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Dephosphorylation of cell-surface phosphoproteins of goat spermatozoa

M. BARUA and G. C. MAJUMDER
Indian Institute of Chemical Biology, 4, Raja S. C. Mullick Road, Jadavpur, Calcutta 700 032, India

Abstract. Multiple ecto-phosphoproteins of the goat cauda-epididymal intact spermatozoa have been shown to undergo dephosphorylation *in vitro* by endogenous phosphoprotein phosphatase(s) located on the sperm outer surface. The major ecto-phosphoproteins that are dephosphorylated have molecular masses of 27, 40, 70, 116 and 205 kDa. The cell surface dephosphorylation reaction is not dependent on bivalent metal ions. Mg^{2+} (5 mM), Mn^{2+} (5 mM), orthovanadate (200 μM) and cAMP (5 μM) have no effect on this surface reaction whereas it is inhibited nearly 50% by Co^{2+} or Zn^{2+} (1 mM). Spermidine (5 mM), or Ca_{2+} (1mM) inhibited to a small extent (approx. 25%) the cell surface dephosphorylation of proteins.

Keywords. Ecto-protein; spermatozoa; phosphoproteins; protein dephosphorylation; Proteinphosphatase; cell-surface.

Introduction

Phosphoproteins have been demonstrated on the surface of the mammalian cells and they have been implicated to have important role in regulating cell functions (Majumder and Turkington, 1972; Rubin and Rosen, 1973; Uno *et al.*, 1977; Kübler *et al.,* 1982; Blackshear *et al.*, 1988). Goat epididymal spermatozoa possess a cAMP-independent protein kinase (CIK) on the outer cell surface that causes phosphorylation of multiple endogenous ectò-phosphoproteins of the intact cells (Haldar and Majumder, 1986). A phosphoprotein phosphatase (PPase) has also been demonstrated on the sperm outer surface that causes dephosphorylation of exogenous [^{32}P]labelled proteins such as histones, protamine, casein and phosvitin (Barua *et al.*, 1985; Barua and Majumder, 1987). Several lines of evidence have been provided to show that the observed ecto-PPase activity is not due to leaky/damaged spermatozoa (Barua *et al.*, 1985; Haldar and Majumder, 1986; Barua and Majumder, 1987). The specific activity of ecto-PPase was markedly higher in the intact forward-motile spermatozoa than the composite cells from where the former cells were isolated, suggesting thereby that the ecto-enzyme may have an important role in regulating sperm motility (Barua and Majumder, 1987). Recently it has been observed that the ecto-phosphoprotein(s) of the intact sperm undergo dephosphorylation by the endogenous ecto-PPase (Barua *et al.*, 1990). The present study describes some characteristics of the sperm ecto-PPase(s) and identifies their physiological substrates located on the sperm surface. The present study shows that the sperm ecto-PPase is capable of dephosphorylating multiple sperm ecto-phosphoproteins.

Abbreviations used: CIK, cAMP-independent protein kinase; PPase, phosphoprotein phosphatase; SDS, sodium dodecyl sulphate; TCA, trichloroacetic acid.

Materials and methods

Chemicals

cAMP, ATP (horse muscle), sodium dodecyl sulphate (SDS), spermidine, EGTA, SDS-marker proteins (cross-linked haemoglobins and albumins, SDS-70L and SDS-200) were obtained from Sigma Chemical Company, St. Louis, Missouri, USA, Spermatozoa were extracted from the goat cauda-epididymides within 2–4 h after slaughter of the animals in the local slaughter houses. [γ-^{32}P]ATP was prepared as described earlier (Majumder and Biswas, 1979).

Isolation of epididymal spermatozoa

Highly motile spermatozoa were extracted from goat cauda-epididymides in medium A (119 mM NaCl, 5 mM KCl, 1·2 mM $MgSO_4$, 10 mM glucose, 16·3 mM K-phosphate, pH 6·9 and 50 U/ml penicillin) as described earlier (Barua *et al.*, 1985). Spermatozoa were sedimented at 500 *g* for 5 min at room temperature (29±2°C) and the pellet was washed twice with medium A. The washed cells were finally dispersed in the same medium.

Dephosphorylation of sperm outer surface proteins

The sperm outer surface phosphoproteins were phosphorylated by the endogenous ecto-CIK (Haldar and Majumder, 1986) prior to estimating the degree of dephosphorylation of the surface proteins by the intact-cell ecto-PPase. Intact spermatozoa ($15–30\times10^7$ cells) were incubated with 75 nmol of [γ-^{32}P]ATP (approx. 2×10^7 cpm), 30 μmol of $MgCl_2$, 3 μmol of EGTA in a total volume of 3 ml of medium A. Incubation was carried out at 37°C for 3 min and the kinase reaction was arrested with 5 mM non-radioactive ATP. Spermatozoa were sedimented immediately by low speed centrifugation and the cells were washed with RTS medium which is same as medium A except that it is buffered with 20 mM Tris-HC1, pH 7·2 rather than K-phosphate. The [^{32}P]labelled cells were finally dispersed in RTS medium and immediately incubated at 37°C for 10 min for the dephosphorylation reaction. The reaction was stopped with the addition of 5 ml of 10% trichloroacetic acid (TCA) and the protein precipitates were processed for assay of [^{32}P]radioactivity (Haldar and Majumder, 1986). One zero-min tube for the dephosphorylation reaction served as control showing initial total [^{32}P] radioactivity of the sperm ecto-proteins. The degree of dephosphorylation of the sperm ecto-proteins was measured by estimating the amount of loss of [^{32}P] (pmol) from the cell surface proteins.

Analysis of sperm ecto-phosphoproteins which undergo dephosphorylation

The intact-sperm ecto-proteins were first phosphorylated by the ecto-CIK and then dephosphorylated by the ecto-PPase as described above. As shown earlier under this condition of protein kinase reaction, only the sperm outer surface proteins undergo phosphorylation (Haldar and Majumder, 1986). The TCA-precipitates of

the [^{32}P]labelled proteins were collected and washed with 5% TCA. The protein pellet was dissolved in SDS-sample buffer and centrifuged in an Eppendorf centrifuge for 15 min. Protein contents of the resulting supernates were estimated according to Bensadoun *et al.* (1976). The [^{32}P]labelled proteins were analysed by gel electrophoresis and autoradiography (Haldar, 1988). The labelled proteins were subjected to 10% SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). Duplicate gels were run for each sample. One gel was stained with Coomassie blue whereas the other was subjected to fluorographic treatment, dried in an automatic gel dryer and finally exposed to X-ray film for autoradiography. Marker proteins: myosin (205 kDa), β-galactosidase (116 kDa), Phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa), were also electrophoresed side by side.

Results

The intact spermatozoa with the [^{32}P]labelled proteins on the outer cell surface, have been found to lose radioactivity when incubated at 37°C. Analysis of the [^{32}P]labelled products of incubation of the labelled cells, in the TCA-soluble fraction by paper electrophoresis (Barua and Majumder, 1987) showed that ^{32}Pi is released from the surface protein(s) indicating that the sperm ecto-PPase(s) cause dephosphorylation of the ecto-phosphoprotein(s) (Barua *et al.,* 1990).

As shown in table 1, the sperm ecto-PPase is not dependent on any bivalent metal ions for its activity to cause dephosphorylation of the surface proteins. Mg^{2+} or Mn^{2+} (5 mM) had little effect on the PPase activity. However, Co^{2+} or Zn^{2+} (1 mm) caused nearly 45% inhibition of the enzymic activity whereas Ca^{2+} at 5 mM level inhibited approx. 40% of the dephosphorylation reaction, and all these metal ion-mediated inhibitions were statistically significance (P <0·05). cAMP (5 μM)

Table 1. Effect of various reagents on the dephosphorylation of intact-sperm ecto-proteins by the endogenous ecto-PPase.

Exp.	Additions	^{32}Pi liberated from protein (pmol) Mean ± SEM
I	Nil (Control)	2·5 ± 0·4
	+ cAMP, 5 μM	2·4 ± 0·3
	+ Sodium orthovanadate, 200 μM	2·3 ± 0·7
	+ Spermidine, 5 mM	1·8 ± 0·1
II	Nil (Control)	1·8 ± 0·3
	+ $MgCl_2$, 5 mM	1·7 ± 0·3
	+ $CaCl_2$, 1 mM	1·4 ± 0·3
	+ $CaCl_2$, 5 mM	1·1 ± 0·2
	+ $MnCl_2$, 5 mM	1·5 ± 0·3
	+ $CoCl_2$, 1 mM	1·0 ± 0·2
	+ $ZnCl_2$, 1 mM	1·1 ± 0·2

Standard assay method was used for the dephosphorylation of sperm ecto-proteins except for the additions indicated above (Exp. I). For Exp. II, the [^{32}P]labelled cells were dispersed in RTS medium devoid of bivalent metal ions. The data shown are for 4 experiments.

and orthovanadate (200 μM) had no appreciable effect on the ecto-PPase activity whereas spermidine (5 mM), a polyamine inhibited to a small extent (approx. 20%) the ecto-enzymic activity.

As shown earlier using gel electrophoretic and autoradiographic techniques (Haldar and Majumder, 1986; Haldar, 1988), multiple sperm surface proteins are phosphorylated by the intact-cell ecto-CIK (Haldar and Majumder, 1986), Investigation was carried out to examine the specificity of the ecto-PPase(s) for the surface protein dephosphorylation (figure 1). All the [^{32}P]labelled sperm surface proteins of the intact cells were dephosphorylated by the endogenous ecto-PPase(s). The major ecto-phosphoproteins undergoing dephosphorylation have molecular masses of 205, 116, 70, 40 and 27 kDa.

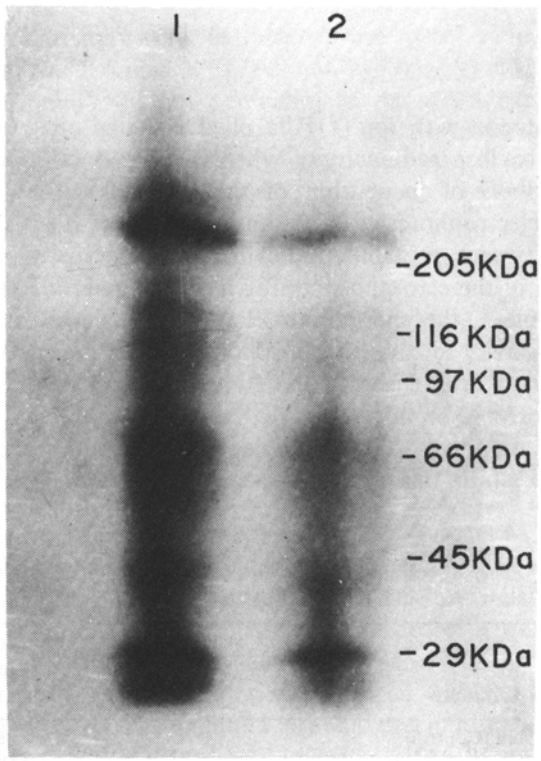

Figure 1. Autoradiogram of sperm ecto-[^{32}P] proteins (lane 1) dephosphorylated for 10 min (lane 2) by the endogenous ecto-PPase(s) of intact cauda-spermatozoa. Lane 1, [^{32}P]labelled sperm protein (200 μg; 27,000 cpm); lane 2, partially dephosphorylated [^{32}P]labelled sperm protein (200 μg; 14,000 cpm).

Discussion

Ecto-phosphoproteins have been demonstrated on the surface of several mammalian cells and they are phosphorylated by the endogenous ecto-protein kinases of the intact cells (Kübler *et al.*, 1982; Kang *et al.*, 1978; Sommarin *et al.*, 1981). Ecto-PPase has been localized in fibroblasts that dephosphorylates

exogenous histones (Makan, 1979). As mentioned above, previous study from our laboratory demonstrated an ecto-PPase on the goat sperm surface that dephosphorylates exogenous [^{32}P]labelled protein substrates (Barua *et al.*, 1985; Barua and Majumder. 1987). This study shows that the sperm ecto-PPase also causes dephosphorylation of multiple ecto-phosphoproteins of the sperm surface. The ecto-PPase may thus modulate the phosphorylated states of the sperm surface proteins and thereby serve an important role in regulating the structure and functions of the sperm plasma membrane. Exogenous Ca^{2+} has been implicated to have an important role in sperm motility (Feng *et al.*, 1987; McGrady *et al.,* 1974) and acrosomal reaction (Yanagimachi and Usui, 1974), although the biochemical basis of this action of Ca^{2+} is largely unknown. It is possible that Ca^{2+} modulation of the phosphorylated state of ecto-phosphoproteins (table 1), may mediate at least in part the regulatory role of this metal ion in sperm physiology.

At present it is difficult to ascertain as to whether the same ecto-PPase is responsible for the dephosphorylation of the exogenous protein substrates (Barua and Majumder, 1987) and the endogenous ecto-phosphoproteins. Both the PPase activity profiles in response to various reagents were similar. A significant difference between these enzymic activities is that the PPase causing dephosphorylation of histone was more sensitive to Zn^{2+} (Barua and Majumder, 1987), than the enzyme that dephosphorylates endogenous ecto-proteins (table 1). The data suggest that there may be more than one PPase species on the sperm surface differing in their enzymic properties with special reference to substrate specificity.

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