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Extracellular Calcium Negatively Modulates Tyrosine Phosphorylation and Tyrosine Kinase Activity during Capacitation of Human Spermatozoa¹

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ABSTRACT

Capacitation of spermatozoa, a complex process occurring after sperm ejaculation, is required to obtain fertilization of the oocyte *in vivo* and *in vitro*. Although most of the biochemical/biophysical events that occur during capacitation *in vitro* have been characterized, the molecular mechanisms underlying these complex events are still obscure. Increases of intracellular free Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) and protein tyrosine phosphorylation have previously been demonstrated during *in vitro* capacitation of human spermatozoa. In the present study we investigated the relationship between extracellular/intracellular Ca^{2+} , protein tyrosine phosphorylation, and tyrosine kinase and phosphatase activities during sperm capacitation. We report that the increase in tyrosine phosphorylation of several protein bands that occurs during sperm capacitation is independent of the presence of Ca^{2+} in the external medium and, at least partially, of the increase in $[\text{Ca}^{2+}]_i$ occurring during the process. Indeed, the spontaneous increase in phosphorylation was still present in Ca^{2+} -free/EGTA-containing-medium and in the presence of the intracellular Ca^{2+} chelator BAPTA/AM. Moreover, phosphorylation of proteins and protein tyrosine kinase (PTK) activity was enhanced if spermatozoa were incubated in Ca^{2+} -free medium, suggesting the presence of Ca^{2+} -inhibited tyrosine kinase(s) in human sperm. This hypothesis is further substantiated by the lower tyrosine phosphorylation observed after incubation with the ionophore A23187 and the endoplasmic Ca^{2+} -ATPase inhibitor thapsigargin, which promote Ca^{2+} influx in human sperm. The ability of the cells to undergo acrosome reaction in response to progesterone, which can be considered a functional endpoint of capacitation, was highly compromised when spermatozoa were incubated in Ca^{2+} -free medium or in the presence of EGTA, confirming that Ca^{2+} is required for sperm capacitation. Conversely, in the presence of erbstatin, a inhibitor of tyrosine kinase activity, which blunts tyrosine phosphorylation during capacitation, response to progesterone was maintained, suggesting that tyrosine phosphorylation must be kept at a low level (physiologically by the presence of Ca^{2+} in the external medium, or pharmacologically by the presence of erbstatin) in order to obtain response to progesterone. This mechanism may be important *in vivo* during sperm transit in the female genital tract to ensure appropriate timing of full capacitation in the proximity of the oocyte.

INTRODUCTION

The process of capacitation is an event that is essential in order for mammalian spermatozoa to acquire their ability to fertilize the oocyte (for review see [1]). During this process, spermatozoa become actively motile (expressing a

distinct motility pattern called hyperactivation) and responsive to physiological stimuli of the acrosome reaction, such as zona pellucida proteins and progesterone [1]. Although physiological capacitation occurs in the female genital tract, the process can be reproduced *in vitro* by incubation in defined media (whose composition is similar to oviductal fluid) and thus appropriately studied [1]. To date, it is known that capacitation is characterized by a series of complex biochemical/biophysical events involving regional modifications of sperm membrane sterols and phospholipids, changes in ionic movements, and other events that for the most part have been well documented [1]. However, the precise molecular mechanisms underlying these complex events are still far from being completely clear. In addition, the lack of a defined assay that can identify capacitated spermatozoa (for review see [2]) limits the interpretation of *in vitro* studies. Increasing evidence indicates that Ca^{2+} ions are of fundamental importance in this process: an increase of intracellular free Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) during capacitation has been indeed demonstrated in spermatozoa from several mammalian species [3–6]. Such increase is accompanied by an enhancement of sperm responsiveness to physiological stimuli of the acrosome reaction such as progesterone [6–9] and zona pellucida proteins [10], which have been shown to increase $[\text{Ca}^{2+}]_i$ [6, 11–13] in capacitated spermatozoa only. In addition to an increase in $[\text{Ca}^{2+}]_i$, capacitation is also characterized by an increase in phosphorylation of sperm proteins, particularly in tyrosine residues [14–17], indicating activation of sperm tyrosine kinases during this process. Recent evidence suggests a role for tyrosine kinase(s) in the process of fertilization [17, 18]. Moreover, a role of tyrosine kinase(s) in the initiation of flagellar movement has been demonstrated in the rainbow trout sperm [19]. These studies indicate that these enzymes, in addition to their well-characterized role in cell proliferation and differentiation [20], may also play an important role in the biology of highly specialized, mature cells such as spermatozoa.

However, little is known about the signals that promote activation of tyrosine kinase during capacitation in human sperm, or about the role, if any, of tyrosine kinase activation for capacitation of spermatozoa. In a recent paper, Aitken et al. [21] reported that the ionophore A23187 enhances tyrosine phosphorylation of human sperm during capacitation, suggesting a positive correlation between the two phenomena.

The increases in $[\text{Ca}^{2+}]_i$ and protein tyrosine phosphorylation during *in vitro* capacitation of human spermatozoa show a similar time course [6, 16, 22], suggesting the possible existence of a close relationship between the two events.

In the present study we investigated whether the increase in tyrosine phosphorylation is dependent on the increase in $[\text{Ca}^{2+}]_i$ by using an experimental approach involving Western blot analysis of tyrosine phosphorylation and assays of sperm tyrosine kinase and phosphatase activities in sper-

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matozoa incubated in the absence of extracellular Ca^{2+} and/or in the presence of various Ca^{2+} chelators. In addition, in order to verify the occurrence of capacitation in the different conditions, we evaluated progesterone-stimulated acrosome reaction. We report that 1) extracellular Ca^{2+} is not required for the increase in tyrosine phosphorylation during capacitation, 2) extracellular calcium negatively modulates tyrosine kinase activity in human sperm, and 3) progesterone can stimulate the acrosome reaction of human sperm when capacitation is obtained in the presence of an inhibitor of tyrosine kinase activity.

MATERIALS AND METHODS

Chemicals

Sodium orthovanadate (Na_3VO_4), progesterone, fatty acid-free BSA, gelatine, glycine, trizma base, Temed, ammoniumpersulfate, Triton X-100, PMSF, soybean trypsin inhibitor, *O*-phospho-DL-tyrosine, Ponceau S and Coomassie R250, EGTA, and EDTA were from Sigma Chemical Company (St. Louis, MO). Thapsigargin, erbstatin, genistein, tyrphostin A47, 1,2-bis(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetra-(acetoxymethyl)-ester (BAPTA/AM), ionomycin, fura-2/AM, A23187, and fluorescein isothiocyanate (FITC)-labeled *Arachis hypogea* (peanut) lectin were from Calbiochem (La Jolla, CA). Unconjugated and peroxidase-conjugated monoclonal (PY20) antiphosphotyrosine antibodies were from ICN (Costa Mesa, CA). Reagents for SDS-PAGE were from Bio-Rad Laboratories (Hercules, CA). Protein molecular weight standards, Tween 20, and Nonidet P-40 (NP-40) were from FLUKA Chemie AG (Bucks, Switzerland). Percoll was obtained from Pharmacia LKB (Uppsala, Sweden). Enhanced chemiluminescence (ECL) Western blotting detection reagents were from Amersham (Buckinghamshire, UK). Medi-Cult (Copenhagen, Denmark) was the supplier of human serum albumin (HSA)-free sperm preparation medium (229.5 mg/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 346.55 mg/L KCl, 173.27 mg/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5891.28 mg/L NaCl, 2200 mg/L NaHCO_3 , 137.15 mg/L $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 866.36 mg/L D-glucose, 8.66 mg/L phenol red, 97.0 mg/L Na pyruvate, 1000 mg/L HEPES sodium salt, 3010 mg/L HEPES acid, 1.0 ml/L SSR2 (comp. A), 1.0 ml/L SSR2 (comp. B), 50 mg/L streptomycin, 50 000 IU/L penicillin), and Ca^{2+} -free sperm preparation medium.

Preparation and Incubation of Spermatozoa

Human semen was obtained from normospermic men undergoing semen analysis for couple infertility in our lab. Semen was collected according to the procedure recommended by WHO [23] via masturbation after 3–4 days of sexual abstinence. Samples with a linear progressive motility of less than 50% at 60 min and with leukocytes and/or an immature germ cell concentration greater than 10^6 /ml were not included in the study. Semen samples were processed as previously described [6, 24] with the exception that sperm preparation medium (SPM) was used instead of Biggers, Whitten and Whittingham (BWW) medium [6]. Briefly, spermatozoa were separated on 40% and 80% Percoll gradients, combined, washed in BSA-free SPM, and resuspended in a small volume of the same medium. All these initial steps were performed in complete absence of BSA in order to avoid initial capacitation of the samples. Indeed, we have previously demonstrated that spermatozoa prepared in these conditions are not able to

respond to progesterone, indicating absence of capacitation, and that they can acquire this ability after reincubation in BSA-containing medium [6]. Spermatozoa were counted and resuspended in BSA-free SPM (noncapacitated samples) and in SPM with added BSA (3 mg/ml; capacitating samples) at the concentration of 3×10^6 /ml in the different conditions tested. Samples were incubated at 37°C in a CO_2 atmosphere, and aliquots were collected at different incubation times and processed as described below. For experiments in which the effect of progesterone was to be evaluated, spermatozoa were capacitated for 2 h in complete SPM medium and then stimulated with progesterone (4 μg /ml, final concentration) for 5 min.

Evaluation of Sperm Motility and Acrosome Reaction

Sperm motility was evaluated with use of a fully automated computer-assisted semen analyzer (Hamilton-Thorne motion analyzer; Hamilton-Thorne, Danvers, MA) as previously described [25]. Sperm acrosome reaction was evaluated through use of the fluorescent probe FITC-labeled *Arachis hypogea* (peanut) lectin as previously described [25, 26]. Briefly, spermatozoa (10×10^6 /ml), after capacitation, were stimulated for 1 h with progesterone (4 μg /ml), centrifuged at $400 \times g$ for 10 min, incubated in 0.5 ml of hypotonic swelling medium for 1 h at 37°C, centrifuged, and finally resuspended in 50 μl of ice-cold methanol. The sperm suspension was layered on a slide, air-dried at room temperature, and stored at -20°C . Fluorescence was observed under a fluorescent microscope (Leitz, type 307–148002; Wetzlar, Germany), and the acrosome reaction was evaluated on a total of 100 spermatozoa per slide. As outlined by Aitken et al. [27], only curly-tailed sperm were considered viable and thus scored.

SDS-PAGE and Western Blot Analysis

After the different incubations, the samples were processed for SDS-electrophoresis as previously described [16]. Briefly, sperm samples, containing 3×10^6 cells/ml, were centrifuged at $400 \times g$ at 4°C for 10 min, washed in HSA-free SPM, and resuspended in 10 μl lysis buffer (20 mM Tris [pH 7.4], 150 mM NaCl, 0.25% NP-40, 1 mM Na_3VO_4 , 1 mM PMSF). After measurement of proteins, the sperm extracts, containing approximately 20 μg of proteins, were diluted in equal volume of double-strength concentrated Laemmli sample buffer (double-strength sample buffer = 62.5 mM Tris [pH 6.8] containing 10% glycerol, 4% SDS, 2.5% pyronin, and 200 mM dithiothreitol), vortexed, incubated at 95°C for 5 min, and then loaded onto 7.5% polyacrylamide/bisacrylamide gels. After SDS-PAGE, proteins were transferred to nitrocellulose (Sigma) and stained with Ponceau to verify equal protein loading. In some experiments, equivalent protein loading was verified by staining parallel gels with Coomassie. If protein loading was not equivalent in each lane, sperm proteins were newly run. The nitrocellulose was immunostained with peroxidase-conjugated monoclonal antiphosphotyrosine antibody (PY20), and the phosphotyrosine antibody-reacted proteins were revealed by the ECL system (Amersham). To obtain immunoabsorption, the antibody was reacted with 40 mM *O*-phospho-DL-tyrosine for 1 h at 4°C. Quantification of the bands was made directly on the films by image analysis with a C3077/01 video camera connected to the video frame grabber M4477 (Hamamatsu Photonics, Hamamatsu City, Japan). This video frame grabber is a plug-in board used in a Macintosh IIsi PC (Apple, Carpin-

teria, CA). Acquisition of image was performed by Image Quest IQ Base software (Hamamatsu Photonics). Image processing and analysis were obtained with IMAGE free software, kindly provided by Wayne Rasband (NIMH, Bethesda, MD). For technical reasons, quantifications were performed in ECL low-exposed films.

Evaluation of Tyrosine Phosphatase and Kinase Activities

Tyrosine phosphatase and kinase activities were evaluated with an ELISA (tyrosine phosphatase assay kit, tyrosine kinase assay kit; Boehringer Mannheim GmbH, Mannheim, Germany). After 1 h of incubation in complete medium or in Ca^{2+} -free, or A23187 (10 μM)-containing medium, sperm extracts were washed and resuspended in modified TN buffer [18, 28] (150 mM NaCl, 20 mM Tris [pH 7.2], 0.5% NP-40, 1 mM PMSF, and 1 mM Na_3VO_4 for protein tyrosine kinase [PTK] or 50 $\mu\text{g}/\text{ml}$ erbstatin for protein tyrosine phosphatase [PTP] assay) and then vortexed for 2 min to obtain membrane preparations [18, 28]. Since both methods may be subjected to interferences due to tyrosine kinase activity (for PTP) and phosphatase activity (for PTK) present in the extracts, the assays were conducted in the presence of 50 $\mu\text{g}/\text{ml}$ of the tyrosine kinase inhibitor erbstatin (for PTP) or 1 mM Na_3VO_4 (for PTK). To evaluate PTP activity, the enzyme reaction was performed in duplicate at 30°C for 15 min in the presence of 1 nM tyrosine phosphatase substrate (corresponding to amino acids 1–17 of human gastrin phosphorylated on tyrosine 12). The reaction was stopped with the tyrosine phosphatase inhibitor Na_3VO_4 (100 μM); the unmetabolized substrate, immobilized on microtiter plates, was determined immunochemically with ABTS (2–2'-azino-di-[3-ethylbenzothiazoline sulfonate]) via antiphosphotyrosine antibody, directly conjugated to peroxidase. The rate of dephosphorylation was determined with a phosphopeptide standard curve. For the tyrosine kinase assay, the enzyme reaction was conducted in duplicate at 30°C for 30 min in an assay buffer (20 mM Tris-HCl [pH 7.2], 0.1% 2-mercaptoethanol, 1 mM ATP, 10 mM MgCl_2 , 1 mg/ml BSA, and 1 nM enzyme substrate [corresponding to the amino acid sequence 1–17 of gastrin biotinylated at the N-terminal glutamate]). The reaction was stopped with 0.5 mM of the tyrosine kinase inhibitor piceatannol (provided in the kit from Boehringer Mannheim GmbH); then tyrosine phosphorylated and unphosphorylated substrates were immobilized on microtiter plates, and tyrosine phosphorylated substrate was determined as described above for the PTP assay. The rate of phosphorylation was quantitated with a phosphopeptide standard curve.

Measurement of $[\text{Ca}^{2+}]_i$ Concentration

$[\text{Ca}^{2+}]_i$ was measured in fura-2-loaded spermatozoa after 2-h incubation in complete and Ca^{2+} -free medium by a fluorimetric method. Spermatozoa were loaded with fura-2/AM as previously described [6, 26], and fluorescence was measured with use of a single-wavelength spectrofluorometer (University of Pennsylvania Biomedical Group, Philadelphia, PA) set at 340 nm excitation with emission at 510 nm. Basal $[\text{Ca}^{2+}]_i$ was calculated according to Grynkiewicz et al. [29], using ionomycin (60 μM) for evaluation of maximal fluorescence (F_{max}) followed by EGTA (7.5 mM) for evaluation of minimal fluorescence (F_{min}), and assuming a K_d of fura-2 for Ca^{2+} of 224 nM [29]. In spermatozoa incubated in Ca^{2+} -free medium, F_{min} was evaluated by adding ionomycin directly to the sample or after incubation with

0.5 mM EGTA to chelate residual calcium present in the medium; F_{max} was evaluated by addition of 2.5 mM CaCl_2 .

Measurement of Proteins

Protein concentrations were evaluated by the Bio-Rad protein assay reagent exactly as indicated by the manufacturer, with BSA used as standard.

Statistical Analysis

Data are expressed as mean \pm SEM. Statistical comparisons were made with Student's *t*-test for paired data.

RESULTS

Role of Extracellular Calcium in Time-Dependent Changes in Tyrosine Phosphorylation of Sperm Proteins during Capacitation

As expected, after incubation of human spermatozoa for 1 and 2 h in complete, Ca^{2+} -containing, capacitating medium, tyrosine phosphorylation of several sperm protein bands of molecular mass ranging between 40 and 100 kDa was increased at 2 h as compared to 1 h (compare lane 1 with lane 5 in Figs. 1 and 4). In most of the experiments, the higher degree of tyrosine phosphorylation was observed in two protein bands with apparent molecular masses of 75 and 97 kDa. Sperm proteins were specifically phosphorylated on tyrosine residues, since preimmunoabsorption of the PY20 antibody with *O*-phospho-DL-tyrosine completely abolished immunoreactivity (data not shown). These data confirm previous results obtained by our group showing progressive increase of tyrosine phosphorylation of sperm proteins at different times of capacitation [16, 22]. When spermatozoa were incubated in a medium devoid of Ca^{2+} (nominally Ca^{2+} -free medium) for 1 and 2 h, tyrosine phosphorylation of sperm proteins was enhanced as compared to that seen in complete medium at both incubation times (compare lane 2 with lane 1, and lane 6 with lane 5, in Figs. 1 and 4; compare also lane 2 with lane 1 in Fig. 3B); and the spontaneous increase of phosphorylation at 2 h vs. 1 h was still present (compare lane 6 with lane 2 in Figs. 1 and 4). The average percentage increases of tyrosine phosphorylation in Ca^{2+} -free vs. complete medium for the 97- and the 75-kDa bands were, respectively, 50.71 ± 3.4 ($n = 7$) and 151.3 ± 64.8 ($n = 7$) at 1-h incubation and 51 ± 10.1 ($n = 8$) and 205 ± 74.6 ($n = 5$) at 2-h incubation. In contrast, the presence of EDTA (3 mM) in the incubation medium abolished tyrosine phosphorylation of proteins at both the 1- and 2-h time points (compare lane 3 with lane 2, and lane 7 with lane 6, in Fig. 1). Since EDTA chelates both Mg^{2+} and Ca^{2+} , we tested the effect of EGTA, which predominantly chelates Ca^{2+} . As shown in Figures 1 and 2, EGTA (3 mM) only slightly affected tyrosine phosphorylation of sperm proteins (compare lane 4 with lane 2 in Fig. 1; lane 8 with lane 6 in Fig. 2). The concentration of free Mg^{2+} in the EDTA- and EGTA-containing media, measured according to Fabiato [30], was, respectively, 230 μM and 370 μM , while the concentration of Ca^{2+} (assumed to be 10 μM due to contamination) was found to be less than 10^{-8} M with both chelating agents; thus the decrease in tyrosine phosphorylation observed in the presence of EDTA was likely to be due to chelation of Mg^{2+} . This result is in agreement with findings of Hayashi et al. [19], who showed that cAMP-dependent tyrosine phosphorylation of a rainbow trout sperm protein of 15 kDa was completely inhibited in EDTA-containing medium but

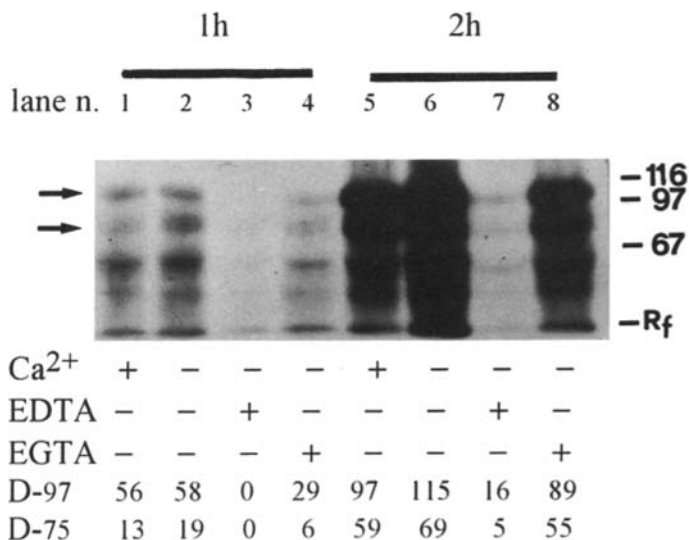


FIG. 1. Western blot analysis of the reactivity of antiphosphotyrosine antibody with human sperm proteins at 1 and 2 h of *in vitro* incubation in the presence or absence of added Ca²⁺, EDTA (3 mM), and EGTA (3 mM). Spermatozoa were incubated for the indicated times at 37°C in an atmosphere of CO₂, collected, and run on 7.5% acrylamide/bisacrylamide gel; tyrosine phosphorylation was evaluated by Western blot analysis using a peroxidase-conjugated antiphosphotyrosine antibody (PY20) followed by the ECL detection system. Densitometric analysis (arbitrary units) for the 97 (D-97)- and 75 (D-75)-kDa tyrosine phosphorylated bands is reported below each lane. Molecular weight markers ($\times 10^3$) are indicated to the right of the blot; Rf = running front. Representative of three similar experiments.

was only slightly affected by EGTA, indicating the role of Mg²⁺ in PTK activation in rainbow trout sperm. Furthermore, we found that the spontaneous increase in tyrosine phosphorylation observed during capacitation in complete medium was still present when EGTA was included in the Ca²⁺-free medium (compare lane 8 with lane 4 in Fig. 1); there was an increase in relation to that seen at 1 h that was similar to the increase found in complete medium (compare densitometric analysis for lanes 8 vs. 4 and lanes 5 vs. 1 in Fig. 1). To evaluate whether incubation in Ca²⁺-free medium was consistent with a decrease in [Ca²⁺]_i, spermatozoa were incubated for 2 h in capacitating conditions (complete medium) or in Ca²⁺-free medium, and basal [Ca²⁺]_i was then evaluated by a spectrofluorometric method. We found that when incubation was conducted in Ca²⁺-free medium, basal [Ca²⁺]_i was reduced by $44.7 \pm 2.3\%$ ($n = 3$) from the value obtained after incubation in complete medium. When [Ca²⁺]_i measurement was conducted in the presence of 0.5 mM EGTA to chelate residual calcium present in the medium, basal [Ca²⁺]_i was reduced by $81.4 \pm 1.8\%$ ($n = 3$).

To further investigate the relationship between extracellular Ca²⁺ and sperm tyrosine phosphorylation during capacitation, spermatozoa were incubated for 2 h with various concentrations of added Ca²⁺ (0.03, 0.15, 0.3, 1.5 [Ca²⁺ concentration in SPM], and 3 mM). As shown in Figure 2, incubation of sperm with increasing concentrations of Ca²⁺ progressively blunted sperm tyrosine phosphorylation of proteins in a concentration-dependent manner, suggesting that Ca²⁺ ions negatively modulate tyrosine phosphorylation of sperm proteins during capacitation. To explore this possibility further, we evaluated the effect of two known stimulators of Ca²⁺ influx and acrosome reaction, the Ca²⁺ ionophore A23187 and the endoplasmic Ca²⁺-ATPase in-

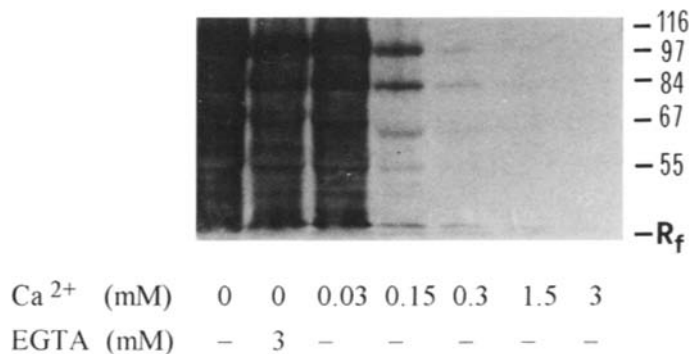


FIG. 2. Effect of different Ca²⁺ concentrations in the capacitation medium on sperm tyrosine phosphorylation. Spermatozoa were incubated at 37°C for 2 h without Ca²⁺ in the presence or absence of EGTA (3 mM), or with increasing Ca²⁺ concentrations; they were then collected and processed as described for Figure 1 for detection of tyrosine phosphorylation of proteins. Molecular weight markers ($\times 10^3$) are indicated to the right of the blot; Rf = running front. Representative of two similar experiments.

hibitor thapsigargin [31, 32]. As shown in Figure 3A, the presence, during the 2-h incubation, of the Ca²⁺ ionophore A23187 (10 μ M) in complete medium inhibited tyrosine phosphorylation of 97- and 75-kDa proteins (compare lane 3 with lane 1 in Fig. 3A) as well as of other proteins (not shown). The average percentage decrease in tyrosine phosphorylation after A23187 incubation was $47 \pm 4.9\%$ for the 97- and $76.6 \pm 9.2\%$ for the 75-kDa ($n = 3$) protein band. In this respect, it must be mentioned that in a recent paper, Aitken et al. [21] showed that incubation with the ionophore A23187 leads to an enhancement of tyrosine phosphorylation of proteins in human sperm. However, the pattern of tyrosine phosphorylated proteins shown in that study [21] differs from that reported by several other groups ([15–17]; present paper), thus pointing to the possibility that methodological differences might be responsible for the conflicting results. In addition, a preliminary report by Moos et al. [33] indicates that the treatment of human sperm with A23187 decreases sperm phosphorylation. Results similar to those obtained with A23187 were observed with thapsigargin (10 μ M), which inhibited tyrosine phosphorylation of the 97- and 75-kDa protein bands and of other bands (not shown) in complete medium (compare lane 3 with lane 1 in Fig. 3B) but was less effective in Ca²⁺-free medium (compare lane 4 with lane 2 in Fig. 3B). Since thapsigargin has been shown to be effective in increasing sperm [Ca²⁺]_i only when Ca²⁺ is present in the extracellular medium [31], the latter result is not surprising. We also evaluated the effect of A23187 in spermatozoa incubated in the presence of the tyrosine phosphatase inhibitor Na₃VO₄ (1 mM) in order to examine whether tyrosine phosphatases may contribute to the inhibitory effect of the ionophore. The inhibitory effect of A23187 was still evident when Na₃VO₄ was present in the medium (compare lane 4 with lane 2 in Fig. 3A). Although Na₃VO₄ is not a specific inhibitor of tyrosine phosphatases, this result suggests that PTP activity does not contribute to the inhibition of tyrosine phosphorylation induced by A23187. Indeed, if the inhibitory effect of A23187 on tyrosine phosphorylation was due to activation of tyrosine phosphatases, it should not have persisted in the presence of Na₃VO₄.

Effect of the Intracellular Ca²⁺ Chelator BAPTA on Sperm Tyrosine Phosphorylation

To further investigate whether Ca²⁺ increase during capacitation was involved in the enhancement of tyrosine

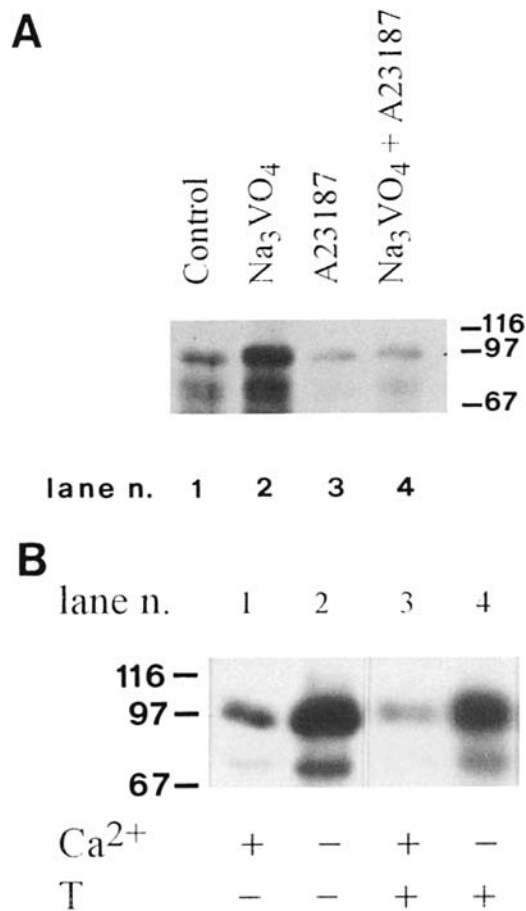


FIG. 3. **A**) Effect of the Ca²⁺ ionophore A23187 on tyrosine phosphorylation of sperm proteins. Spermatozoa were capacitated for 2 h at 37°C in an atmosphere of CO₂ in Ca²⁺-containing medium with or without A23187 (10 μM). The effect of A23187 was also tested in the presence of the tyrosine phosphatase inhibitor Na₃VO₄ (1 mM). Representative of three similar experiments. **B**) Effect of the endoplasmic Ca²⁺-ATPase inhibitor thapsigargin (T; 10 μM) on tyrosine phosphorylation of 97-kDa and 75-kDa protein bands of spermatozoa. Spermatozoa were allowed to capacitate for 2 h at 37°C in an atmosphere of CO₂ with or without added Ca²⁺ and in the presence or absence of thapsigargin for 1 h, and tyrosine phosphorylation was evaluated. Representative of two similar experiments. Tyrosine phosphorylated proteins were revealed as described for Figure 1. Molecular weight markers (×10³) are indicated to the right (A) or to the left (B) of the blot.

phosphorylation, spermatozoa were incubated with the intracellular Ca²⁺ chelator BAPTA/AM (10 μM) in either the presence or absence of Ca²⁺ in the external medium, and protein tyrosine phosphorylation was evaluated at 1 and 2 h. In preliminary experiments, loading of spermatozoa with 10 μM BAPTA/AM was found to completely abolish the [Ca²⁺]_i increase in response to progesterone in fura 2-loaded sperm (results not shown), indicating that BAPTA effectively chelates intracellular Ca²⁺ in these conditions. As shown in Figure 4, when spermatozoa were incubated in the presence of BAPTA, sperm tyrosine phosphorylation was reduced both at 1- and 2-h incubation either in the presence (compare lane 3 with lane 1 for 1-h incubation; lane 7 with lane 5 for 2-h incubation) or in the absence (compare lane 4 with lane 2 for 1-h incubation; lane 8 with lane 6 for 2-h incubation) of Ca²⁺ in the external medium; this result, consistently observed, might suggest a partial Ca²⁺-dependence of sperm tyrosine kinase activity during capacitation. Alternatively, the result might be explained,

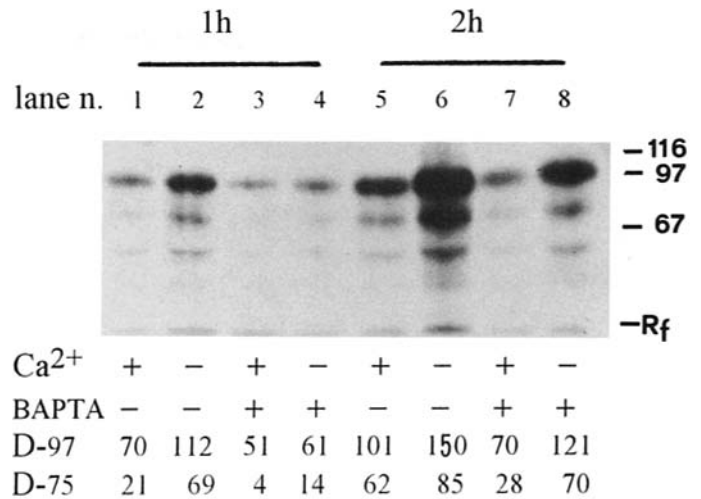


FIG. 4. Effect of the intracellular Ca²⁺ chelator BAPTA (10 μM) on sperm protein tyrosine phosphorylation at 1- and 2-h in vitro incubation in the presence or absence of added Ca²⁺ in the medium. Spermatozoa were incubated in the different conditions for the indicated times at 37°C in an atmosphere of CO₂, collected, and run on 7.5% acrylamide/bisacrylamide gel; tyrosine phosphorylation was evaluated by Western blot analysis as described for Figure 1. Densitometric analysis (arbitrary units) for the 97 (D-97)- and 75 (D-75)-kDa tyrosine phosphorylated bands is reported below each lane. Molecular weight markers (×10³) are indicated to the right of the blots; Rf = running front. Representative of two similar experiments.

as in the case of EGTA incubation (Fig. 1), by a possible chelating effect of BAPTA on intracellular Mg²⁺. Although the affinity of BAPTA for Mg²⁺ is much lower than that for Ca²⁺, the possibility of a chelating effect of BAPTA on intracellular Mg²⁺ cannot be excluded. On the other hand, in the presence of BAPTA, the spontaneous increase in tyrosine phosphorylation at 2-h incubation (compared to 1 h) was still present both in complete (compare lane 7 with lane 3 in Fig. 4) and in Ca²⁺-free (compare lane 8 with lane 4 in Fig. 4) medium. Moreover, densitometric analysis of the 97- and 75-kDa phosphoprotein bands indicates that the relative increase in tyrosine phosphorylation of this protein at 2-h incubation in the presence of BAPTA in Ca²⁺-free medium was similar to, if not higher than, that observed in complete medium (compare densitometric analysis in lane 8 vs. 4 for BAPTA and lane 5 vs. 1 for complete medium in Fig. 4).

The results presented so far indicate that Ca²⁺ ions negatively modulate tyrosine phosphorylation in human sperm. It has been shown that an influx of Ca²⁺ decreases PTP activity [34]. We therefore investigated the possible role of tyrosine phosphatase(s) activity in the tyrosine phosphorylation increase during capacitation. Spermatozoa were incubated for 2 h with the tyrosine phosphatase inhibitor Na₃VO₄ (1 mM) in the presence or absence of Ca²⁺. As shown in Figures 3A and 5, Na₃VO₄ increased tyrosine phosphorylation of the 97- and 75-kDa as well as (not shown) other protein bands, in complete medium (compare lane 3 with lane 1 in Fig. 5; lane 2 and lane 1 in Fig. 3A), whereas no apparent effect was observed in Ca²⁺-free medium (compare lane 4 with lane 2 in Fig. 5). This result suggests that the increase of tyrosine phosphorylation observed in Ca²⁺-free medium is not mediated by inhibition of tyrosine phosphatase(s).

To investigate further the relative contributions of sperm tyrosine phosphatase and tyrosine kinase activities in the

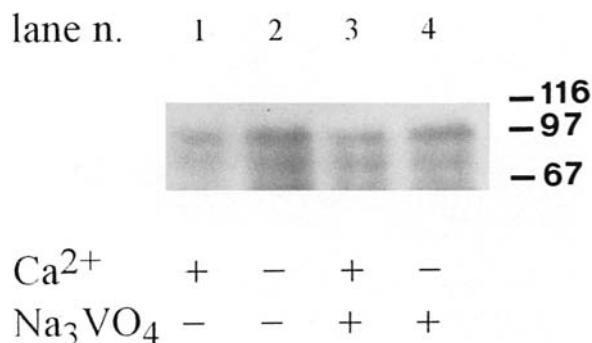


FIG. 5. Effect of incubation for 2 h with the tyrosine phosphatase inhibitor Na₃VO₄ (1 mM) on tyrosine phosphorylation of 97-kDa and 75-kDa protein bands in human sperm. Spermatozoa were incubated in complete medium with or without added Ca²⁺ in the external medium at 37°C in an atmosphere of CO₂ in the presence or absence of Na₃VO₄. Tyrosine phosphorylation of the two proteins at 2-h capacitation with or without added Ca²⁺ is shown in lanes 1 and 2, respectively. Molecular weight markers (×10³) are indicated to the right of the blots; Rf = running front. Representative of three similar experiments.

increase in tyrosine phosphorylation observed in the absence of extracellular Ca²⁺, we evaluated these activities in spermatozoa incubated for 1 h in complete medium, in the absence of Ca²⁺, and in the presence of A23187. The activity of the two enzymes was evaluated by measuring the rate of phosphorylation (for kinase) and dephosphorylation (for phosphatase) of peptide substrates by a photometric assay. Tyrosine phosphatase activity at 1-h incubation in complete medium was 2.24 ± 0.04 pmol/μg protein/min (n = 3) and was not modified in the absence of Ca²⁺ in the external medium or in the presence of A23187 (10 μM) (Table 1), suggesting that these enzymes are not involved in the increase in tyrosine phosphorylation observed in Ca²⁺-free medium. Tyrosine phosphatase activity was inhibited, in all the conditions tested, by the concomitant presence of the tyrosine phosphatase inhibitor Na₃VO₄ (not shown). Sperm tyrosine kinase activity after 1-h incubation in complete medium was 85 ± 10.7 fmol/μg protein/min (n = 4). When spermatozoa were incubated in a medium devoid of Ca²⁺, the rate of substrate phosphorylation was significantly increased (Table 1), thus suggesting higher tyrosine kinase activity in this condition that may be responsible for the higher tyrosine phosphorylation observed. However, the results obtained with A23187 (10 μM) were conflicting, since in 2 of 4 experiments the tyrosine kinase activity was reduced by about 30%, while a slight (10–20%) increase was observed in the other 2 experiments, with an average percentage inhibition of about 20% that was not significant (Table 1). At present we have no explanation for these conflicting results; they may be attributable, however, to variability among the different subjects studied.

Effect of Different Incubation Conditions on Motility and on Basal and Stimulated Acrosome Reaction of Spermatozoa

The effect of the different incubation conditions on motility parameters (and, for comparison, on protein tyrosine phosphorylation) of human spermatozoa are reported in Table 2. Total and progressive motilities were not significantly affected by incubation in Ca²⁺-free medium both at 1- and 2-h incubation, while tyrosine phosphorylation was increased. Incubation in BAPTA-containing Ca²⁺-free medi-

TABLE 1. Effect of incubation in Ca²⁺-free medium and in presence of A23187 on PTP and PTK activity during capacitation of human spermatozoa.^a

Activity ^b	n	Ca ²⁺ -free medium	A23187
PTP	3	109 ± 6	74 ± 9.2
PTK	4	154 ± 14*	81 ± 29

^a Spermatozoa were capacitated for 1 hr in complete medium without added Ca²⁺ and in complete medium in the presence of A23187 (10 μM).

^b Total enzyme activities, measured with use of a photometric method as described in the text, are expressed as percentage of control (capacitation in complete medium = 100%). Values represent means ± SEM of the indicated number of experiments.

* $p < 0.05$ vs. values in complete medium.

um (which slightly decreased tyrosine phosphorylation over that in Ca²⁺-free medium alone) did not affect motility after 1 h and slightly decreased it after 2 h. In contrast, treatment with Na₃VO₄ reduced all motility parameters while increasing tyrosine phosphorylation. EGTA inhibited motility and tyrosine phosphorylation, whereas the addition of EDTA (not shown in the table) totally inhibited motility and tyrosine phosphorylation. Incubation with erbstatin, which dose-dependently blunted tyrosine phosphorylation during capacitation in both the presence [16] and absence (Fig. 6) of Ca²⁺, had a variable effect on sperm motility at 1 h and inhibited it at 2 h. Sperm viability, evaluated by staining with eosin [23], was not affected by incubation with these agents, with the exception of EDTA, which reduced viability by about 20–30%.

We next evaluated spontaneous and progesterone-stimulated acrosome reaction in spermatozoa incubated in the different conditions used to detect tyrosine phosphorylation. As shown in Table 3, spontaneous acrosome reaction was not modified in spermatozoa capacitated in Ca²⁺-free, BAPTA-, EGTA-, and Na₃VO₄-containing media. In contrast, incubation with erbstatin (25 μg/ml) consistently induced an increase in spontaneous acrosome reaction (Table 3). Similar results were also obtained with two different tyrosine kinase inhibitors, genistein and tyrphostin A47 (results not shown), which have been shown to decrease sperm tyrosine phosphorylation [18, 35]. This finding is difficult to explain, since erbstatin does not affect sperm viability and had a slight inhibitory effect on sperm motility (Table

TABLE 2. Effect of incubation in different conditions on total and progressive motility of human spermatozoa.*

Treatment	Total motility: Capacitation time		Progressive motility: Capacitation time		Tyrosine phosphorylation: Capacitation time
	1 h	2 h	1 h	2 h	
Ca ²⁺ -free medium	↔	↔	↔	↔	↑↑
Na ₃ VO ₄ 1 mM	↓	↓	↓	↓	↑
BAPTA 10 μM	↔	↓	↔	↓	↔↓
EGTA 3 mM	↓	↓↓	↓	↓↓	↔↓
Erbstatin 25 μg/ml	↔↓	↓	↔↓	↓	↓↓

* Spermatozoa were incubated for 1 or 2 h in the indicated conditions, and motility was evaluated by using a fully automated motion analyzer. Arrows indicate changes in sperm motility and tyrosine phosphorylation of proteins in comparison with values obtained after incubation in complete medium or, in the case of BAPTA and EGTA, in Ca²⁺-free medium. Results shown represent the average effect of motility in at least 5 different experiments for each condition. ↔, No change; ↑, increase; ↓, decrease; ↔↓, variable results.

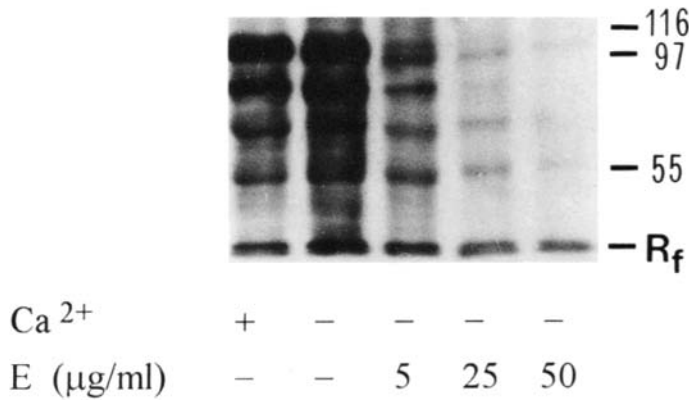


FIG. 6. Effect of erbstatin (E) on the levels of tyrosine phosphorylation of sperm proteins at 2-h incubation in the absence of added Ca²⁺. Spermatozoa were incubated at 37°C in an atmosphere of CO₂ with or without Ca²⁺ in the presence of 5, 25, and 50 μg/ml erbstatin. Tyrosine phosphorylated proteins were revealed as described for Figure 1. Molecular weight markers (×10³) are indicated to the right of the blot. Rf = running front. Representative of two similar experiments.

2). Moreover, the acrosome reaction was evaluated by scoring only curly-tailed (and thus viable) spermatozoa [27], further implying that the acrosome reaction-inducing activity of tyrosine kinase inhibitors is not attributable to toxic effects. In addition, tyrosine kinase inhibition with genistein did not affect sperm [Ca²⁺]_i [35], excluding the involvement of this pathway in the acrosome reaction-inducing activity of these compounds. As for response to progesterone, when spermatozoa were incubated in capacitating, complete medium, progesterone increased the acrosome reaction (Table 3), as indicated by the difference between percentage progesterone-induced and percentage spontaneously acrosome-reacted spermatozoa (considered to be the percentage of spermatozoa in the population capable of responding to progesterone—thus showing acrosome reaction following progesterone challenge [ARPC]). In contrast, the steroid was ineffective when preincubation was conducted in Ca²⁺-free medium or in the presence of EGTA (Table 3), confirming that the presence of Ca²⁺ in the external medium is required to obtain capacitation of human spermatozoa [1, 36]. When spermatozoa were incubated in the presence of the phosphatase inhibitor Na₃VO₄, the acrosome reaction in response to progesterone was lower than that obtained in complete medium (Table 3). On the other hand, the presence of erbstatin during capacitation did not apparently modify the ability of progesterone to induce the acrosome reaction (Table 3).

TABLE 3. Effect of capacitation in different conditions on basal acrosome reaction and in response to progesterone.^a

Exp	Condition of capacitation ^b														
	Complete medium			Without Ca ²⁺			Without Ca ²⁺ + EGTA			+ Erbstatin			+ Na ₃ VO ₄		
	C	P	ARPC ^b	C	P	ARPC	C	P	ARPC	C	P	ARPC	C	P	ARPC
1	8	21	13	7	10	3	4	8.5	4.5	27	42	15	6	11	5
2	5	15	10	9	10	1	5	6	1	—	—	—	7	14	7
3	7	25	18	9	9	0	7	9	2	33	51	18	7	9	2
4	14	26.5	12.5	16	18	2	6	13	7	38	65	27	—	—	—
5	17	42	25	17	29	12	10	11	1	50	66	16	—	—	—

^a Spermatozoa were capacitated for 2 h in the different conditions, centrifuged, resuspended in complete medium, and incubated for 1 h in presence (P) or absence (C) of progesterone (4 μg/ml). Results of single experiments obtained in different sperm samples are shown. Values represent percentage acrosome-reacted sperm.

^b ARPC = acrosome reaction following progesterone challenge (difference between percentage progesterone-induced and percentage spontaneous acrosome-reacted spermatozoa).

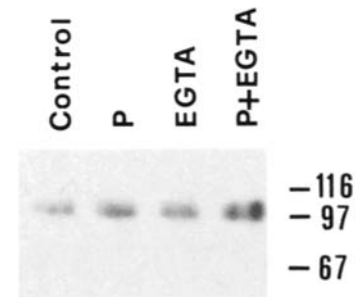


FIG. 7. Effect of progesterone (P; 4 μg/ml, 5-min incubation) on tyrosine phosphorylation of sperm proteins. Spermatozoa were capacitated for 2 h in Ca²⁺-containing medium, washed, reincubated in Ca²⁺-free medium, and stimulated with progesterone in the presence or absence of EGTA (E; 3 mM). Tyrosine phosphorylated proteins were revealed as described for Figure 1. Molecular weight markers (×10³) are indicated to the right of the blot. Representative of two similar experiments.

Effect of Progesterone on Sperm Protein Tyrosine Phosphorylation in Absence of Extracellular Ca²⁺

To test the role of Ca²⁺ in the progesterone-mediated increase in tyrosine phosphorylation of sperm proteins, spermatozoa that had been capacitated for 2 h in complete medium were incubated with progesterone for 5 min in Ca²⁺-free medium in the presence or absence of EGTA (3 mM). As shown in Figure 7, in both conditions progesterone was able to induce an increase in tyrosine phosphorylation of the 97-kDa protein band that was similar in magnitude (1.5-fold over basal) to that observed in Ca²⁺-containing medium [16, 37].

DISCUSSION

In the present study we investigated the interrelationship between the well-documented increases of [Ca²⁺]_i [3–6] and protein tyrosine phosphorylation [14–17] occurring during the process of capacitation of spermatozoa. We clearly demonstrate that the spontaneous increase in tyrosine phosphorylation of several protein bands during in vitro capacitation of human sperm was still present when the cells were incubated in Ca²⁺-free/EGTA-containing medium or in the presence of the intracellular Ca²⁺ chelator BAPTA, thus implying that extracellular Ca²⁺ and the spontaneous increase in [Ca²⁺]_i during capacitation are not necessary for the increase in tyrosine phosphorylation. At the same time, we show that incubation in (nominally) Ca²⁺-free medium resulted in an increase in sperm tyrosine phosphorylation and tyrosine kinase activity. Although this

condition cannot be considered an absolute absence of Ca^{2+} , since contamination from water and other sources (including extrusion of Ca^{2+} from spermatozoa) cannot be excluded, this result, consistently observed in several experiments conducted in different sperm samples, leads to the hypothesis that Ca^{2+} ions restrain the increase in tyrosine phosphorylation during capacitation. Indeed, in the virtual absence of Ca^{2+} in the external medium, the uptake of the ion that occurs during sperm capacitation is apparently reduced as demonstrated by the decreased $[\text{Ca}^{2+}]_i$ observed in this condition. Thus, the higher tyrosine phosphorylation observed in Ca^{2+} -free medium is likely to be due to the lower levels of Ca^{2+} in the cell. This hypothesis is further substantiated by our experimental results showing a concentration-dependent decrease in tyrosine phosphorylation in the presence of increasing concentrations of added Ca^{2+} as well as after incubation with agents that promote an influx of Ca^{2+} in human sperm, such as the Ca^{2+} ionophore A23187 and the endoplasmic Ca^{2+} -ATPase inhibitor thapsigargin.

Moreover, since tyrosine kinase activity during capacitation was higher in Ca^{2+} -free medium than in Ca^{2+} -containing medium (Table 1), the presence of Ca^{2+} -inhibited tyrosine kinase in human sperm is suggested. It is worth noting that in two recent studies conducted in epididymal mouse spermatozoa, Visconti et al. [38, 39] reported that the increase in tyrosine phosphorylation during capacitation in this species is dependent on the presence of Ca^{2+} in the external medium [38] and is mainly regulated by cAMP-dependent protein kinase [39]. However, the pattern of tyrosine phosphorylated proteins that increase during capacitation in this species is quite different from the pattern for human sperm. In particular, in human sperm, an increase in tyrosine phosphorylation has been reported in a protein band in the 95–97-kDa molecular mass range, among others ([15–17]; present paper). Such protein, which has been recently sequenced [17], has been shown to be the sperm receptor for ZP3, the zona pellucida protein responsible for inducing the acrosome reaction [14, 17, 18]. Conversely, in the studies reported by Visconti et al. [38, 39], a 97-kDa protein band had a similar tyrosine phosphorylation intensity in both noncapacitated and capacitated sperms and appeared to be different from the ZP3 receptor [17]. It is possible that epididymal spermatozoa have a different mode of capacitation than ejaculated sperm and/or that important species-related differences exist among mammals in this process.

So far, the possible role of Ca^{2+} ions in phosphorylation of sperm proteins in processes other than capacitation has been investigated in few studies [14, 19, 32, 40, 41]. These studies suggest that sperm kinase activity is poorly modulated or, as in the case of bovine sperm [40], even inhibited by an increase in $[\text{Ca}^{2+}]_i$, in agreement with our findings. In that report, however, total sperm kinase activity was evaluated. Tesarik et al. [37] reported that the increase in tyrosine phosphorylation induced by progesterone in human sperm was independent of the increase in Ca^{2+} . In the present paper we confirm these studies, showing that the increase in tyrosine phosphorylation in response to progesterone also occurs in Ca^{2+} -free/EGTA-containing medium—thus suggesting that progesterone affects $[\text{Ca}^{2+}]_i$ and protein phosphorylation by two distinct pathways. In agreement with this hypothesis, recent data obtained by our group indicate an involvement of tyrosine kinase in the generation of the sustained phase of $[\text{Ca}^{2+}]_i$ increase induced by progesterone [35].

An important, yet unresolved, question regarding sperm capacitation is which event, among all the events described to occur during this process, is absolutely required to achieve capacitation. In the past, Ca^{2+} has been indicated to be essential for capacitation, motility, and the acrosome reaction of human sperm [1, 36]; however, recent data demonstrate that in particular conditions, the acrosome reaction can also be obtained in Ca^{2+} -free medium [25, 42, 43] and that the capacitative effect of the phorbol ester TPA (tetradecanoylphorbol 13-acetate) can be demonstrated also in the absence of Ca^{2+} [44]. More recently, Bielfeld et al. [45] have reported that the acrosome reaction in response to zona pellucida proteins necessitates both $[\text{Ca}^{2+}]_i$ increase and kinase activation. We evaluated the acrosome reaction in response to progesterone as an index of capacitation. Indeed, although a clear assay for identifying capacitation has not been defined (for review see [2]), we [6] and others [7–9] have shown that response to progesterone does not occur in uncapacitated sperm and that thus this parameter can be used as a measure of the occurrence of capacitation. Our findings on progesterone-stimulated acrosome reaction clearly confirm that Ca^{2+} is essential to achieve sperm capacitation *in vitro*. Indeed, if sperm are preincubated in Ca^{2+} -free medium or in the presence of EGTA, the acrosome reaction in response to progesterone is absent or highly compromised, suggesting lack of capacitation in these conditions, as already indicated in previous studies [6–9]. Conversely, inhibition of tyrosine kinase during capacitation does not prevent the response to progesterone, and conditions that increase tyrosine phosphorylation (presence of Na_3VO_4 , absence of Ca^{2+}) impair or prevent the response to progesterone, indicating that capacitation does not likely occur in these conditions. It appears that tyrosine phosphorylation must be kept at a low level (by the presence of Ca^{2+} in the medium or by pharmacological inhibition of its activity with erbstatin) to produce a response to progesterone. In view of the fact that tyrosine kinase activation is necessary for exocytosis in response to physiological stimuli such as zona proteins [14, 18] and progesterone [16, 37], it is conceivable that low levels of tyrosine phosphorylation are required in order to obtain a response to these stimuli. An intriguing possibility is that Ca^{2+} -inhibited tyrosine kinase may provide a mechanism for the control of tyrosine phosphorylation during capacitation. Although the *in vitro* condition is much different from the *in vivo* one, our results suggest that the Ca^{2+} -dependent decrease in tyrosine phosphorylation might be important *in vivo* during sperm transit in the female genital tract for maintaining low levels of tyrosine phosphorylation in spermatozoa in order to ensure full responsiveness to the physiological agents (progesterone, zona pellucida proteins) at the moment of interaction with the oocyte.

Another important issue concerns the role of Ca^{2+} in maintenance of sperm motility. Recent data indicate a negative correlation between Ca^{2+} and motility [39, 41, 46]. Furthermore, the increase in motility induced by the phorbol ester, TPA, in human sperm was found to be independent of extracellular Ca^{2+} [47]. In our study, we found that motility was not affected by incubation in (nominally) Ca^{2+} -free medium (which resulted in increased tyrosine phosphorylation), was slightly affected by the intracellular Ca^{2+} chelator BAPTA, and was blunted by incubation with EGTA after 2-h incubation, suggesting that Ca^{2+} ions are at least partially involved in maintenance of sperm motility *in vitro*. Conversely, no apparent correlation appears to exist between motility and level of phosphorylation in tyro-

sine (see Table 2), although a 2-h incubation with erbstatin decreased motility. However, factors controlling sperm motility and capacitation in vivo are probably multiple and are unknown, thus making it difficult to compare the in vitro condition with the physiological one.

Spermatozoa do not appear to be the only cell type in which Ca^{2+} negatively affects phosphorylation. Indeed, an increase in intracellular Ca^{2+} has been shown to affect phosphorylation of proteins and phosphatase activity in other cell systems [34, 48–50]. In particular, A23187 has been shown to inhibit epidermal growth factor-induced tyrosine phosphorylation in HER14 cells [50]. However, the ionophore has also been shown to inhibit PTP activity in the same cells [51], suggesting that the inhibition of epidermal growth factor-mediated phosphorylation by A23187 in HER14 cells is due to an inhibitory action of Ca^{2+} on tyrosine kinase as in our system. The possible molecular mechanism(s) involved in Ca^{2+} -mediated inhibition of sperm tyrosine kinase activity is at present under investigation in our lab. In particular, we are studying the possible involvement of Ca^{2+} -regulated kinases in this phenomenon.

In conclusion, our results show that tyrosine phosphorylation in human sperm during capacitation in vitro is, at least partially, independent of the concomitant increase of Ca^{2+} , and that this ion negatively modulates tyrosine phosphorylation and tyrosine kinase activity. This mechanism might be relevant in vivo during physiological capacitation of sperm in the female genital tract.

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