 10 μg/ml and 100 μg/ml of C3 exoenzyme were incubated at room temperature for 6 h. Thereafter, sperm motility was determined by the use of a Makler counting chamber and a Stroemberg-Mika cell motion analysis computer (Stroemberg-Mika medical equipment, Bad Feilnbach, Germany).

For the evaluation of time depending effects of C3 exoenzyme, spermatozoa were incubated without and with 10 μg/ml C3 exoenzyme at room temperature for 8 h. The percentage of motile bovine spermatozoa to total sperm count was calculated. Concurrently to sperm motility measurements, the viability of C3 exoenzyme-treated and control spermatozoa was determined by dye exclusion with eosin Y. The percentage of immotile and live spermatozoa to total sperm count was calculated. For dye exclusion test, 5 μl of the spermatozoa suspension (3×10^7 cells/ml) was mixed with 5 μl of eosin Y solution (0.15% (v/v) in Ringer's solution).

2.4. *Control experiments*

Spermatozoa were treated without and with C3 exoenzyme (10 μg/ml) for 7 h. The immotile spermatozoa were obtained by sedimentation and washed two times with phosphate-buffered saline. A medium containing 1 mM dithiothreitol, 1 mM EDTA, 2 mM $MgCl_2$, 10 mM thymidine, 1 mM ATP, 100 μM GDP, 0.01% SDS, 0.3 μM [^{32}P]NAD, 0.4 μg/ml C3, 50 mM triethanolamine-HCl (pH 7.5) was added to the pelleted spermatozoa in a final volume of 200 μl. Pellets were lysed by sonication for 1 min on ice. After incubation for 30 min at 37°C, proteins were solubilized in Laemmli sample buffer, heated at 95°C in the presence of 10 mM dithiothreitol and analyzed on a 12.5% SDS-polyacrylamide gel. The Coomassie Blue-stained and dried gels were subjected to autoradiography for 12 h.

3. RESULTS AND DISCUSSION

In a course of experiments to identify low-molecular-mass GTP-binding proteins in bovine spermatozoa, we prepared fractions of enriched bovine spermatozoal tail membranes and cytosol [14] and performed Western blots and subsequent [α-^{32}P]GTP-binding. Autoradiography exhibited the labelling of at least three proteins with apparent molecular masses of 20–25 kDa (Fig. 1). To characterize low-molecular-mass GTP- binding protein further, we made use of *Clostridium botulinum* ADP-ribosyltransferase C3 [20] which selectively modifies Rho proteins [2–5]. As shown in Fig. 2A, C3 exoenzyme ADP-ribosylated protein bands with apparent molecular masses of 22 kDa in enriched bovine spermatozoa tail membranes and, to a smaller extent, in sper-

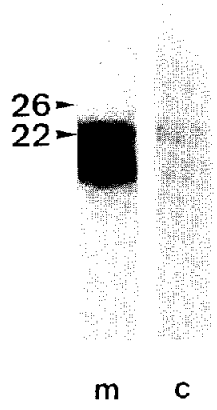


Fig. 1. [α-^{32}P]GTP-binding to enriched bovine spermatozoal tail membrane (m) and cytosol proteins (c). Spermatozoal membrane proteins (40 μg/lane) and cytosol proteins (40 μg/lane) were separated on SDS-PAGE, blotted onto nitrocellulose, incubated with [α-^{32}P]GTP and subsequently subjected to autoradiography. Apparent molecular masses (kDa) of labelled proteins are indicated on the left.

matozoal cytosol. No further substrates of different molecular masses were detected by the C3 exoenzyme-catalyzed labelling. Two-dimensional gel electrophoresis of the [^{32}P]ADP-ribosylated proteins revealed two C3 exoenzyme-substrates of identical molecular masses but different isoelectric points (Fig. 2B, panel 1). Based on reports that human platelet membranes contain exclusively RhoA [21], two-D gel electrophoresis was performed with platelet membrane proteins that were ADP-ribosylated by C3 exoenzyme (Fig. 2B, panel 2). A combination of platelet membrane proteins and enriched bovine spermatozoa tail membrane proteins exhibited no additional C3 exoenzyme-substrate (not shown), suggesting that one of the two spermatozoal C3 exoenzyme-substrates is identical with RhoA. The other C3 exoenzyme-substrate is suggested to represent RhoB or RhoC and remains to be identified.

Progressive swimming of spermatozoa is essential for successful fertilization. By using C3 exoenzyme-like toxins as tools, we studied whether Rho proteins are involved in the regulation of spermatozoa motility. Bovine spermatozoa were treated for up to 8 h with various concentrations of C3 exoenzyme. The effects upon spermatozoa movement were determined by using computer-assisted cell motion analysis. Treatment of spermatozoa with C3 exoenzyme inhibited the cell motility in a time and concentration dependent manner. As shown in Fig. 3A, depending on the concentration of C3 exoenzyme, the number of motile spermatozoa decreased by 50% at about 20 μg/ml after 6 h of incubation. At the highest concentration (100 μg/ml) studied, only 19% of spermatozoa were motile. In order to exclude that C3 exoenzyme was merely lethal for spermatozoa, we studied the viability of the treated cells by eosin Y staining and concurrently measured the sperm

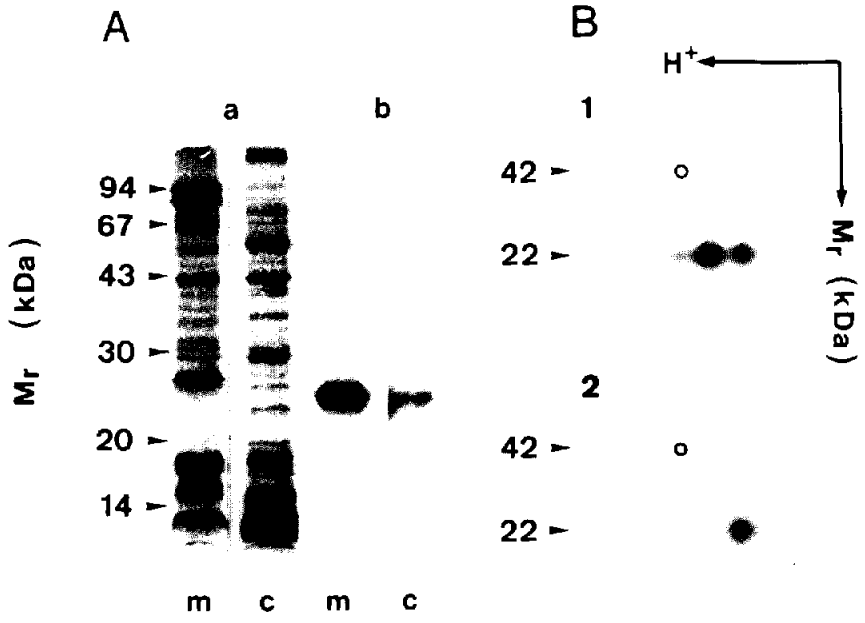
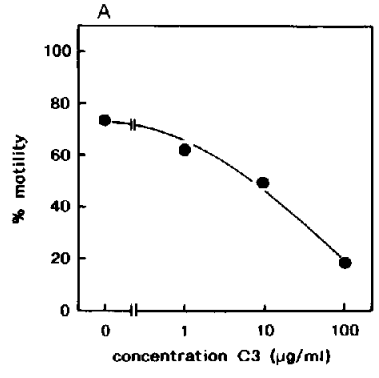


Fig. 2. (A) [^{32}P]ADP-ribosylation of Rho proteins in enriched bovine spermatozoal tail membranes (m) and cytosol (c) by C3 exoenzyme and analysis of the labelled proteins by SDS-PAGE. Gels were stained with Coomassie blue (a) and subsequently autoradiographed (b). The position of proteins run as standards are indicated on the left. (B) Autoradiography of 2D gel electrophoresis performed with [^{32}P]ADP-ribosylated enriched spermatozoal tail membrane proteins (panel 1) and human platelet membrane proteins (panel 2). The open circle gives the position of platelet actin (M_r = 42 kDa, isoelectric point of 5.4) which was used as internal marker protein.

motility. Fig. 3B shows that C3 induced a time dependent decrease in motile spermatozoa and concomitantly an increase in vital but immotile spermatozoa. The first functional effects of C3 exoenzyme upon sperm motility were observed after a lag period of about 3 h. Treatment of spermatozoa with 10 μg/ml of C3 exoenzyme for 8 h induced a decrease of motile spermatozoa from 75% to 22%, whereas motile control spermatozoa decreased only from 77% to 65%. The time dependent decrease of motile spermatozoa correlated with an increase in the percentage of immotile but viable spermatozoa. After an incubation for 8 h with C3 exoenzyme, 48% of immotile spermatozoa were still vital, whereas only 18% of control spermatozoa were immotile but alive. After 8 h of C3 exoenzyme treatment, light microscopy did not reveal any significant morphological changes between



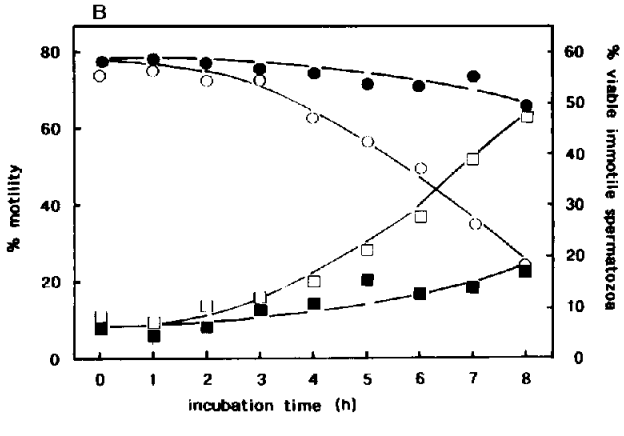


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Fig. 3. (A) Effects of increasing concentrations of C3 exoenzyme on bovine sperm motility. The percentage of motile bovine spermatozoa to total sperm count is shown on the ordinate, the concentration of C3 exoenzyme is given on the abscissa. (B) The time course of motility (◐) and viability of bovine spermatozoa (◧) in the absence (filled symbols) and presence of C3 exoenzyme (open symbols) was determined. Given are the mean values of three measurements of one of seven experiments with comparable results. Concurrently to sperm motility measurements, the viability of C3 exoenzyme-treated (□) and control (■) spermatozoa was determined by dye exclusion with eosin Y. The percentage of immotile and live spermatozoa to total sperm count is indicated on the right ordinate. Given are the mean values of three independent measurements; shown is one of five experiments with comparable results.

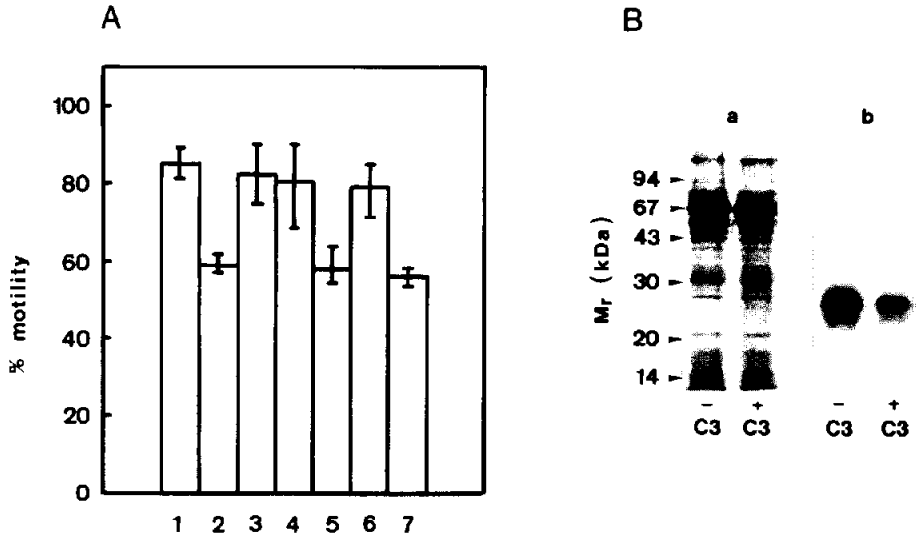

Fig. 4. (A) Influence of *Clostridium botulinum* C3 exoenzyme and *Clostridium limosum* exoenzyme on sperm motility. The motility of spermatozoa (3×10^7 cells) was studied after incubation for 7 h without (lane 1) and with C3 exoenzyme (20 μg/ml; lane 2), heat-inactivated C3 exoenzyme (30 min, 95°C; lane 3), C3 plus polyclonal affinity purified anti-C3-exoenzyme antibody (preincubated for 1 h at room temperature, lane 4), C3 exoenzyme plus heat-inactivated anti-C3-exoenzyme antibody (95°C, 15 min; lane 5), *Clostridium limosum* exoenzyme control buffer (lane 6) and *Clostridium limosum* exoenzyme (20 μg/ml, lane 7). (B) ADP-ribosylation of Rho proteins by C3 exoenzyme in intact spermatozoa. For determination of Rho proteins ADP-ribosylated in intact cells, lysates of C3 exoenzyme-treated (+C3) and control spermatozoa (−C3) were [^{32}P]ADP-ribosylated with C3 exoenzyme. The labelled proteins were analyzed by SDS-PAGE and subsequent Coomassie blue stain (a) and autoradiography (b).

spermatozoa treated with C3 exoenzyme or control spermatozoa.

Several control experiments were performed. It is shown in Fig. 4A that the effect of C3 exoenzyme was blocked by heat treatment of the toxin and by preincubation of C3 exoenzyme with polyclonal affinity purified anti-C3 exoenzyme-antibody. Furthermore, we studied the effects of *Clostridium limosum* exoenzyme, a C3 exoenzyme-like ADP-ribosyltransferase, which is about 70% homologous to C3 exoenzyme and also selectively modifies Rho proteins [6]. Fig. 4A shows that *Clostridium limosum* exoenzyme exhibited similar inhibitory effects on spermatozoa motility as observed with C3 exoenzyme. These findings obtained with two different ADP-ribosyltransferases largely exclude the possibility that the observed effects on spermatozoa were caused by any undetectable contamination of the exoenzyme preparations. Moreover, we studied whether the toxin-induced effects on spermatozoa motility are related to ADP-ribosylation of the Rho proteins (Fig. 4B). For this purpose, intact spermatozoa were treated with C3 exoenzyme (10 μg/ml) for 8 h. Thereafter, the cells were extensively washed, lysed and ADP-ribosylated in the presence of freshly added C3 exoenzyme and [^{32}P]NAD. Fig. 4B shows that pretreatment of vital spermatozoa with C3 exoenzyme significantly reduced the [^{32}P]ADP-ribosylation in the lysates, indicating the modification of Rho proteins in intact cells.

Our data that C3 exoenzyme-like enzymes, which selectively ADP-ribosylate Rho proteins, inhibit spermatozoa motility are in line with the view that Rho proteins play a role in the regulation of cell movement. It has been suggested that the microtubules play the major role in sperm motility [22]. Because Rho proteins are apparently not involved in the regulation of the microtubule system [3,7,23] our data extend the current concept of flagellar motility. We suggest that the regulation of the microfilamental system by Rho proteins is basically involved in the control of sperm motility. The precise functions of Rho proteins in the regulation of sperm motility are unclear; whether Rho regulates sperm motility via the actin filament system, remains to be elucidated.

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