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Phosphorylation of endogenous and TEST-yolk buffer proteins by intact human sperm

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Summary

Protein kinase activity of intact, motile sperm was assessed by measuring the transfer of the terminal phosphate from [^{32}P]ATP to tricholoroacetic acid (TCA)-precipitable casein. The action of TEST (TES and Tris) yolk buffer (TYB) treatment on phosphorylation of sperm and TYB proteins was studied by detecting labelled phosphoproteins by autoradiography of polyacrylamide gel electrophoresis (PAGE). Results demonstrate that intact, forward-motile sperm have cell surface protein kinase activities. Although the difference between the kinase activity of freshly ejaculated sperm and sperm incubated in TYB was not significant, the protein phosphorylation during incubation in TYB showed that: (i) specific sperm surface proteins were phosphorylated to different degrees during the course of treatment; (ii) TYB proteins were phosphorylated by sperm during incubation; (iii) solubilised [^{32}P]-labelled surface proteins were similar in molecular weight to TYB-labelled proteins. Taking into account that specific proteins on the human sperm surface undergo phosphorylation during incubation in TYB and that the sperm enzyme also acts specifically on some TYB proteins that become attached to the surface of the sperm, working hypotheses are proposed that suggest some correlation between the preservation of semen in TYB and the phosphorylation of proteins by intact human sperm.

Keywords: Phosphoproteins, Protein kinase, Sperm, TEST yolk buffer

Introduction

The ability of human sperm to penetrate zona-pellucida-free hamster eggs has been extensively used to predict the fertilising potential of the sperm as well as to investigate the mechanism of fertilisation (Yanagimachi *et al.*, 1976; Tarin *et al.*, 1993). High rates of human sperm penetration of hamster eggs have been achieved by incubating the sperm in a TES-Tris (TEST) buffer containing hen egg yolks before incubation with hamster eggs (Bolanos *et al.*, 1983; Johnson *et al.*, 1984). Using this method, penetration levels in the sperm penetration assay (SPA) can be increased (Johnson *et al.*, 1984; Chan *et al.*, 1987). TEST-yolk buffer (TYB) treatment of sperm has also been used for hemizona assays (Lazendorf *et al.*, 1992) and human *in vitro* fertilisation.While some reports suggest an enhancement of *in vitro* fertilisation rates after TYB treatment (Katayama *et al.*, 1989; Paulson *et al.*, 1992), results of other authors indicate that this treatment does not show clinically significant improvement (Gamzu *et al.*, 1997). While the mechanism of action of TYB is unclear (Jacobs *et al.*, 1995), its activity seems to be related to its ability to preserve sperm motility over prolonged periods, during which increased capacitation of the total sperm population is achieved (Hirsch *et al.*, 1986). Recently it was demonstrated that even in the presence of high degree of autoimmunisation against the sperm head, sperm fusion with oolemma is not impaired after sperm preincubation with TyB (Francavilla *et al.*, 1997).

Sperm forward motility may be regulated by phosphoproteins, as suggested by previous work (Tash *et al.*, 1986; Horowitz *et al.*, 1988). Moreover, a cell protein kinase activity associated with the sperm cell surface has been described in the sperm of several mammalian

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species (Majumder, 1981; Pariset *et al.*, 1983; Atherton *et al.*, 1985; Haldar *et al.*, 1986), suggesting the possibility that protein phosphorylation may be involved in the process of sperm capacitation or sperm–egg interaction (Tash & Means, 1982; Horowitz *et al.*, 1988). Given that phosphoproteins could be involved in the motility and viability of sperm and that TYB preserves these characteristics of human sperm, the present study was conducted to examine the effect of human sperm storage in TYB on both sperm and TYB protein phosphorylation.

Materials and methods

Media and chemicals

Chemicals and all the salts included in the media were purchased from Sigma (St Louis, MO). TEST-yolk buffer (TYB) was purchased from Irvine Scientific (Santa Ana, CA). [γ^{32}P]ATP was purchased from Amersham.

Motile sperm preparation

Human semen samples from healthy donors were collected by masturbation following 3 days of abstinence, and allowed to liquefy at 37 °C for 30–40 min. They were examined according to the World Health Organization guidelines (1991) and samples with normal concentration and motility were retained. Unless otherwise noted, motile sperm were used in all assays. The motile sperm were prepared as follows: liquefied semen was diluted 1:1 in Tyrode buffer (99.4 mM NaCl, 1.42 mM KCl, 0.47 mM $MgCl_2$, 1.78 mM $CaCl_2$, 12.1 mM $HaHCO_3$, 5.56 glucose, pH 7.5) containing 0.3% human serum albumin (HSA; fraction V) and centrifuged for 5 min at 650 *g*. The supernatant was removed, the sperm pellet resuspended in 3 ml of Biggers, Whitten and Wittingham buffer (BWW) (Biggers *et al.*, 1971) containing 0.3% HSA, recentrifuged for 10 min at 650 *g* to form a loose pellet, aspirated, and overlaid with 0.6 ml BWW supplemented with 1.0% HSA. During incubation for 90 min at 37 °C under a 95% air / 5% CO_2 atmosphere only motile sperm migrate from the pellet, as verified by microscopic observation. Layers of medium containing 'swim-up' sperm were collected for use in the phosphorylation assays.

Capacitation of human sperm was obtained according to Johnson *et al.* (1984), as follows. After liquefaction, semen samples were mixed with an equal volume of TYB (211 mM TES [*n*-tris(hydroxymethyl)methyl-2-amino-ethane sulphonic acid), 96 mM Tris (hydroxymethyl-aminomethane), 11 mM dextrose and 1% penicillin–streptomycin solution to which was added 20% fresh hen egg yolk). Samples were then slowly cooled to 4 °C and allowed to capacitate for 12 or 43 h. At the end of the incubation period spermatozoa were washed twice in 6 ml BWW. The sperm pellet was resuspended in 3 ml of BWW containing 0.3% HSA, recentrifuged for 10 min at 650 *g* and submitted to the same procedure described previously for obtaining 'swim-up' sperm.

For obtaining acrosome-reacted sperm, the suspension obtained as above was incubated for 60 min at 37 °C in the presence of 0.01 mM A23187. The latter was added as a dimethylsulphoxide solution (0.002 ml for 1 ml of sperm suspension). The same volume of solvent was added to control samples incubated without A23187 (Bennet *et al.*, 1987). The incubation of sperm with A23187 produced a complete reaction in 50–55% of the cells whereas only 15–17% appeared to be still intact and 30–33% of them displayed a swollen acrosome.

Assay of sperm ecto-protein kinase activity

The activity of sperm external surface protein kinase was assayed by the procedure described by Day & Majumder (1987). The standard assay medium contained 20 nmol of [^{32}P]ATP ($60–80 \times 10^4$ cpm) in a total volume of 0.2 ml of a reaction mixture: 10 mM sucrose, 3.6 mM $MgCl_2$, 16.3 mM K_2HPO_4, 1.2 mM $MgSo_4$ and bovine casein (250 µg), pH 6.8, Cyclic AMP (3.25 µM) was added to the reaction mixture to measure cAMP-dependent protein kinase activity. The reaction was started by addition of freshly prepared motile sperm suspension (7×10^6 cells) and stopped by addition of 0.1 ml of a solution containing 1.5% casein, 10 mM ATP, 250 mM potassium phosphate and 2 ml of cold 20% trichloroacetic acid (TCA). After incubation in ice for 30 min, the samples were filtered through Whatman GF / C glass fibre filters, and washed with 40 ml of cold 5% TCA. When the sperm previously capacitated in TYB were tested for kinase activity, the glass fibre filters were washed three times with cold 5% TCA (5 ml each), 15 ml 100% ethanol, 5 ml ethanol:diethyl ether (3:1) and 5 ml diethyl ether to remove lipids. [^{32}P] associated with the precipitated protein was measured by Cerenkov counting. For detecting labelled proteins in the reaction media, the TCA precipitates were dissolved and protein contents of the samples were estimated according to Lowry *et al.* (1951) using bovine serum albumin as the standard and submitted to polyacrylamide gel electrophoresis (PAGE).

Preparation of sperm ^{32}P-labelled ecto-proteins

Swim-up sperm were incubated in the reaction medium described previously but with no substrate. At the end of the incubation period the sperm suspension was centrifuged at 1300 *g* for 10 min to sediment

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the ^{32}P-labelled cells. The pellet of sperm was washed five times in Tyrode buffer containing 1% HSA, dispersed in Tyrode buffer containing 0.2% Triton X-100 and kept 5 min in ice in order to solubilise [^{32}P]-labelled surface proteins. The suspension was centrifuged and the supernatant containing the radiolabelled proteins, which had been solubilised by the Triton X-100 treatment, was precipitated by addition of 20% cold TCA. Protein contents of the precipitate were estimated. The precipitate was dissolved and submitted to PAGE.

Phosphorylation of TYB protein

Motile sperm suspension (7 x 10^6 cells) was treated with TYB (0.2 ml) for 12 h or 43 h before the addition of 20 nmol of [^{32}P]ATP (60–80 x 10^4 cpm) for 1 h. The reaction was stopped by addition of 10 volumes of a solution containing 10 mM non-isotopic ATP and 10 mM NaF to inhibit phosphate activity, and the samples were centrifuged in order to separate the sperm pellets from the TYB. Spermatozoa were then washed five times in 6 ml BWW containing 0.3% HSA. Preparation of ^{32}P-labelled ecto-proteins was as described previously. TCA precipitates were collected by centrifugation and washed three times with 5% trichloroacetic acid (5 ml each). The precipitate was finally washed successively with 5 ml each of ethanol, ethanol / diethyl ether (3:1, v / v) and diethyl ether to remove lipids. Protein contents were estimated as indicated previously and the PAGE of the precipitates was carried out as described below.

Polyacrylamide gel electrophoresis

Samples were dissolved in 10 mM Tris-HCl (pH 8.0) containing 1% sodium dodecyl sulphate (SDS) and 140 mM 2-mercaptoethanol and then boiled for 5 min prior to application to acrylamide gel (7.5%). The gels were stained with the silver-based colour stain (Sammons *et al.*, 1981) and then equilibrated with acetic acid / glycerol / methanol / water (10:3:50:57). ^{32}P incorporation was detected by autoradiography using Kodak XS-5 film and DuPont Quanta III enhancer screens (DuPont, Wilmington, DE).

Results

Effect of TYB protein and acrosome reaction on human spermatozoa ecto-protein kinase

Protein kinase activity of intact motile human spermatozoa caused phosphorylation of casein in the presence and the absence of cAMP. P'chloromercuriphenyl sulphonic acid (PCMPS), a protein kinase inhibitor that cannot penetrate the sperm plasma membrane, was used as a surface probe to investigate whether the cAMP-independent kinase activity was located on the outer surface of human sperm. PCMPS at a concentration of 50 mM caused nearly 50% inhibition of the enzymatic activity, thereby supporting the notion that spermatozoa possess cAMP-independent ecto-protein kinase activity. This view was further supported by the finding that the activity was markedly reduced (80–90%) when the entire cells were pretreated with trypsin or pronase, each 250 µg / ml, at 37 °C for 5 min.

The experiments designed to study the effect of capacitation by TYB treatment and the acrosome reaction on the ecto-protein kinase activity indicated that the activity was not changed either by the TYB treatment or by induction of the acrosome reaction by the calcium inophore A23187. The amount of enzyme activity in motile 'swim-up' sperm collected from freshly liquefied semen (uncapacitated sperm) and that from semen incubated for 43 h in TYB (capacitated sperm) as shown in Fig. 1*A* was almost identical. Likewise, the enzyme activity after treatment of the capacitated 'swim-up' sperm with the calcium ionophore was not different from that of the samples incubated with the same volume of solvent without A23187 (Fig. 1*B*).

Phosphorylation of endogeneous protein by vigorously forward-motile sperm

The ecto-enzyme of intact sperm was detected to phosphorylate its own proteins when the enzyme assay was performed with no substrate. Fresh sperm obtained from liquefied semen were incubated with casein as a substrate or without casein under the standard assay conditions, with no cAMP. The incubation medium (included sperm), precipitated with TCA as indicated in Materials and Methods, was assessed by gel electrophoresis (Fig. 2*A*) and autoradiography (Fig. 2*B*). The results indicated that cAMP-independent protein kinase of sperm surface was able to use casein as a substrate (Fig. 2*B*, lane 2). Labelled proteins detected in a standard reaction mixture lacking casein as a substrate indicated that in the absence of casein the sperm were able to phosphorylate their own proteins (Fig. 2*B*, lane 3). As shown in lane 1 (Fig. 2*A*, *B*) the reaction mixture incubated without sperm did not show radioactive proteins, thus indicating that the phosphorylated proteins observed in the absence of casein did not belong to any contaminants of the incubation medium. However, the small number of broken spermatozoa initially present in the sperm preparation of the former experiments may be responsible for the observed phosphorylation of 81 kDa, 45 kDa, 36 kDa and 21 kDa endogenous proteins. To investigate this possibility, vigorously forward-motile spermatozoa that moved upward were separated from the rest of the cell

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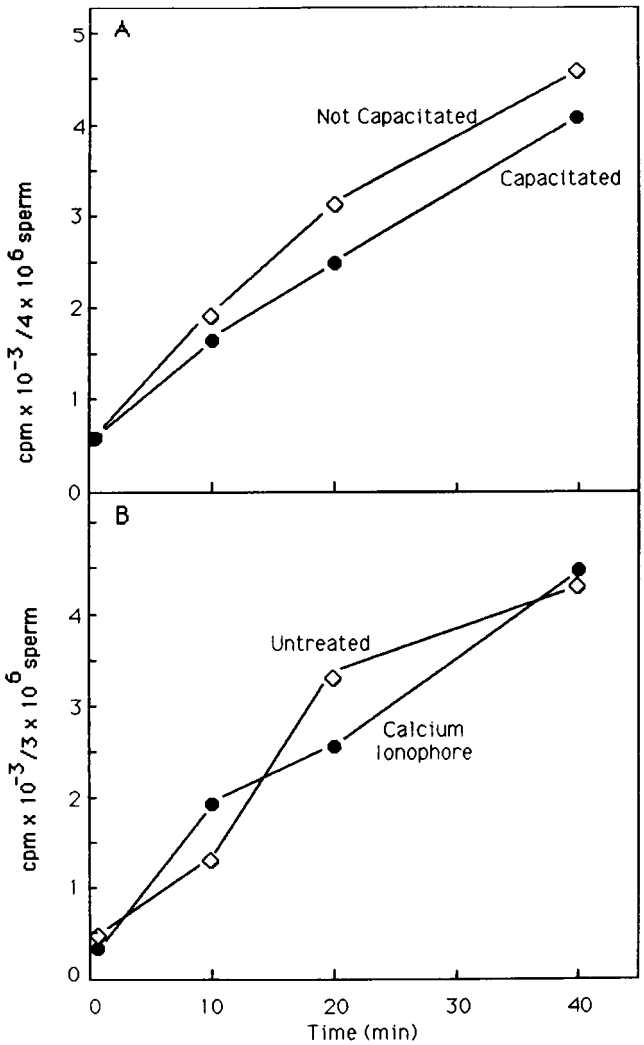

Figure 1 Effect of TEST-yolk buffer (TYB) treatment (*A*) and acrosome reaction induced by calcium ionophore (*B*) or cAMP-independent protein kinase activity. Sperm were assayed for enzyme activities using casein as substrate. See details in Materials and Methods.

population. Both sperm maintained in TYB for 43 h and fresh sperm were collected by the 'swim-up' procedure and incubated with [^{32}P]ATP under the standard assay conditions but in the absence of substrate (the reaction being arrested with 2 mM non-radioactive ATP and 5 mM NAF). Because Triton X-100 is a well-known detergent that solubilises proteins from the plasma membrane of mammalian cells, including spermatozoa (Tash & Means, 1982), sperm pellets were dispersed in Tyrode buffer containing 0.2% Triton X-100 and incubated for 5 min in ice in order to extract the membrane proteins, as indicated in Materials and Methods. The radiolabelled sperm ecto-proteins that had been solubilised by Triton treatment were assessed by gel electrophoresis (Fig. 3*A*) and autoradiography (Fig. 3*B*). The data above showed that even though the total amount of kinase activity was unaffected by TYB treatment (see Fig. 1), specific sperm surface proteins were phosphorylated to different degrees by fresh of TYB-treated sperm. Although 38 kDa protein was the protein phosphorylated to the greatest extent by both types of sperm, it appeared more phosphorylated in the spermatozoa incubated in TYB than in the non-incubated sperm.

Sperm and TYB Protein phosphorylation during incubation in TYB

Because TYB-treated sperm showed more phosphorylated ecto-proteins than fresh sperm, our purpose was to find out whether or not the sperm changed the phosphorylation of its own proteins during its incubation in TYB. Semen samples were diluted 1:2 in TYB and incubated at 4 °C for 12 h and 43 h before the addition of 20 µM [^{32}P]ATP for 1 h. At the end of the incubation period sperm were separated from TYB by centrifugation and motile sperm were washed and collected by the 'swim-up' procedure, as described in Materials and Methods. In order to study sperm phosphorylated proteins, motile sperm were washed several times to remove non-specifically attached proteins derived from TYB, before mild treatment with Triton X-100 as indicated previously. The incorporation of ^{32}P into the sperm proteins was assessed by gel electrophoresis (Fig. 4*A*) and autoradiography (Fig. 4*B*). Sperm incubated for 12 or 43 h (lanes 2 and 3, respectively) showed similar patterns of 48 kDa, 41–42 kDa, 36 kDa, 24 kDa and 19 kDa solubilised labelled proteins. However, it is important to note that according to the density of the labelled bands analysed with Molecular Analyst Software (BioRad), the 41 kDa, 36 kDa and 19 kDa proteins were more phosphorylated in sperm incubated for 43 h (41 kDa = 151.38 OD / mm, 36 kDa = 170.13 OD / mm, 19 kDa = 191.76 OD / mm) than in sperm incubated for 12 h (41 kDa = 46.3 OD / mm, 36 kDa = 77.65 OD / mm, 19 kDa = 63.00 OD / mm).

The possibility that sperm could phosphorylate proteins of TYB was also analysed. After removing sperm by centrifugation, the TYB Proteins were precipitated with 20% TCA and washed to remove lipids (see Materials and Methods). The phosphoproteins were studied by gel electrophoresis (Fig. 5*A*) and autoradiography (Fig. 5*B*) and the pattern of radioactive proteins analysed by Molecular Analyst Software (BioRad). Lanes 1 and 2 show the TYB proteins phosphorylated by sperm previously incubated in TYB for 12 h (s12) and 43 h (s43), respectively. The 42 kDa, 36 kDa and 32 kDa TYB proteins were more strongly phosphorylated by s12 than by s43. The density of TYB protein bands phosphorylated by s12 and s43 were as follows: 42 kDa = 146.29 OD / mm and 41.73 OD / mm respectively, 36 kDa = 108.15 OD / mm and 41.76 OD / mm respec-

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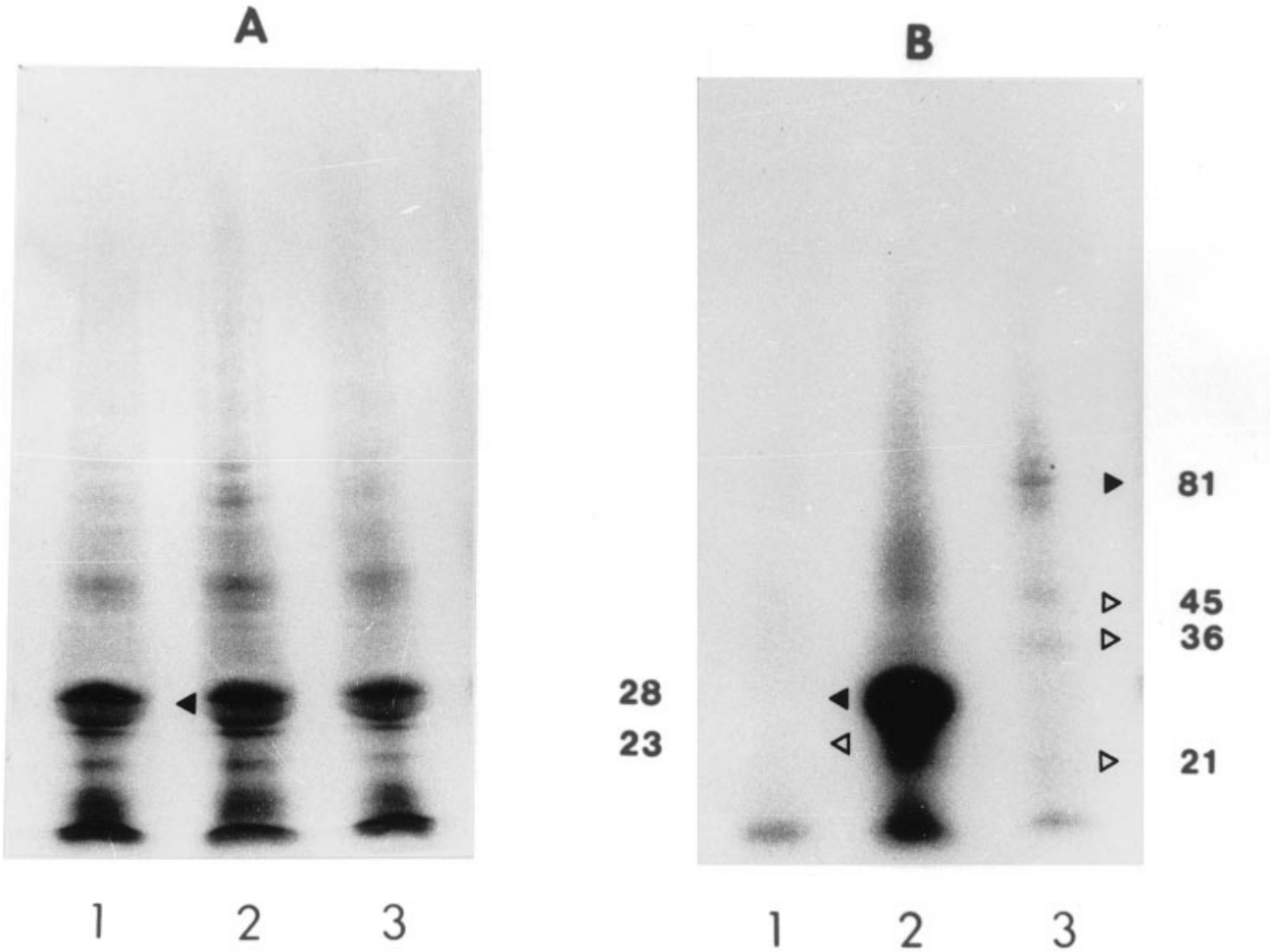

Figure 2 Gel electrophoresis (*A*) and autoradiography (*B*) of trichloroacetic acid (TCA)-precipitable proteins of the reaction medium. Lane 1, the reaction mixture incubated in absence of the sperm; lane 2, the reaction mixture incubated with sperm and casein as substrate; lane 3, the reaction mixture incubated with sperm but with no casein. Relative molecular weights of proteins phosphorylated by sperm are indicated by arrowheads.

tively and 32 kDa = 37.51 OD / mm and 34.01 OD / mm respectively. In contrast the 51 kDa and 19 kDa proteins were less phosphorylated by s12 than by s43: 51 kDa = 9.47 OD / mm and to 43.0 OD / mm respectively, and 19 kDa = 20.87 OD / mm and 43.45 OD / mm respectively. On the other hand, TYB incubated with 43 h sperm showed 91 and 24 kDa labelled proteins that were absent from TYB incubated with 12 h sperm. No endogenous protein kinase activity could be detected in TYB.

Discussion

Evidence has earlier been provided of the localisation of a cAMP-dependent protein kinase on the outer surface of spermatozoa (Sammons *et al.*, 1981; Pariset *et al.*, 1983) that causes phosphorylation of multiple species of endogenous ecto-phosphoproteins. This study indicates that human intact spermatozoa also possess a cAMP-dependent and AMP-independent protein kinase activity on the external surface. Our results are consistent with those reported by earlier investigators who provided extensive evidence that the ecto-enzyme activity of the sperm of other species was not due to the 'leakage' of cells or to contamination with dead or damaged cells. The impermeability of the sperm membrane to [^{32}P]ATP (Halder & Majunder, 1986) and the surface probe PCMPS further support the ecto-nature of the protein kinase activity. The observation that intact vigorously forward-motile spermatozoa possess a relatively high protein phosphorylation capacity also confirms the surface localisation of the protein kinase. Total kinase activity did not change with TYB capacitation or induction of the acrosome reaction of sperm, supporting the view that most of the sperm ecto-protein

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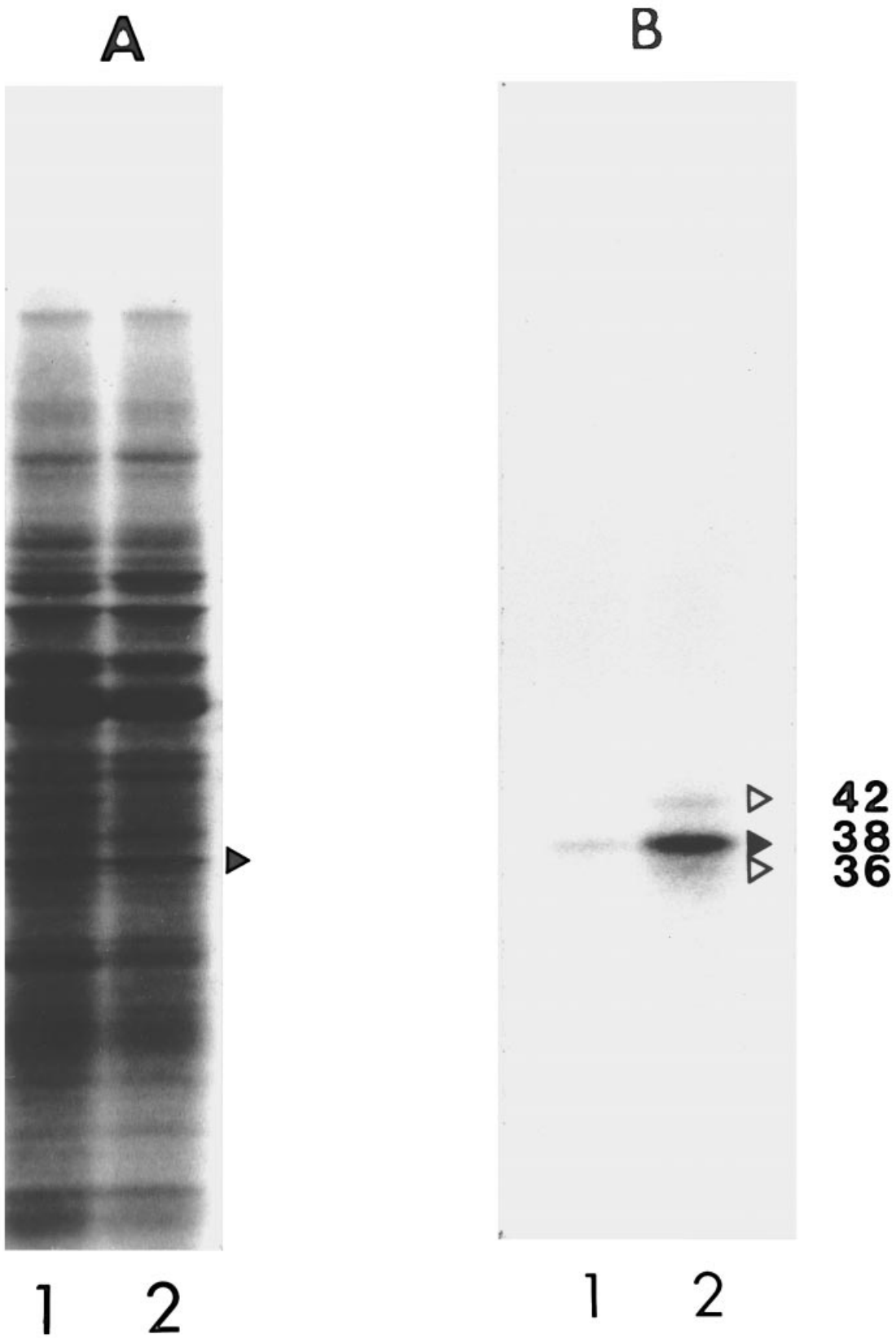

Figure 3 Gel electrophoresis (*A*) and autoradiography (*B*) of TCA-precipitable proteins of sperm ecto-proteins solubilised by Triton X-100 treatment. Fresh sperm (lane 1) and sperm previously incubated in TYB for 43 h (lane 2) were incubated with [^{32}P]ATP under standard assay conditions but in the absence of the substrate. Relative molecular weights of sperm phosphoproteins are indicated by arrowheads.

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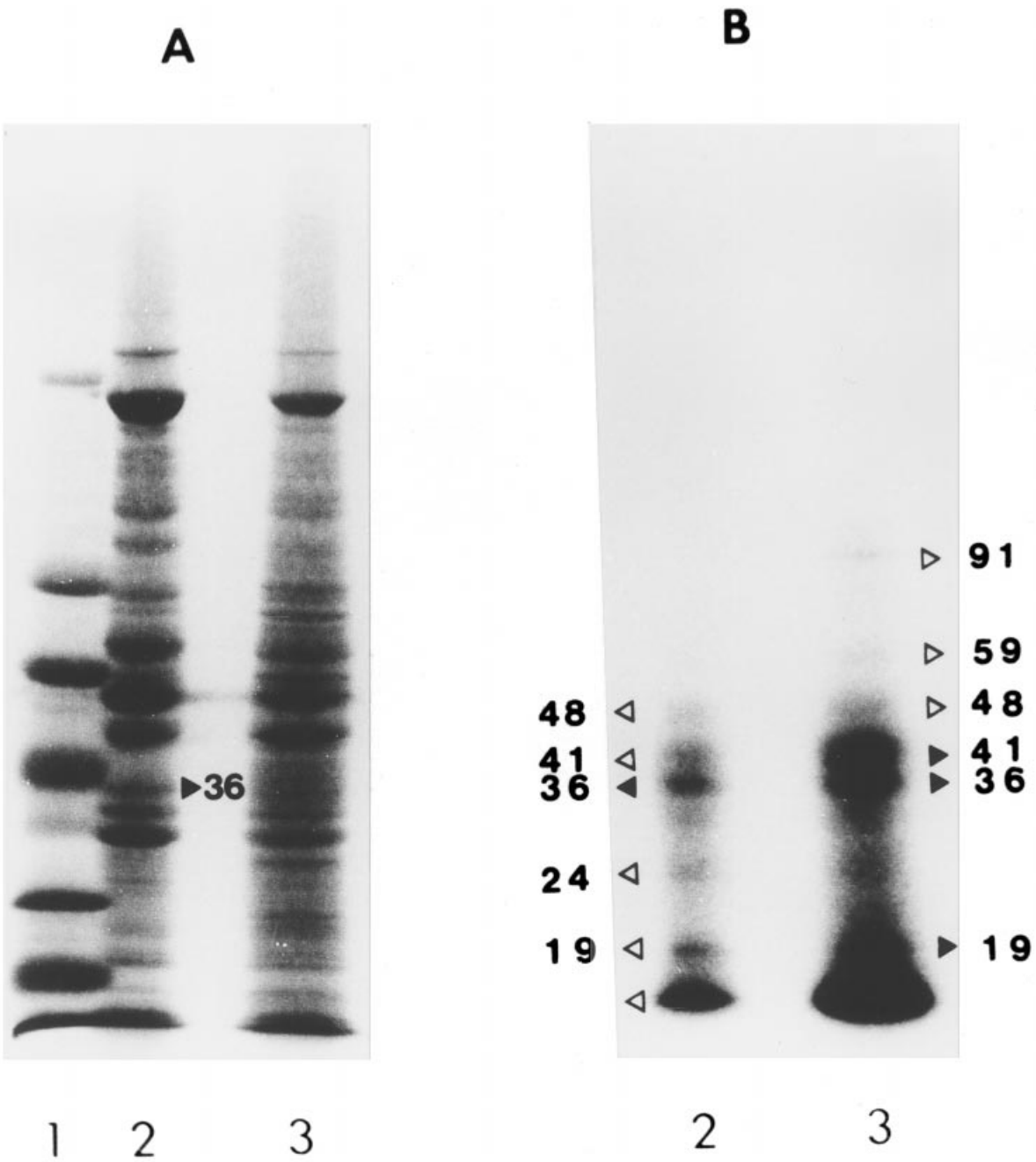

Figure 4 Gel electrophoresis (*A*) and autoradiography (*B*) of TCA-precipitable proteins of swim-up sperm ecto-proteins solubilised by Triton X-100 treatment. Proteins phosphorylated by sperm were maintained in TYB at 4 °C for 12 h (lane 2) or 43 h (lane 3) before the addition of 100 µM [^{32}P]ATP for 1 h. Relative molecular weights of sperm surface phosphoproteins are indicated by arrowheads.

kinase activities seem to be associated with flagellum (Tash & Means, 1982).

The examination of the detergent extract of sperm incubated with no substrate led to the identification of specific surface phosphoproteins that were phosphorylated to different degrees. Vigorously forward-motile sperm capacitated in TYB possessed a markedly higher phosphorylation capacity of their own ecto-proteins than did fresh sperm. When comparing the silver-stained gels that showed the electrophoretic pattern of total proteins solubilised with Triton X-100 with the pattern of labelled proteins detected by autoradiography, it was evident that ^{32}P incorporation occurred only in specific proteins existing in low concentration. These results would indicate that the enzyme is able to recognise and phosphorylate specific sperm surface

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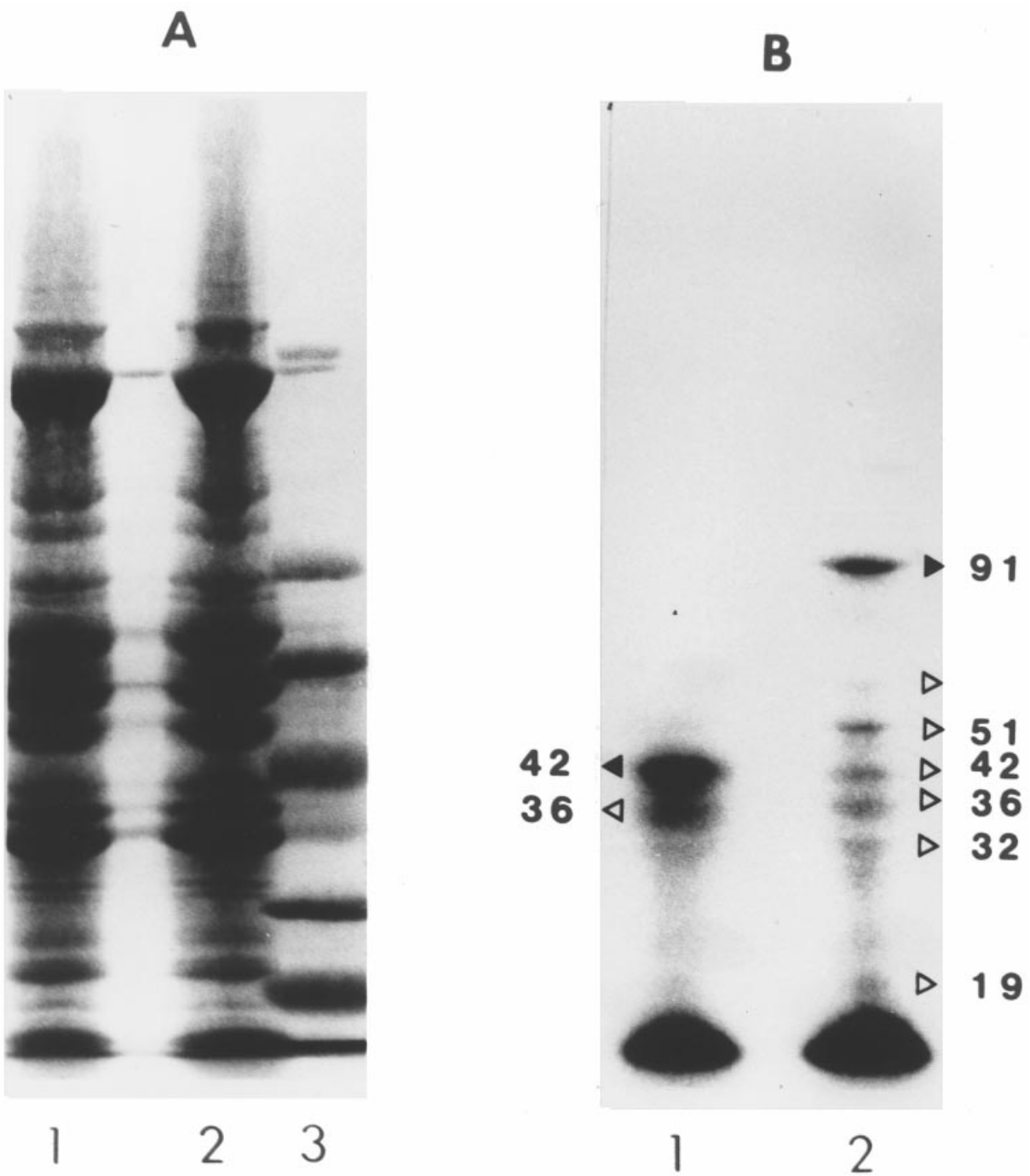

Figure 5 Gel electrophoresis (*A*) and autoradiography (*B*) of TCA-precipitable TYB proteins. Proteins phosphorylated by sperm were previously incubated in TYB at 4 °C for 12 h (lane 1) or 43 h (lane 2). Relative molecular weights of TYB phosphoproteins are indicated by arrowheads.

proteins, such as the 38 kDa protein which was more highly phosphorylated by sperm previously incubated in TYB for 43 h than by fresh sperm. Given that protein phosphorylation has been shown to be an important factor in sperm function (see Ward & Kopf, 1993), including the molecular events mediating sperm activation (Shapiro *et al.*, 1990) and the mechanism of action of acrosome-reaction-inducing molecules (Leyton *et al.*, 1992), it may be that a modulation of the phosphorylation state of these proteins is involved in the action of TYB.

When the protein kinase activity of sperm was measured in TYB instead of in the standard reaction mixture, phosphorylation took place in different ectoproteins, as shown by autoradiograph analyses. Considering that the same proteins of 41–42, 36 and 19 kDa

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appeared more or less phosphorylated according to the time they were kept in the TYB, and that these proteins were different from those phosphoproteins solubilised from sperm incubated in standard assay conditions, we believe that these proteins are TYB proteins adsorbed onto the sperm surface. A fact in support of this view is that a number of TYB proteins became phosphorylated, some of which had the same molecular weight as the sperm-solubilised proteins. The 91, 42, 36 and 19 kDa proteins appeared more strongly phosphorylated in 43 h sperm and also in their respective TYB, indicating that these TYB proteins could be phosphorylated by sperm during incubation and preferentially attach to the sperm. It is important to note that TYB does not have protein kinase activity. Apart from the 91 kDa protein, which was phosphorylated only by TYB-incubated sperm, the TYB proteins phosphorylated by TYB-incubated sperm were also phosphorylated by the non-treated sperm, even though the extent of phosphorylation varied. In the present study at least 12 TYB proteins of molecular weight 17 000 to 91 000 were phosphorylated, thus indicating the existence of several substrate proteins for sperm ecto-protein kinase. The autoradiographic analyses also showed several low-molecular-weight proteins in the front of the PAGE. Some phosphorylated TYB proteins were found in low quantities (e.g. 42 kDa and 36 kDa) or were not detectable by the silver stain in the PAGE (91 and 19 kDa), indicating that sperm ecto-protein kinase acted specifically on these proteins. Coincidentally, TYB proteins phosphorylated by sperm enzyme possess the interesting property of interaction with the sperm plasma membrane. Whether some of these phosphoproteins remain associated with the sperm plasma membrane until fertilisation and confer new biochemical properties on the sperm with regard to reproductive events is a matter for further investigation.

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