BIOLOGY OF REPRODUCTION 58, 1476–1489 (1998)

Extracellular Signal-Regulated Kinases Modulate Capacitation of Human Spermatozoa[1]

Michaela Luconi,[3] Tullio Barni,[4] Gabriella B. Vannelli,[4] Csilla Krausz,[3] Fabio Marra,[5] Pier Alberto Benedetti,[6] Valter Evangelista,[6] Sandro Francavilla,[7] Giuliana Properzi,[7] Gianni Forti,[3] and Elisabetta Baldi[2,3]

Dipartimento di Fisiopatologia Clinica—Unita' di Andrologia,[3] Dipartimento di Anatomia Umana,[4] and Istituto di Medicina Interna,[5] Universita' di Firenze, I-50139 Firenze, Italy
Istituto di Biofisica,[6] CNR, Pisa, Italy
Dipartimento di Medicina Interna,[7] Sezione di Andrologia, Università dell'Aquila, Aquila, Italy

ABSTRACT

Recent evidence indicates the presence of p21 Ras and of a protein with characteristics similar to mitogen-activated protein kinases (MAPKs), also known as extracellular signal-regulated kinases (ERKs), in mammalian spermatozoa, suggesting the occurrence of the Ras/ERK cascade in these cells. In the present study we investigated the subcellular localization of ERKs and their biological functions in human spermatozoa. Immunohistochemistry, immunofluorescence, confocal microscopy, and immunoelectron microscopy demonstrated localization of ERKs in the postacrosomal region of spermatozoa. After stimulation of acrosome reaction with the calcium ionophore A23187 and progesterone, ERKs were mostly localized at the level of the equatorial region, indicating redistribution of these proteins in acrosome-reacted spermatozoa. Two proteins of 42 and 44 kDa that are tyrosine phosphorylated in a time-dependent manner during in vitro capacitation were identified as p42 (ERK-2) and p44 (ERK-1) by means of specific antibodies. The increase in tyrosine phosphorylation of these proteins during capacitation was accompanied by increased kinase activity, as determined by the ability of ERK-1 and ERK-2 to phosphorylate the substrate myelin basic protein. The role of this activity in the occurrence of sperm capacitation was also investigated by using PD098059, an inhibitor of the MAPK cascade. The presence of this compound during in vitro capacitation inhibits ERK activation and significantly reduces the ability of spermatozoa to undergo the acrosome reaction in response to progesterone. Since only capacitated spermatozoa are able to respond to progesterone, these data strongly indicate that ERKs are involved in the regulation of capacitation. In summary, our data demonstrate the presence of functional ERKs in human spermatozoa and indicate that these enzymes are involved in activation of these cells during capacitation, providing new insight in clarifying the molecular mechanisms and the signal transduction pathways of this process.

INTRODUCTION

Spermatozoa are terminally differentiated, highly specialized cells with morphological, structural, biochemical, and functional characteristics all directed toward fertilizing the oocyte. The process of fertilization in mammals is a complex mechanism that requires several biochemical/biophysical events to occur in ejaculated spermatozoa, culminating in sperm membrane fusion with the oolemma and subsequent penetration of the oocyte [1]. Among the events that characterize sperm life in the female genital tract, two are considered crucial for the process of fertilization, namely capacitation and acrosome reaction. During the process of capacitation, spermatozoa acquire the ability to fertilize the oocyte. Capacitation is characterized by a series of profound membrane and metabolic transformations in spermatozoa that increase their ability to respond to physiological stimuli of the acrosome reaction [1]. The acrosome reaction is a specialized exocytotic event consisting of multiple fusions and fenestrations of the outer acrosomal membrane and the overlying plasma membrane that lead to subsequent release of acrosomal enzymes that help the spermatozoa to penetrate the zona pellucida [1]. Although the precise signal transduction pathways involved in sperm capacitation and the acrosome reaction have not been fully elucidated (reviewed in [2]), it is known from in vitro studies that both processes are characterized by increases in intracellular calcium concentrations [3–5], pH [6], and phosphorylation of proteins including that in tyrosine residues [7–14]. In particular, although the increase in tyrosine phosphorylation during capacitation has been well characterized [9–14], only a few sperm phosphoproteins involved in this phenomenon have been identified, and their role during sperm activation is not known. In somatic cells, increased tyrosine phosphorylation may be associated with activation of downstream kinases that in turn phosphorylate specific substrates involved in the regulation of growth, adhesion, secretion, and other events. Among the downstream kinases activated by stimuli inducing protein phosphorylation, a family of serine/threonine kinases named mitogen-activated protein kinases (MAPKs) occupy a focal point in signal transduction, mainly by activating gene transcription via translocation into the nucleus (for review, see [15]).

Among the several members of this family, the most extensively studied are p44 and p42 MAPK, also known as extracellular signal-regulated kinases (respectively, ERK-1 and ERK-2). These enzymes have been recognized as cytoplasmatic and nuclear kinases; they are activated by several stimuli that induce a cascade of events starting from activation of the p21 Ras. Ras binds and stimulates the serine/threonine kinase Raf-1 that phosphorylates and activates MAPK (also named MEK), a dual-specificity kinase known to phosphorylate ERKs both in threonine and in tyrosine residues [15]. ERKs are involved in the regulation of several functions in proliferating [15] as well as terminally differentiated cells such as platelets [16], macrophages [17], and neurons [18]. The presence and the

Accepted January 27, 1998.
Received November 3, 1997.
[1]Supported by Consiglio Nazionale delle Ricerche (CNR-Rome, Targeted Project: FATMA, contract n. 92.00097-PF41), Ministero dell'Universita' e della Ricerca Scientifica e Tecnologica (Rome), and Regione Toscana (Florence).
[2]Correspondence: E. Baldi, Dipartimento di Fisiopatologia Clinica, Unita' di Andrologia, Universita' di Firenze, viale Pieraccini 6, I-50139 Firenze, Italy. FAX: 39.55.4221848; e-mail: e.baldi@mednuc2.dfc.unifi.it

biological activity of p21 Ras has recently been demonstrated in human spermatozoa [19]. In addition, a tyrosine/serine/threonine-phosphorylated protein of about 40 kDa has been detected in capacitated human spermatozoa [20]. These findings, together with the recent characterization of a boar sperm protein kinase with characteristics similar to those of ERK-2 [21], lead to the hypothesis that the Ras/ERK cascade may be present in human spermatozoa. Despite this preliminary evidence, the biochemical characterization, subcellular localization, and biological function(s) of ERKs in human spermatozoa have not been addressed. In the present study, we demonstrate that ERKs are localized in the postacrosomal region of human spermatozoa and that they redistribute in the equatorial segment after induction of the acrosome reaction. In addition, we provide evidence that p44 and p42 ERKs are activated during in vitro capacitation and are involved in the development of this process.

MATERIALS AND METHODS

Chemicals

Percoll was obtained from Pharmacia LKB (Uppsala, Sweden). A23187 was from Calbiochem (La Jolla, CA). All the other chemicals were from Sigma Chemical Company (St. Louis, MO). Enhanced chemiluminescence Western blotting detection reagents and [γ-^{32}P]ATP were from Amersham (Buckinghamshire, UK). Human serum albumin (HSA)-free human tubal fluid (HTF) was from Irvine (Santa Ana, CA). Peroxidase-conjugated monoclonal (PY20) anti-phosphotyrosine antibody was from ICN (Costa Mesa, CA); fluorescein isothiocyanate (FITC)-labeled *Arachis hypogea* (peanut) lectin and monoclonal anti-ERK-2 were from Calbiochem; rabbit and mouse biotinylated IgG were from Pierce (Rockford, IL). 4′,6-Diamidino-2-phenylindole (DAPI), conjugated secondary anti-mouse and anti-rabbit IgG, and streptavidin-colloidal gold particles were from Sigma. Lowicryl K4M was from Agar Sci Ltd (Essex, GB); αIIcp42 (raised against the C-terminal 14 amino acids of ERK-2) and αIcp44 (raised against the C-terminal 16 amino acids of ERK-1) anti-ERK polyclonal antibodies were a kind gift of Prof. M.J. Dunn (Medical College of Wisconsin, Milwaukee, WI). Anti-MEK antibody was a kind gift of J. Pouyssegur (Centre de Biochimie, Université de Nice, Nice, France). Antibody against tyrosine-phosphorylated p42 ERK-2 was obtained from New England Biolabs (Beverly, MA). PD 098059 was a kind gift of Dr. A.R. Saltiel (Parke-Davis, Co., Morris Plains, NJ). Recombinant ERK-2 (ERK-2 fusion protein) was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Testicular Specimens

Testicular specimens were obtained under epidural anesthesia by unilateral testicular biopsy from patients presenting with infertility (n = 7, age range 28–45), who were investigated for suspected obstructive azoospermia. Among the obstructive-azoospermic patients, only specimens from those showing normal spermatogenesis (n = 3) were used as normal controls. Testicular sections were also obtained from patients (n = 4) with azoospermia due to tubular damage. The histological evaluation was established according to standard histological criteria.

Preparation and Incubation of Spermatozoa

Semen was collected from normospermic men according to the procedure recommended by WHO [22]. Samples with a linear progressive motility of less than 50% at 60 min and with leukocytes and/or immature germ cell concentration greater than 10^6/ml were not included in the study. Semen samples were processed as previously described [5, 23]. Briefly, spermatozoa were separated on 40% and 80% Percoll gradients, combined, washed in HSA-free HTF, 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 [5] that spermatozoa prepared in these conditions are not able to respond to progesterone, indicating absence of capacitation, and that they can acquire this ability following reincubation in BSA-containing medium (also referred as complete medium in this paper). Spermatozoa were counted and resuspended in HSA-free HTF (noncapacitated spermatozoa) and in HTF with added BSA (3 mg/ml, capacitating spermatozoa) at the concentration of 3×10^6/ml in the various conditions tested. Samples were incubated at 37°C in a 5% CO_2 atmosphere, and aliquots were collected at different capacitation times and processed as described below.

ERK Immunohistochemistry of Formaldehyde-Fixed Human Spermatozoa and Tissues

Control and A23187-stimulated sperm samples, obtained as described above, were layered on a slide, fixed in 3.7% formaldehyde, permeabilized in 0.1% Triton X-100, and then incubated for 24 h in humidity chamber at 4°C with either the polyclonal (1:500) or the monoclonal (1:100) anti-ERK antibodies. Immunohistochemistry of testis sections was performed as previously described [24]. Briefly, testes were fixed in Bouin's solution for 4 h, embedded in paraffin, and sectioned. After deparaffinization and rehydration, the sections were incubated with either the polyclonal (1:500) or the monoclonal (1:100) anti-ERK antibodies as for the sperm samples. The slides (spermatozoa and tissues) were rinsed with PBS and then incubated with anti-rabbit (for polyclonal anti-ERK) or anti-mouse (for monoclonal anti-ERK) peroxidase-conjugated antibodies (1:1000) for 1 h at room temperature. For immunofluorescence, spermatozoa were double stained with FITC-labeled peanut lectin and anti-ERK2 antibody revealed by tetramethylrhodamine B isothiocyanate (TRITC)-labeled anti-mouse IgG. Three-dimensional confocal microscopy analysis was performed with video-confocal microscopy built at the CNR (Istituto di Biofisica-Pisa). This instrument was designed to perform multiconfocal imaging including UV excitation [25, 26]. For confocal microscopy analysis, spermatozoa were double stained for ERKs (using anti-ERK antibody and FITC-labeled secondary antibody) and DNA (with DAPI). Controls were performed by processing slides only with the secondary antibody or by staining with a nonimmune serum.

Transmission Electron Microscopy

A postembedding technique was used to detect the subcellular localization of ERK-2 and ERK-1 in spermatozoa capacitated for 18 h. Samples were washed by centrifugation (380 $\times$ *g*) for 20 min at 4°C in cold 100 mM cacodylate buffer, pH 7.4, and pellets were resuspended in the

same buffer containing 2.5% (v:v) glutaraldehyde for 1 h at 4°C. After washing, samples were dehydrated over 6 h in graded ethanol at −20°C and embedded in Lowicryl K4M at −20°C under long-wavelength (360 nm) ultraviolet light (according to supplier's instructions). Thin sections recovered on nickel grids were incubated for 15 min in PBS containing 1% BSA at room temperature. Grids were than incubated with anti-ERK2 monoclonal and anti-ERKs polyclonal antibodies diluted 1:100 in PBS/1% BSA overnight at 4°C; after three washes in PBS/1% BSA (5 min each), grids exposed to monoclonal or polyclonal antibodies were incubated for 1 h at room temperature, respectively, with mouse or rabbit biotinylated IgG diluted 1:10 with PBS/1% BSA. After three washes in PBS/1% BSA (5 min each), grids were incubated for 1 h at room temperature with 10% streptavidin-colloidal gold particles (10 nM) diluted 1:10 with PBS/1% BSA. After three washes, grids were washed in distilled water, contrasted with uranyl acetate and lead citrate, and viewed in a Zeiss 10 electron microscope (Zeiss, Oberkochen, Germany). As control, some grids were treated as indicated but incubation with the primary antibody was omitted.

Western Blot Analysis

After the various incubations, the samples were processed for SDS-PAGE as previously described [11, 14]. Briefly, sperm samples containing 3×10^6 cells/ml were added with 1 mM Na_3VO_4, centrifuged at $400 \times g$ at 4°C for 10 min, washed in HSA-free HTF, and resuspended in 10 μl lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 0.25% Nonidet P-40, 1 mM Na_3VO_4, 1 mM PMSF). After protein measurement (see below), the sperm extracts, containing approximately 20 μg of proteins, were diluted in equal volume of double-strength SB (Laemmli sample buffer: 62.5 mM Tris, pH 6.8, containing 10% glycerol, 20% SDS, 2.5% pyronin, and 200 mM dithiothreitol); incubated at 95°C for 5 min; and loaded onto 8%, 10%, or 12% 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. The nitrocellulose was blocked in 5% BSA for 2 h in TTBS solution (Tris-buffered saline containing 0.1% Tween 20, pH 7.4), washed, and then immunostained with peroxidase-conjugated monoclonal anti-phosphotyrosine antibody (PY20, diluted 1:2000 in 2% BSA-TTBS); the phosphotyrosine antibody-reacted proteins were revealed by the enhanced chemiluminescence system (ECL; Amersham). In some experiments the blots were washed for 30 min at 50°C in stripping buffer (10 mM Tris, pH 6.8, 1% SDS, 5 mM β-mercaptoethanol) in order to remove bound anti-phosphotyrosine antibodies; then the immunostaining was performed with anti-ERK antibodies αIIcp42 or αIcp44 (dilution 1:1000), or anti-MEK-1 antibody (dilution 1:1000) followed by incubation with peroxidase-conjugated goat anti-rabbit IgG; finally, the bands were visualized using the ECL system. The immunospecificity of PY20 was determined by preadsorbing the antibody with 40 mM *o*-phospho-DL-tyrosine for 1 h at room temperature. Quantification of the bands was made directly on the films by image analysis with a C3077/01 video camera, connected with the video frame-grabber M4477 (Hamamatsu Photonics, Hamamatsu, Japan). This video frame-grabber is a plug-in board used in a Macintosh Ilsi PC (Apple Computers, Cupertino, CA). Acquisition of images was done with Imagequest IQ Base software (Hamamatsu Photonics). Image processing and analysis were performed with IMAGE free software, kindly provided by Wayne Rasband (NIMH, Bethesda, MD). For technical reasons, quantifications were performed on ECL low-exposure films.

Immunokinase Assay

ERK activity was determined as the myelin basic protein (MBP) kinase activity of ERK immunoprecipitates [27, 28]. Briefly, stimulated spermatozoa were extracted in lysis buffer, sonicated three times for 15 sec each, at 8 bursts, and then centrifuged. After preclearing of the supernatants with 20 μl of protein A-sepharose, 100 μg of total cell lysates was immunoprecipitated with 2 μl of αIIcp42 antibody for 1 h on ice followed by overnight incubation at 4°C with 20 μl of protein A-sepharose. The immunobeads were washed three times in lysis buffer and then resuspended in 30 μl of kinase buffer (10 mM Hepes, pH 7.4, 20 mM $MgCl_2$, 1 mM DTT, 1 mM Na_3VO_4, 25 μM ATP, 0.5 mg/ml MBP, 1.5 μCi [γ-^{32}P]ATP). After 30 min of incubation at 30°C, 25 μl of the reaction mixture was spotted onto 1-cm^2 pieces of phosphocellulose paper, which were subsequently washed with five changes of 1% phosphoric acid and finally with 95% ethanol. The amount of radioactivity retained on the papers was determined by liquid scintillation counting, and the kinase activity was expressed as cpm/μg of total lysate proteins per minute of incubation.

In-Gel Kinase Assay

To evaluate the effects of capacitation on the activity of sperm ERK-1 and ERK-2, we determined the ability of kinases present in total cell lysates to phosphorylate MBP (0.5 mg/ml) copolymerized in SDS-polyacrylamide gels [29] as previously described [28]. After the treatments, spermatozoa were lysed and sonicated as for the immunokinase assays (see above). After centrifugation, aliquots of the supernatants containing 10 μg of proteins were boiled in double-strength Laemmli sample buffer and separated on a 10% SDS-polyacrylamide gel containing 0.5 mg/ml MBP. SDS was removed from the gel by washing with two changes of 20% 2-propanol in buffer A (50 mM Hepes, pH 7.4, 5 mM 2-β-mercaptoethanol) for 1 h at room temperature. The gel was subsequently denatured with 6 M guanidine-HCl in buffer A for 1 h, and then renatured in buffer A containing 0.04% Tween 20 for 16 h at 4°C. After 1-h preincubation in buffer B (25 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 100 μM Na_3VO_4, 5 mM 2-β-mercaptoethanol, 0.5 mM EGTA), the kinase assay was carried out by incubating the gel at 25°C for 2 h in 5 ml buffer B containing 40 μM ATP and 50 μCi of [γ-^{32}P]ATP. The gel was then extensively washed with 5% trichloroacetic acid containing 1% sodium pyrophosphate, dried under vacuum, and subjected to autoradiography. Quantification of the bands was performed as described above.

Evaluation of Sperm Acrosome Reaction

Sperm acrosome reaction was evaluated using the fluorescent probe FITC-labeled *Arachis hypogea* (peanut) lectin as previously described [30]. Spermatozoa were selected by swim-up procedure for 2 h at 37°C in 0.3% BSA-containing medium in the presence or absence of the MAPK cascade inhibitor PD098059 (100 μM) [31, 32], centrifuged, and

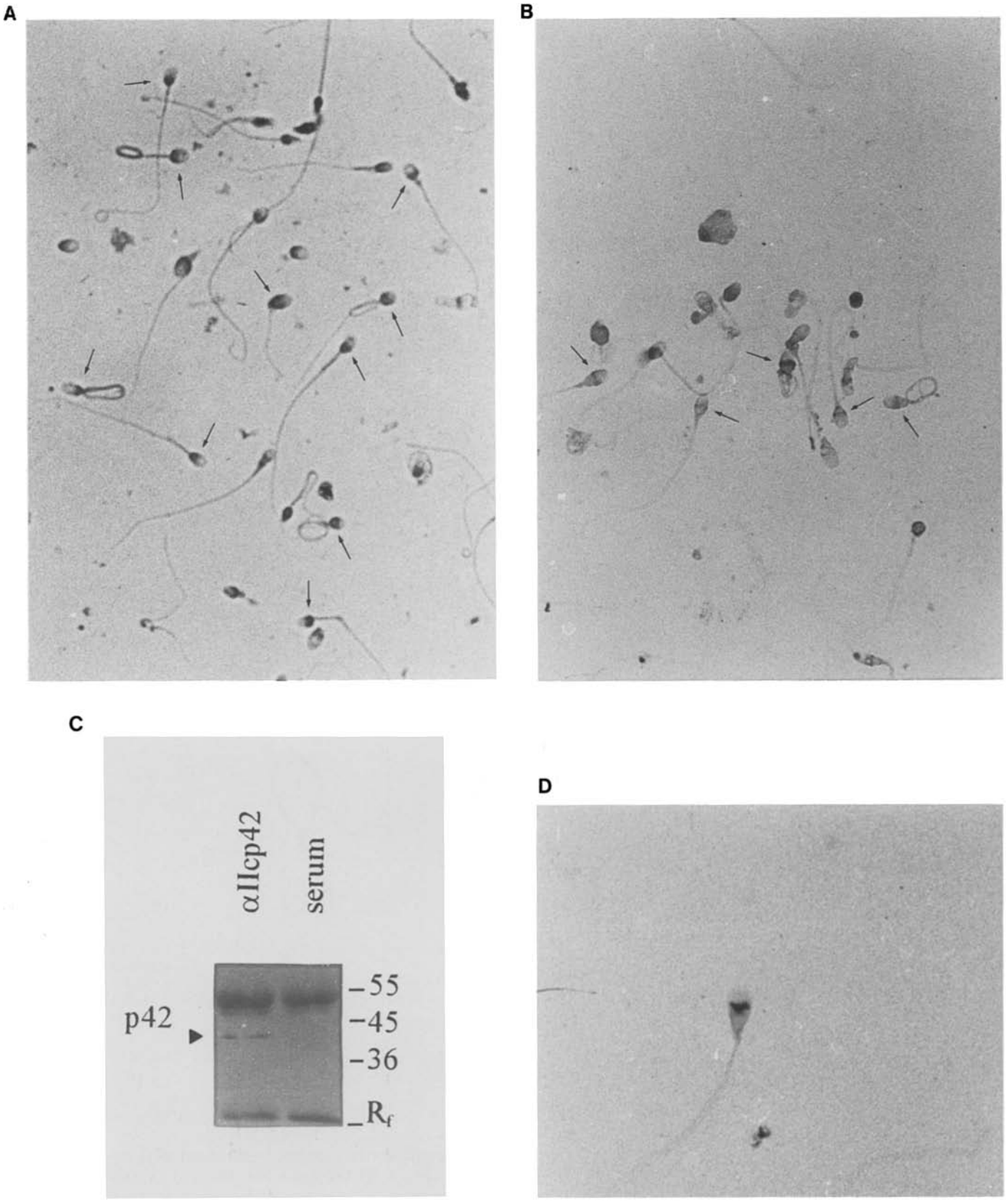

FIG. 1. Localization of ERKs in human spermatozoa. Capacitated spermatozoa were treated for 1 h at 37°C with control solvent (**A**) or A23187 (10 μM) to stimulate the acrosome reaction (**B, D**). The cells were then fixed and analyzed by staining with polyclonal αIIcp42 (**A, B**) or monoclonal (**D**) anti-ERK antibodies and peroxidase-conjugated secondary antibodies. Arrows indicate selective localization of ERKs to the postacrosomal region of acrosome-intact spermatozoa (**A**) and to the equatorial segment of acrosome-reacted spermatozoa (**B, D**). Representative of 5 similar experiments. **C**) Western blot analysis (8% SDS-PAGE) of sperm proteins immunoprecipitated with the polyclonal αIIcp42 antibody and revealed with the same antibody, showing a specific band of 42 kDa. This band was not present when immunoprecipitation was conducted with control serum. Molecular weight markers ($\times 10^3$) are indicated to the right of the blot. Rf: running front. The protein band at about 55–60 kDa present is the heavy chain of the antibody used to immunoprecipitate. **A** and **B**, ×500; **D**, ×750 (reproduced at 82%).

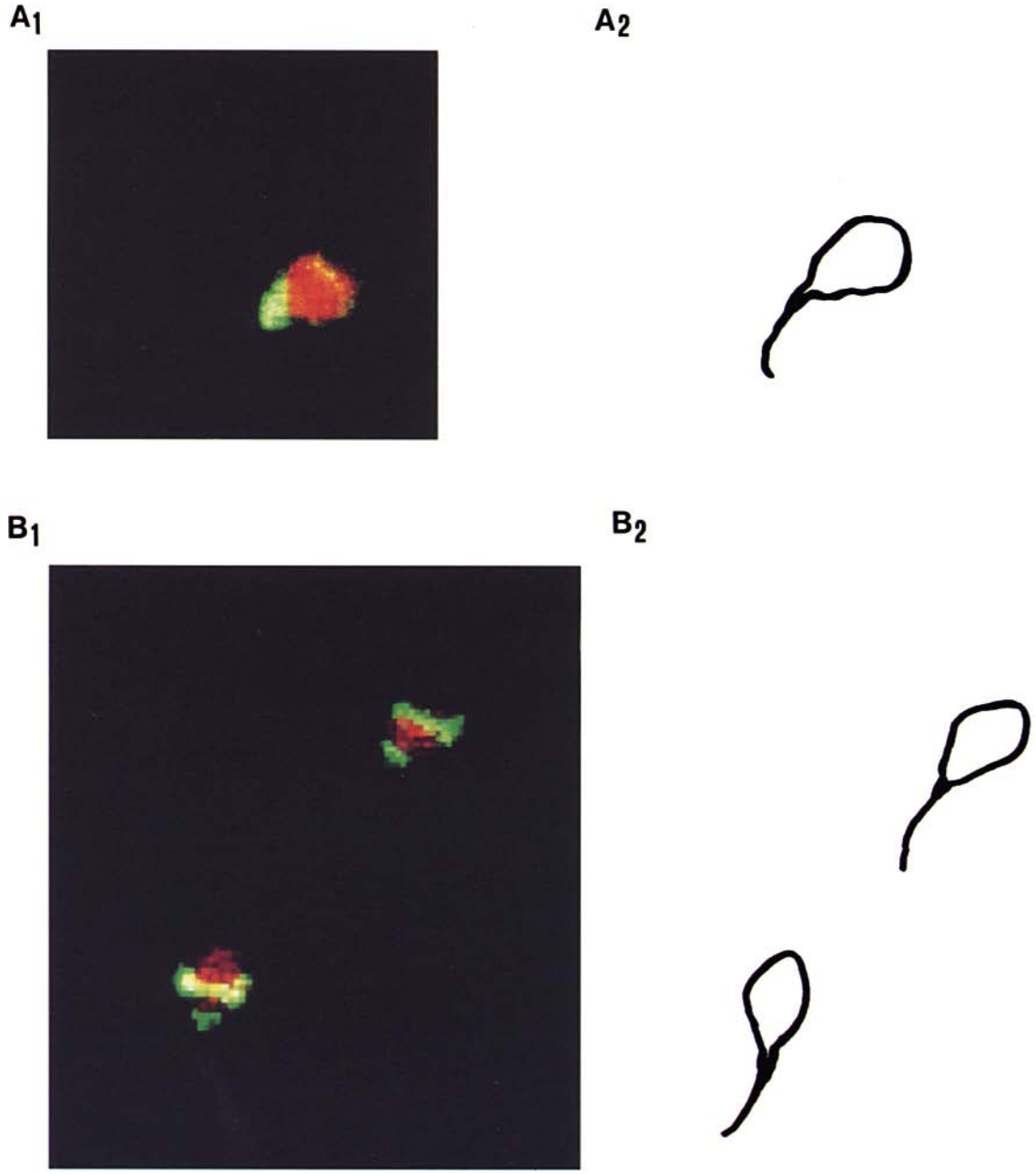

FIG. 2. Confocal microscopy analysis of ERKs in spermatozoa. Spermatozoa double stained with anti-ERK antibody (αIIcp42) and DAPI for DNA (red staining) show strong positivity for ERKs (green-white staining) at the level of postacrosomal region in an acrosome-intact spermatozoon (**A**) and of equatorial segment in two spermatozoa treated for 1 h with A23187 (10 μM) (**B**). Indirect immunofluorescence labeling procedure was performed as described in *Materials and Methods*. DAPI staining was printed in red because of the improved contrast. A drawing showing orientation of the three spermatozoa is seen on the right of each panel. **A1** and **B1**, ×2000.

stimulated for 1 h with progesterone (10 μM). Incubation in hyposmotic swelling medium for 1 h at 37°C was performed. Fluorescence was observed under a fluorescence microscope (Leitz, Type 307–148002; Wetzlar, Germany), and the acrosome reaction was evaluated on a total of 100 spermatozoa per slide. According to Aitken et al. [33], only curly-tailed spermatozoa were considered viable and thus scored. Acrosome reaction following progesterone challenge (ARPC) represents the difference between the percentage acrosome reaction obtained in progesterone-stimulated spermatozoa and the percentage spontaneous acrosome reaction.

Measurement of Proteins

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

Statistical Analysis

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

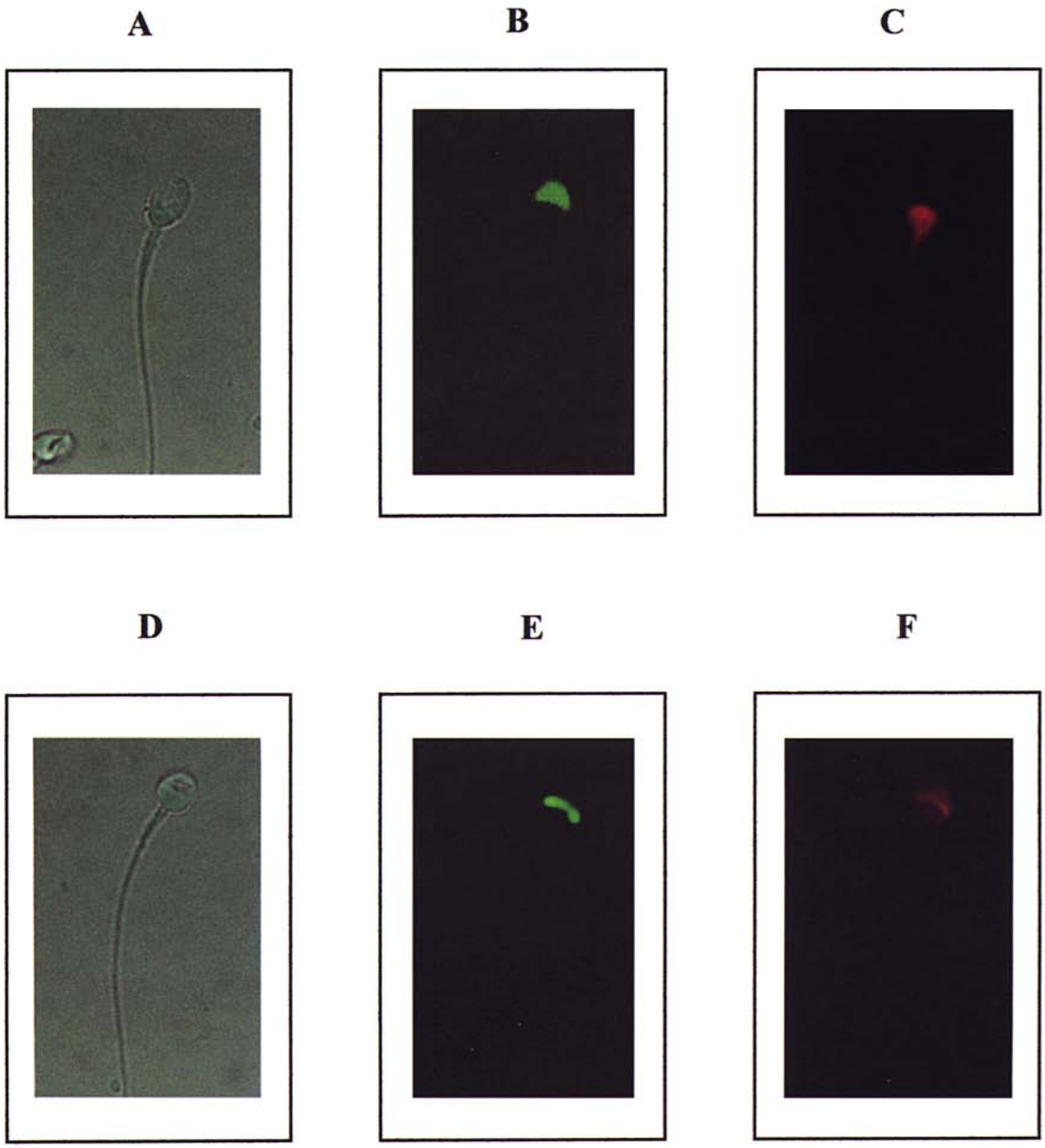

FIG. 3. Immunofluorescence analysis of ERK and acrosomal status in human spermatozoa. Paired views of spermatozoa showing actual acrosomal status, examined by FITC-labeled peanut lectin (**B, E**) and ERK immunostaining by TRITC-labeled secondary antibody (**C, F**). **A** and **D** show light transmission microscopy photographs of the spermatozoa in **B** and **C** and **E** and **F**, respectively. The upper panels show an acrosome-intact spermatozoon as evidenced by the staining at the acrosomal level of FITC-labeled peanut lectin; the lower panels show an acrosome-reacted one as evidenced by staining of FITC-labeled peanut lectin at the equatorial level. Note ERK staining in the neck region in the acrosome-intact sperm and at the equatorial level in the acrosome-reacted sperm. **A–F**, ×750.

RESULTS

Localization of ERKs in Human Spermatozoa and Testis

Immunohistochemical staining of fixed and permeabilized ejaculated human spermatozoa with the polyclonal antibody αIIcp42 revealed a selective localization of ERKs to the postacrosomal region of the sperm head (Fig. 1A), while the acrosome and the tail were unreactive. Similar results were obtained with the monoclonal anti-ERK-2 antibody (data not shown). Western blot analysis of sperm proteins immunoprecipitated with αIIcp42 antibody, and blotted with the same antibody, detected a single band of approximately 42 kDa (Fig. 1C), which was not present in the control sample (immunoprecipitation with nonimmune rabbit serum [Fig. 1C]). We also performed immunohistochemistry of human spermatozoa after stimulation with A23187, which induced the acrosome reaction in a high percentage of live, reacted spermatozoa (ranging from 30% to 50%). Under this condition, αIIcp42 antibody staining was mostly localized to the equatorial region of the plasma membrane in the central region of the sperm head (Fig. 1B). These results, confirmed also using the monoclonal anti-ERK-2 antibody (Fig. 1D), and consistently observed in 5 experiments, suggest a translocation of the enzyme during the acrosome reaction. Similar results were obtained when the acrosome reaction was stimulated with progesterone [34], a well-known physiological inducer of the acrosome reaction [35].

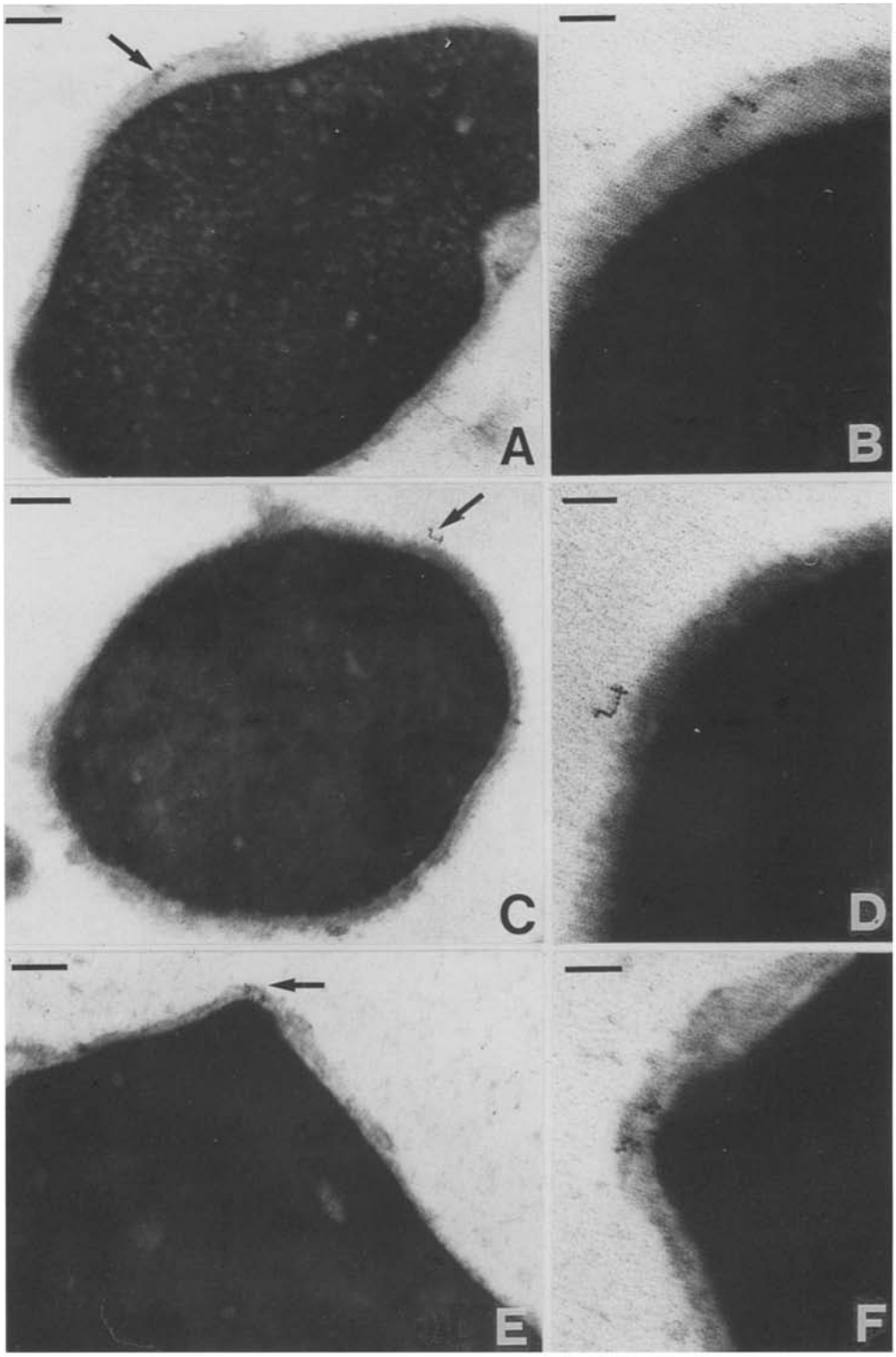

FIG. 4. Electron microscopy of human spermatozoa. Transmission electron micrographs of human sperm heads submitted to 18 h of capacitation. **A** shows a longitudinal section of a sperm head and **C** a cross section at the level of the equatorial segment. Gold particles are associated with the surface of the cell overlying an intact acrosome (**A**) or with the remnants of plasma membrane in a head with fuzzy acrosomal matrix (**C**). **B** and **D** show enlargements of regions indicated by arrows in **A** and **C**, respectively. **E**) Longitudinal section of an acrosome-reacted head that shows gold particles associated with the surface overlying the anterior part of the postacrosomal region at the equatorial level. **F** shows an enlargement of the region indicated by arrow in **E**. Gold particles (10 nm) were used in **A–F**. Scale bar = 0.2 μm in **A, C, E**; 0.1 μm in **B, D, F**.

Indirect immunofluorescence confocal microscopy analysis of spermatozoa double stained for ERKs and DNA confirmed a high density of ERK staining in the postacrosomal region of untreated spermatozoa (Fig. 2A) and in the equatorial segment of A23187-treated spermatozoa (Fig. 2B). Despite the presence of immunoreactivity for ERK at the nuclear level in germ cells (see Fig. 5A), confocal microscopy scanning did not reveal any positive staining of the nucleus of mature sperm and indicated surface localization of the antigen.

Confirmation that ERK localization at the equatorial segment occurs in acrosome-reacted spermatozoa was obtained in experiments in which these cells were double-stained with peanut lectin and anti-ERK2 antibody. The upper panels of Figure 3 show an acrosome-intact spermatozoon with FITC-labeled peanut lectin localized to the acrosome (panel B) and ERK2 in the neck (panel C), while the lower panels show an acrosome-reacted spermatozoon with both FITC-labeled peanut lectin (panel E) and ERK2 (panel F) localized in the equatorial segment. Panels A and D show light transmission microscopy photographs of the spermatozoa in panels B–C and E–F, respectively.

Immunoelectron microscopy was performed in 18-h-capacitated unstimulated spermatozoa. These studies demonstrated that anti-ERK antibodies were associated with the surface of the sperm heads, although the omission of sam-

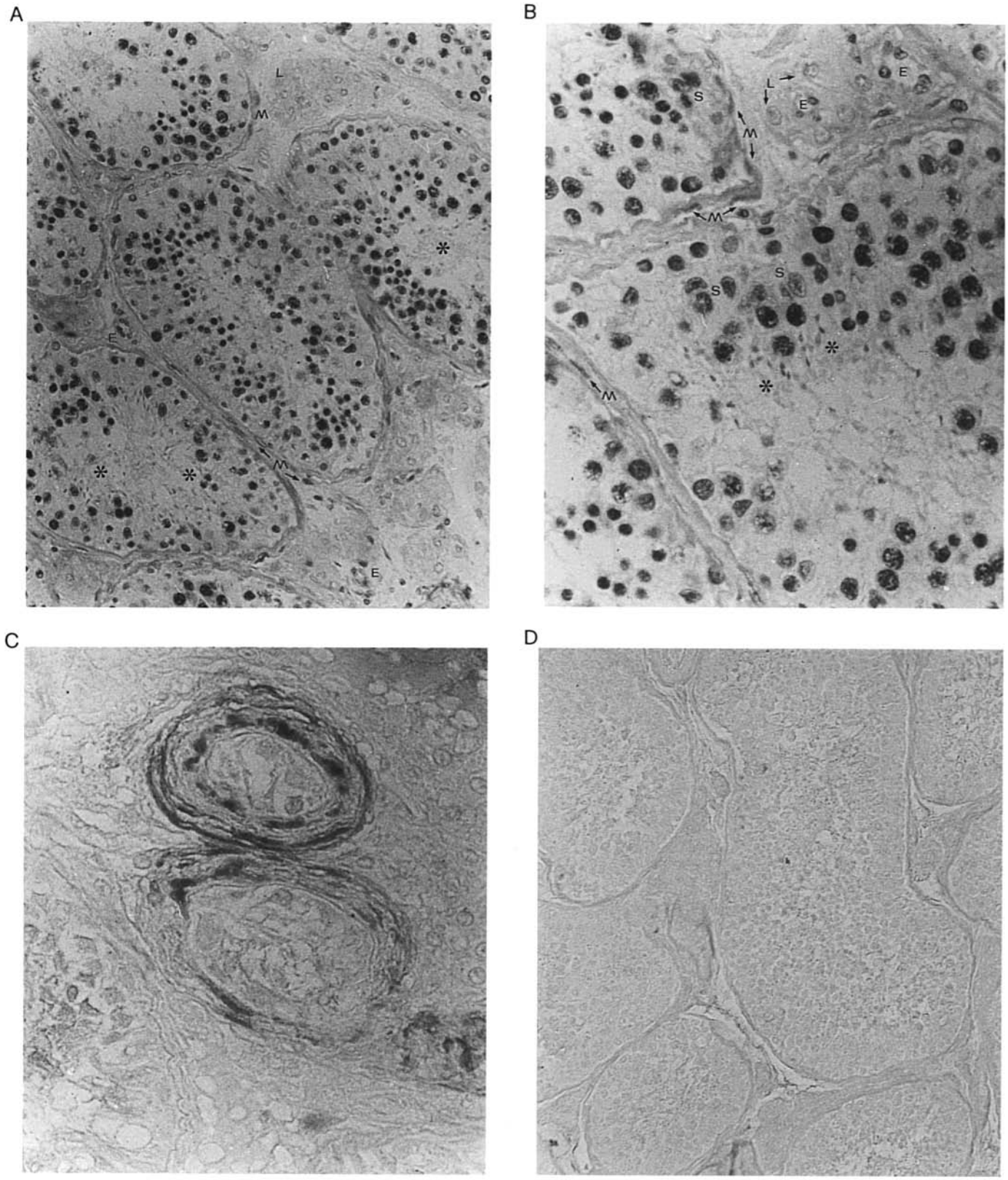

FIG. 5. Localization of ERK immunoreactivity in human testis. **A**) Testis section from a man with obstructive azoospermia and normal spermatogenesis showing high positivity for ERKs in germ cells within the tubules, including spermatozoa (*). Note the presence of positive staining in endothelial cells (E) and myoid cells (M) and absence of staining in Leydig cells (L). Representative of similar experiments performed in different subjects (n = 3). **B**) In this detail, results similar to those in **A** are shown. Note the absence of staining in Leydig cells (L) and presence of staining in a few Sertoli cells (S). Peritubular myoid (M) and endothelial (E) cells are also positive for ERKs. Note the presence of positive mature spermatozoa (*). **C**) Testis section from a man with azoospermia due to tubular damage, showing ERK positivity in connective elements around tubular walls. Immunostaining was performed with anti-ERK αIIcp42 antibody and peroxidase-conjugated secondary antibody as described in *Materials and Methods*. Representative of similar results obtained from different subjects (n = 4). **D**) Control section stained with normal rabbit serum. **A** and **D**, ×80; **B** and **C**, ×250 (reproduced at 81%).

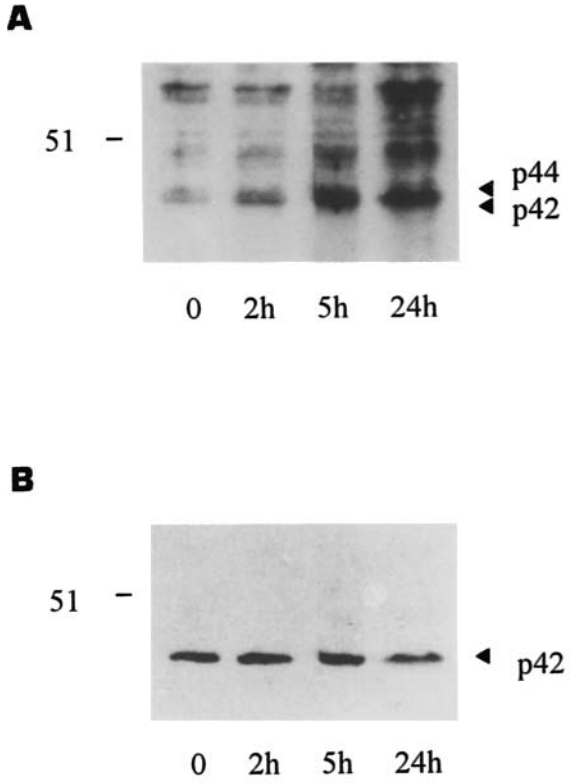

FIG. 6. Comigration of tyrosine-phosphorylated p42 with ERK-immunoreactive proteins in capacitating spermatozoa. Sperm cells were incubated under capacitating conditions for the indicated times, and proteins in cell extracts were separated by 10% SDS-PAGE. The blot was first probed for tyrosine-phosphorylated proteins with peroxidase-conjugated PY20 antibody (**A**) and then for ERK-immunoreactive proteins with αIIcp42 antibody (**B**) followed by ECL detection. An exact alignment of the blots indicates a comigration of the p42 tyrosine-phosphorylated band with the p42-immunoreactive band. Molecular weight markers (× 10^3) are indicated to the left of the blots.

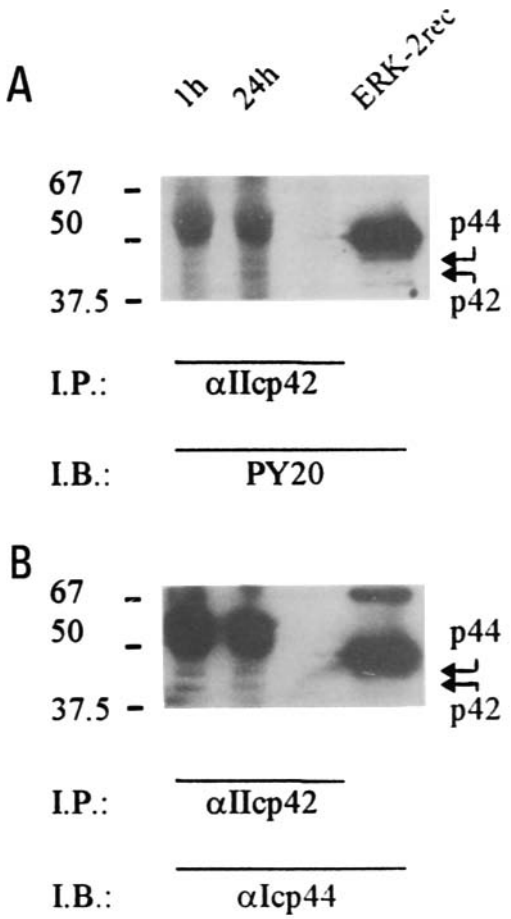

FIG. 7. Immunoprecipitation of phosphorylated p42 and p44 ERKs in capacitating spermatozoa. The αIIcp42 antibody was used to immunoprecipitate (I.P.) p42 and p44 ERKs from spermatozoa at 1- and 24-h capacitation, and the blot was first probed with PY20 antiphosphotyrosine antibody (immunoblotting = I.B.) (**A**), then washed thoroughly and reprobed with αIcp44 antibody (I.B.) (**B**). As control, 2 μg ERK-2 recombinant protein was run in the lane marked ERK-2rec, showing a band at the expected 50–55-kDa molecular mass. The protein band at about 55–60 kDa present in both blots is the heavy chain of the antibody used to immunoprecipitate. Molecular weight markers (× 10^3) are indicated to the left of the blots. Fold increase in the ratio between autoradiographic signals of PY20 and αIcp44 decorated bands at 24-h vs. 1-h capacitation was, respectively, 1.73 for the p44 and 4.6 for the p42. Representative of 2 similar experiments.

ple osmification did not allow an appropriate visualization of cellular membranes. Clusters of gold particles were associated with the surface of cells overlying the intact acrosome at the level of the equatorial region (Fig. 4, A and B) and with the remnants of plasma membrane at the level of the equatorial region in sperm heads that showed an initial dispersion of acrosome matrix (Fig. 4, C and D). In acrosome-reacted spermatozoa, gold particles were associated with the surface of sperm heads at the level of the equatorial segment in the anterior part of the postacrosomal region (Fig. 4, E and F). No labeling was observed in the sperm tail (data not shown). These results confirm localization of ERKs at the level of the equatorial region of acrosome-reacted spermatozoa and indicate, in agreement with the results of confocal microscopy analysis, a surface localization of the antigen.

In testis sections from three different men with obstructive azoospermia but normal spermatogenesis that were stained with anti-ERK αIIcp42 antiserum, specific and intense immunoperoxidase staining was detected mainly in germ cells localized to the nuclei (Fig. 5A). Mature spermatozoa also showed positive staining. A higher-magnification detail of a testis section from the same man as in Figure 5A is shown in Figure 5B. In testis sections from men (n = 4) with azoospermia due to tubular damage, staining for ERKs was restricted to connective elements around the tubular walls (Fig. 5C). No positivity was observed in control sections treated with nonimmune rabbit serum (Fig. 5D).

Capacitation Stimulates Tyrosine Phosphorylation and Activity of ERKs in Human Spermatozoa

Previous investigators reported a progressive increase in tyrosine phosphorylation of several proteins in a wide

FIG. 8. Western blot analysis of the ERK-2 tyrosine-phosphorylated form during capacitation. Sperm cells were incubated under capacitating conditions for the indicated times, and proteins in cell extracts were separated by 10% SDS-PAGE. The blot was first probed with an antibody for the tyrosine-phosphorylated form of ERK-2 (**A**, indicated by the arrows) and then for ERK-2-immunoreactive proteins with αIIcp42 antibody (**B**, indicated by the arrows) followed by ECL detection. Molecular weight markers (× 10^3) are indicated to the left of the blots.

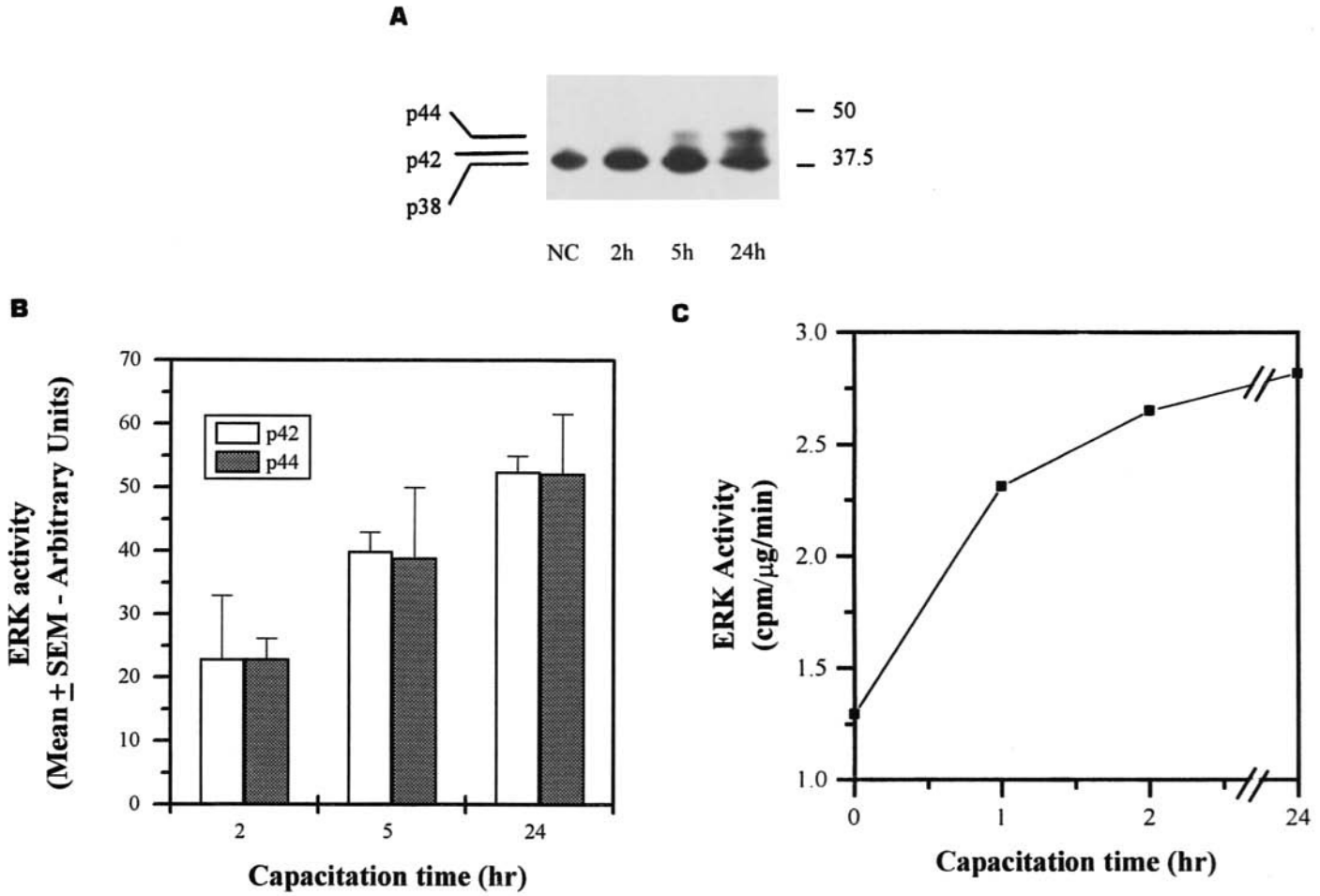

FIG. 9. ERK activity during capacitation of spermatozoa. **A**) Sperm cell extracts, obtained at the indicated capacitation times, were run in 10% SDS-PAGE containing the ERK substrate MBP (in-gel kinase renaturation assay). ERK activity was measured as described in *Materials and Methods*. Molecular weight markers (× 10^3) are indicated to the right of the blot. An increase of p44 and p42 MBP phosphorylation is evident at any time in capacitation as compared with noncapacitated sperm cells (NC). **B**) Average phosphorylating activity of p42 and p44 bands at different times of capacitation in 4 different MBP-containing gel experiments. **C**) MBP kinase activity at various capacitation times in immunocomplexes obtained after immunoprecipitation of ERKs with αIcp44 antibody (immunokinase assay) as described in *Materials and Methods*.

molecular weight range during in vitro capacitation of human [10–12, 14] and mouse spermatozoa [9, 13], including proteins in the 40–50-kDa molecular mass range. Since ERKs are activated by phosphorylation both on tyrosine and threonine residues [15], we evaluated whether the increase in tyrosine phosphorylation during sperm capacitation was present in these proteins by Western blot analysis using anti-phosphotyrosine antibodies and anti-ERK antibodies. As shown in Figure 6A, among the protein bands that showed a capacitation-dependent increase in tyrosine phosphorylation, two were located at about 42 and 44 kDa (indicated by the arrowheads) and thus were in the expected range of ERK isoforms [15]. In order to ascertain whether the phosphoprotein at 42-kDa molecular mass corresponds to ERK-2, the same blot was stripped and reprobed with αIIcp42 antibody, showing the presence of a single band at 42 kDa (ERK-2) comigrating with the p42 phosphorylated on tyrosine during capacitation (Fig. 6B). The αIIcp42 antibody has been shown to recognize mainly or only the ERK-2 isoform in Western blot analysis [27, 36]. After the same blot was washed and reprobed with αIcp44 antiserum, which recognizes both ERK-1 and ERK-2 isoforms [27, 36, 37], the staining showed two bands, respectively, at 42- and 44-kDa molecular mass (not shown), that comigrated with those revealed by the antiphosphotyrosine antibody—indicating that both ERK-1 and ERK-2 isoforms are present in human spermatozoa (see also Fig. 10, right panel, for presence of ERK-1). These data were confirmed by immunoprecipitation experiments in which sperm proteins obtained at 1- and 24-h capacitation were immunoprecipitated with αIIcp42 antibody and then blotted with PY20 antibody (Fig. 7A), stripped, and reprobed with αIcp44 antibody (Fig. 7B). In these conditions, two tyrosine-phosphorylated bands of 42- and 44-kDa molecular mass corresponding to ERK-2 and ERK-1 (Fig. 7, A and B) were detected. This result is in agreement with those of Wang et al. [27], who showed the presence of both ERK-1 and ERK-2 after immunoprecipitation with this antibody. Incubation in capacitating conditions increased tyrosine phosphorylation in both proteins (Fig. 7A), as indicated the increase in the ratio between autoradiographic signals of PY20 and αIcp44 decorated bands at 24-h versus 1-h capacitation (respectively, 1.7-fold increase for p44 and 4.6-fold increase for p42). For comparison, recombinant ERK-2 was run in the lane marked ERK-2rec (Fig. 7), showing a band at the expected 50–55-kDa molecular mass range. The presence of tyrosine-phosphorylated ERK-2 during capacitation was further confirmed by using an antibody directed against the tyrosine-phosphorylated form of ERK-

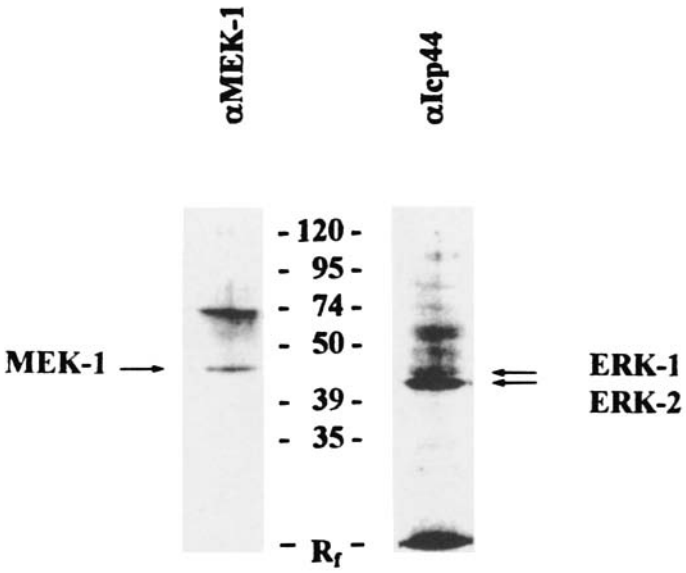

FIG. 10. Western blot analysis of MEK-1 in human sperm lysates. Sperm cells lysates, prepared as described, were separated by 10% SDS-PAGE. The blot was first probed with an antibody for MEK-1 (left panel, indicated by the arrow) and then for ERK-1- and ERK-2-immunoreactive proteins with αIcp44 antibody (right panel, indicated by the arrows) followed by ECL detection. Molecular weight markers (× 10^3) are indicated in the center of the blots.

2 [38]. Figure 8A shows a capacitation-dependent increase in the tyrosine-phosphorylated form of ERK-2. No other protein bands were detected (not shown). When the same blot, after stripping, was reprobed with αIIcp42 antibody, a single band comigrating with the tyrosine-phosphorylated form of ERK-2, with equal intensity in each lane, was observed (Fig. 8B).

To determine whether the increased tyrosine phosphorylation observed during sperm capacitation was associated with an increase in ERK activity, we carried out in-gel renaturation kinase assays with MBP as substrate (in-gel kinase assay). We observed a time-dependent increase in MBP kinase activity migrating at 42 and 44 kDa (Fig. 9A, see also Fig. 11B2) during sperm capacitation, which was similar to the time-dependent increase in tyrosine phosphorylation seen in Figures 6A and 8A. Mean MBP kinase activity of these protein bands, detected in four different in-gel experiments performed in different subjects, is shown in Figure 9B. An additional MBP kinase activity migrating at 38 kDa was observed in these experiments (Figs. 9A and 11B); however, this kinase does not appear to be modulated during capacitation, showing similar activity in noncapacitated and capacitated spermatozoa (Figs. 9A and 11B). The time-dependent increase in ERK activity during capacitation of human spermatozoa was also confirmed by immunokinase assays of sperm lysates immunoprecipitated with αIcp44 antibody (Fig. 9C). Since this antibody recognizes both ERK-1 and ERK-2 [27], MBP kinase activity detected with this method is likely to be due to both ERK isoforms.

Effect of Inhibition of ERK Activation on Human Sperm Capacitation

PD098059 is a recently developed compound, able to inhibit MEK activity and therefore phosphorylation and activation of ERKs [31, 32]. Figure 10 shows Western blot analysis using an antibody directed against MEK-1 of human sperm lysates. A band migrating at the expected 45-kDa molecular mass was observed (Fig. 10, left panel), indicating that MEK is present in human sperm. The additional band at ≈70 kDa (Fig. 10, left panel) could be a high molecular form of the enzyme and has been detected also in mesangial cells by Wang et al. [39]. After washing, the blot was reprobed with αIcp44 antibody, demonstrating the presence of ERK-1 and ERK-2 as expected (Fig. 10, right panel). To determine the inhibiting activity of PD098059 on activation of ERKs in spermatozoa, in-gel MBP kinase activity of sperm lysates in the presence of the inhibitor was evaluated at 24-h capacitation (since high activity of both ERKs was observed at this time point). As shown in Figure 11, PD098059 (10 μM) inhibited the increase of phosphorylation in ERK-2 (panel A), as well as the MBP kinase activity of ERK-1 (panels B1 and B2) and ERK-2 (evident in higher-exposure films, not shown) at 24-h sperm capacitation. Therefore, we used PD098059 as a pharmacological tool to study the involvement of ERKs in the induction of the capacitation state in human spermatozoa. To this end, spermatozoa were selected by swim-up procedure for 2 h at 37°C in complete, capacitating medium or in the presence of PD098059 (10 and 100 μM). No differences were observed in motility or number of recovered spermatozoa in control versus PD098059-treated samples (data not shown). The occurrence of capacitation in the two different conditions was evaluated by measuring the ability of spermatozoa to undergo ARPC. Indeed, it has been reported by several groups that response to progesterone does not occur in noncapacitated samples [5, 40, 41] and thus this parameter can be used as an assay to identify the occurrence of capacitation [14]. As expected, spermatozoa incubated in capacitating complete medium were able to undergo the acrosome reaction in response to progesterone, as indicated by the ARPC value (Fig. 11C). In contrast, the steroid was almost ineffective when the swim-up procedure was conducted in the presence of two different concentrations of PD098059 (Fig. 11C). The presence of the inhibitor of ERK activation during the swim-up procedure did not significantly modify spontaneous acrosome reaction (14.4 ± 2.4% acrosome reaction in control vs. 14.8 ± 5.7% in 10 μM PD098059- and 18.0 ± 3.8% in 100 μM PD098059-treated samples). This result strongly suggests that activation of ERKs plays an important role in the development of the capacitation state of human spermatozoa.

DISCUSSION

The present paper shows the presence of ERKs in human spermatozoa and establishes a biological role for these enzymes in sperm capacitation. Indeed, we demonstrate here that in vitro capacitation stimulates a sustained and concomitant increase in tyrosine phosphorylation and kinase activity of ERKs, indicating activation of these enzymes during capacitation. Furthermore, our results suggest that activation plays a biological role in the development of capacitation of human spermatozoa, since the ability of these cells to undergo the acrosome reaction in response to progesterone is strongly inhibited when capacitation is performed in the presence of the MAPK cascade inhibitor PD098059. Our data also demonstrate the selective localization of ERKs to the postacrosomal region of permeabilized human spermatozoa and, after stimulation of the acrosome reaction with ionophore (present study) and progesterone [34], to the extracellular face of the equatorial region. Taken together, these findings are consistent with a

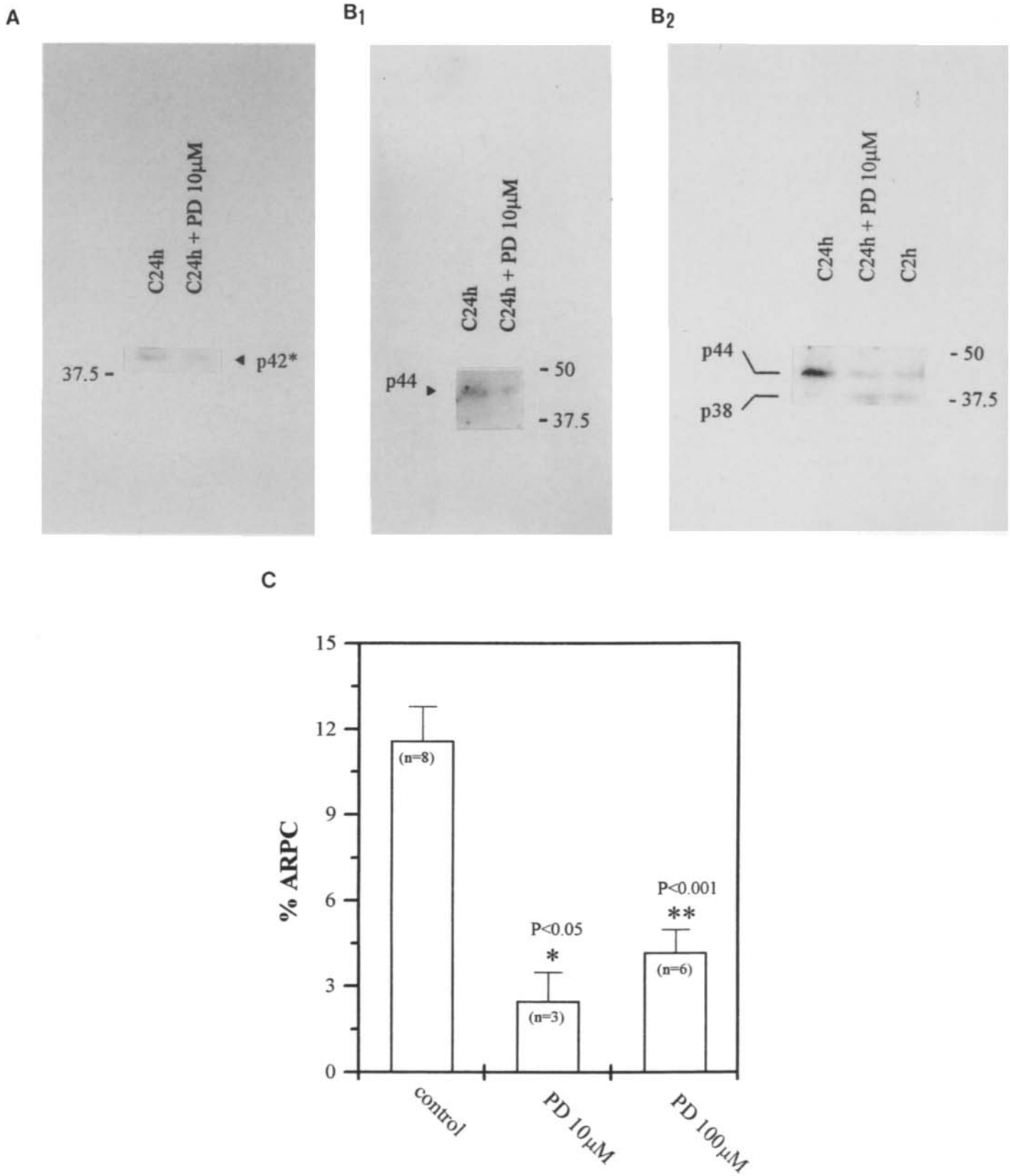

FIG. 11. Effect of the MAPK cascade inhibitor PD098059 on human sperm capacitation. In **A**, the inhibitory effect of 10 μM PD098059 on ERK-2 phosphorylation at 24-h capacitation is shown. Spermatozoa were capacitated for 24 h in the presence or absence of PD098059; after 10% SDS-PAGE, the blot was probed with an antibody against the tyrosine-phosphorylated form of ERK-2 (p42*). Molecular weight markers (× 10^3) are indicated to the left of the blot. **B1** and **B2** show the inhibitory effect of PD098059 (10 μM) on MBP-phosphorylating activity of p44 at 24-h capacitation (evaluated by in-gel kinase renaturation assay) in two different subjects. In **B2**, a time point at 2-h capacitation was also examined. Similar results were obtained in a third independent experiment. Molecular weight markers (× 10^3) are indicated to the right of the blots. **C**) Spermatozoa were capacitated by swim-up procedure for 2 h in the presence or absence of PD098059 (10 μM and 100 μM), washed, and reincubated with progesterone (10 μM) for 1 h. Acrosome reaction was evaluated by fluorescence microscopy as described in the text. ARPC: acrosome reaction following progesterone challenge (difference between percentage acrosome reaction after progesterone and basal percentage acrosome reaction).

redistribution of ERKs during sperm activation. The ability to translocate from one cell compartment to another in response to extracellular signals is a common feature of MAPKs [15]. Indeed, it has been shown that they are capable of translocation not only to the nucleus [15], but also to the cell surface membrane [42]. Since MAPK substrates may be located in different (and multiple) cellular compartments, it is conceivable that MAPKs could translocate to multiple cell structures. This assumption may be particularly important when one considers highly differentiated cells such as spermatozoa. In mature spermatozoa, the nucleus plays only a marginal, if any, role in modulation of their function, chromatin being condensed and little or no protein synthesis occurring during the life of mature spermatozoa. In these conditions, a nuclear translocation of ERKs seems unlikely. A similar situation can be found in platelets, where activation of ERKs occurs in response to stimuli that induce aggregation, such as thrombin [16]. In contrast, in view of the fundamental role played by the equatorial segment in sperm-oocyte interaction [1], translocation of ERKs at this level may be expected. Following stimulation of the acrosome reaction, a conformational rearrangement of some proteins at the level of the equatorial segment has been demonstrated [43]. These proteins appear to play an important role in sperm-oocyte fusion [43]. Thus, the presence of ERKs at the level of the equatorial segment after the acrosome reaction might be related to the regulation and/or activation of proteins that mediate binding and fusion between sperm and egg plasma membranes. This process involves interaction between a complex of sperm surface antigens and integrins present on the egg surface (for review, see [44]). In addition, several integrin chains present in mammalian spermatozoa have been shown to play a role in binding of spermatozoa to the oocyte, and the expression of these molecules is regulated during capacitation [44]. The involvement of ERKs in regulation of the integrin affinity state has been reported [45]. In this scenario, sperm ERKs may regulate activity of these molecules during capacitation and the acrosome reaction, possibly by phosphorylation of downstream targets involved in this process such as cytoskeletal molecules (see below).

The possible downstream targets of ERKs during capacitation of spermatozoa remain to be defined. As mentioned above, capacitation is characterized by several biochemical events, most of which require activation of enzymatic activities, as well as rearrangement of cytoskeletal elements [1]. Among the substrates of MAPK identified so far, possible candidates for phosphorylation by activated ERKs in human spermatozoa are phospholipase A2 [46], which has been demonstrated to play a role during sperm capacitation [47], and cytoskeletal elements such as microtubule-associated proteins (MAP), whose phosphorylation may be important in the cytoskeletal rearrangements occurring during capacitation [7]. In particular, MAP-2 has been reported to be phosphorylated in serine in bovine spermatozoa, and its phosphorylation is regulated by agents that modulate capacitation and the acrosome reaction [7].

The occurrence of ERKs in sperm cells appears to be an early event during spermatogenesis. We have demonstrated the presence of ERKs in testicular germ cells at any stage of sperm development. In these cells, ERKs were predominantly localized to the nuclei, where they might regulate gene transcription, maturation, and differentiation. In this connection, the involvement of ERKs in the meiotic maturation of female gametes has been recently demonstrated [48, 49]. In particular, activation of ERKs in oocytes is stimulated by mos oncogene, resulting in the modulation of the various stages of oocyte development [49]. Recently, it has been shown that regulation of expression of mos oncogene may play a role in spermatogenesis and that MAPK is involved in this effect [50]. In addition, it has been recently reported that MAP-2, a possible substrate for ERKs, is abundant in the rat testis and is localized both in the cytoplasm and, as in the case of ERKs in human testis, in the nuclei of germ cells [51]. The lack of staining in tubules of testis sections of azoospermic men is a further indication of the possible role of ERKs in the maturation and differentiation of germ cells.

In conclusion, our data demonstrate the presence of ERKs in human spermatozoa and their direct or indirect role in the capacitation process. Although further studies are needed to clarify the signal transduction pathways involved in their activation and the downstream targets of their action, the present findings shed new light in the effort to clarify the molecular mechanisms involved in capacitation and fertilization processes.

ACKNOWLEDGMENTS

We thank Prof. M.J. Dunn (Medical College of Wisconsin, Milwaukee, WI) for helpful advice. We are indebted to Prof. M. Serio and Dr. M. Maggi (Unita' di Endocrinologia, Universita' di Firenze) for critical reading of the manuscript. We also thank Dr. Sophie Lotersztajn (Hopital Henry Mondor, Creteil, France) for helpful suggestions and Dr. David Chiaramonti for technical help.

REFERENCES

1. Yanagimachi R. Mammalian fertilization. In: Knobil E, Neil JD (eds.), The Physiology of Reproduction, Vol 1, 2nd ed. New York: Raven Press; 1994: 189–317.
2. Baldi E, Luconi M, Bonaccorsi L, Krausz C, Forti G. Human sperm activation during capacitation and acrosome reaction: role of calcium, protein phosphorylation and lipid remodelling pathways. Front Biosci 1996; 1:189–205.
3. White DR, Aitken RJ. Relationship between calcium, cyclic AMP, ATP and intracellular pH and the capacity of hamster spermatozoa to express hyperactivated motility. Gamete Res 1989; 22:163–177.
4. Zhou R, Sky B, Chou KCK, Oswald MD, Hang A. Changes in intracellular calcium of porcine sperm during in vitro incubation with seminal plasma and a capacitation medium. Biochem Biophys Res Commun 1990; 172:47–53.
5. Baldi E, Casano R, Falsetti C, Krausz C, Maggi M, Forti G. Intracellular calcium accumulation and responsiveness to progesterone in capacitating human spermatozoa. J Androl 1991; 12:323–330.
6. Vredenburgh-Wilberg WL, Parrish JJ. Intracellular pH of bovine sperm increases during capacitation. Mol Reprod Dev 1995; 40:490–592.
7. Duncan AE, Fraser LR. Cyclic AMP-dependent phosphorylation of epididymal mouse sperm proteins during capacitation in vitro: identification of an M_r 95 000 phosphotyrosine-containing protein. J Reprod Fertil 1993; 97:287–299.
8. Carr DW, Acott TS. The phosphorylation of a putative sperm microtubule-associated protein 2 (MAP2) is uniquely sensitive to regulation. Biol Reprod 1990; 43:795–805.
9. Leyton L, Saling PM. 95 kd sperm proteins bind ZP3 and serve as tyrosine kinase substrates in response to zona binding. Cell 1989; 57: 1123–1130.
10. Naz RK, Ahmad K, Kumar R. Role of membrane phosphotyrosine proteins in human spermatozoa function. J Cell Sci 1991; 99:157–165.
11. Luconi M, Bonaccorsi L, Krausz C, Gervasi G, Forti G, Baldi E. Stimulation of protein tyrosine phosphorylation by platelet-activating factor and progesterone in human spermatozoa. Mol Cell Endocrinol 1995; 108:35–42.
12. Burks DJ, Carballada R, Moore HDM, Saling PM. Interaction of a tyrosine kinase from human sperm with the zona pellucida at fertilization. Science 1995; 269:83–86.
13. Visconti PE, Bailey JL, Moore GD, Pan D, Olds-Clarke P, Kopf GS.

Capacitation of mouse spermatozoa. I: Correlation between the capacitation state and protein tyrosine phosphorylation. Development 1995; 121:1129–1137.
14. Luconi M, Krausz C, Forti G, Baldi E. Extracellular calcium negatively modulates tyrosine phosphorylation and tyrosine kinase activity during capacitation of human spermatozoa. Biol Reprod 1996; 55: 207–216.
15. Bokemeyer D, Sorokin A, Dunn MJ. Multiple intracellular MAP kinase signaling cascade. Kidney Int 1996; 49:1187–1198.
16. Papkoff J, Chen R-H, Blenis J, Forsman J. p42 mitogen-activated protein kinase and p90 ribosomal S6 kinase are selectively phosphorylated and activated during thrombin-induced platelet activation and aggregation. Mol Cell Biol 1994; 14:463–472.
17. Dong Z, Qi X, Fidler IJ. Tyrosine phosphorylation of mitogen-activated protein kinase is necessary for activation of murine macrophages by natural and synthetic bacterial products. J Exp Med 1993; 177:1071–1077.
18. Marsh HN, Scholz WK, Lamballe F, Klein R, Nanduri V, Barbacid M, Palfrey HC. Signal transduction events mediated by the BDNF receptor gp 145trkB in primary hippocampal pyramidal cell culture. J Neurosci 1993; 13:4281–4292.
19. Naz RK, Ahmad K, Kaplan P. Expression and function of ras-proto-oncogene proteins in human sperm cells. J Cell Sci 1992; 102:487–494.
20. Tesarik J, Moos J, Mendoza C. Stimulation of protein tyrosine phosphorylation by a progesterone receptor on the cell surface of human sperm. Endocrinology 1993; 133:328–335.
21. Berruti G. Biochemical characterization of the boar sperm 42 kilodalton protein tyrosine kinase: its potential for tyrosine as well as serine phosphorylation towards microtubule-associated protein 2 and histone H 2B. Mol Reprod Dev 1994; 38:386–392.
22. World Health Organization. WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interactions. Third edition. Cambridge, UK: Cambridge University Press; 1992.
23. Baldi E, Falsetti C, Krausz C, Gervasi G, Carloni V, Casano R, Forti G. Stimulation of platelet-activating factor synthesis by progesterone and A23187 in human spermatozoa. Biochem J 1993; 292:209–216.
24. Maggi M, Barni T, Orlando C, Fantoni G, Finetti G, Vannelli GB, Mancina R, Gloria L, Bonaccorsi L, Yanagisawa M, Forti G. Endothelin-1 and its receptors in human testis. J Androl 1995; 16:213–224.
25. Benedetti PA, Evangelista V, Guidarini D, Vestri S. Achieving confocal-point performance in confocal-line microscopy. Bioimaging 1994; 2:122–130.
26. Benedetti PA, Evangelista V, Guidarini D, Vestri S. Three-dimensional microscopy: image acquisition and processing II. Proc Reprint Int Soc Optical Engineering 1995; 2412:56–62.
27. Wang Y, Simonson MS, Pouysségur J, Dunn MJ. Endothelin rapidly stimulates mitogen-activated protein kinase activity in rat mesangial cells. Biochem J 1992; 287:589–594.
28. Bonaccorsi L, Luconi M, Maggi M, Muratori M, Forti G, Serio M, Baldi E. Protein tyrosine kinase, mitogen-activated protein kinase and protein kinase C are involved in the mitogenic signaling of platelet-activating factor (PAF) in HEC-1A cells. Biochim Biophys Acta 1996; 1355:155–166.
29. Chao TSO, Byron KL, Lee KM, Villereal M, Rosner MR. Activation of MAP kinases by calcium-dependent and calcium-independent pathways. J Biol Chem 1992; 267:19876–19883.
30. Krausz C, Bonaccorsi L, Luconi M, Fuzzi B, Criscuoli L, Pellegrini S, Forti G, Baldi E. Intracellular calcium increase and acrosome reaction in response to progesterone in human spermatozoa are correlated with in vitro fertilization. Hum Reprod 1995; 10:120–124.
31. Dudley DT, Pang L, Decker SJ, Bridges AJ, Saltiel AR. A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc Natl Acad Sci USA 1995; 92:7686–7689.
32. Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR. PD 098059 is a specific inhibitor of activation of mitogen-activated protein kinase in vitro and in vivo. J Biol Chem 1995; 270:27489–27494.
33. Aitken RJ, Buckingham DW, Fang HG. Analysis of response of human spermatozoa to A23187 employing a novel technique for assessing acrosome reaction. J Androl 1993; 14:132–141.
34. Luconi M, Krausz C, Barni T, Vannelli GB, Forti G, Baldi E. Progesterone stimulates p42 extracellular-signal regulated kinase ($p42^{ERK}$) in human spermatozoa. Mol Hum Reprod 1998; (in press).
35. Baldi E, Krausz C, Forti G. Nongenomic action of progesterone on human spermatozoa. Trends Endocrinol Metab 1995; 6:198–205.
36. Meloche S. Cell cycle reentry of mammalian fibroblasts is accompanied by the sustained activation of $p44^{MAPK}$ and $p42^{MAPK}$ isoforms in the G1 phase and their inactivation at the G1/S transition. J Cell Physiol 1995; 163:577–588.
37. Marra F, Pinzani M, DeFranco R, Laffi G, Gentilini P. Involvement of phosphatidyl inositol 3-kinase in the activation of extracellular-signal-regulated kinase by PDGF in hepatic stellate cells. FEBS Lett 1995; 376:141–145.
38. Fukuda K, Shoda T, Morikawa H, Kato S, Mori K. Activation of mitogen-activated protein kinase by the nociceptin receptor expressed in chinese hamster ovary cells. FEBS Lett 1997; 412:290–294.
39. Wang Y, Schramek H, Dunn MJ. Cytosolic and nuclear mitogen-activated protein kinases are regulated by distinct mechanisms. Exp Cell Res 1996; 225:382–388.
40. Mendoza C, Tesarik J. A plasma-membrane progesterone receptor is switched on by increasing intracellular free calcium. FEBS Lett 1993; 330:57–60.
41. Shi Q-X, Roldan ERS. Evidence that a GABAα-like receptor is involved in progesterone-induced acrosomal exocytosis in mouse spermatozoa. Biol Reprod 1995; 52:373–381.
42. Gonzalez FA, Seth A, Raden DL, Bowman DS, Fay FS, Davis RJ. Serum-induced translocation of mitogen-activated protein kinase to the cell surface ruffling membrane and the nucleus. J Cell Biol 1993; 122:1089–1101.
43. Allen CA, Green DPL. Monoclonal antibodies which recognize equatorial segment epitopes presented de novo following the A23187-induced acrosome reaction of guinea pig sperm. J Cell Sci 1995; 108: 767–777.
44. Bronson RA, Fusi FM. Integrins and human reproduction. Mol Hum Reprod 1996; 2:153–168.
45. Hughes PE, Renshaw MW, Pfaff M, Forsyth J, Keivens VM, Schartz MA, Ginsberg MH. Suppression of integrin activation: a novel function of a ras/arf-initiated MAP kinase pathway. Cell 1997; 88:521–530.
46. Lin LL, Wartmann M, Lin AY, Knopf JL, Seth A, Davis RJ. cPLA2 is phosphorylated and activated by MAP kinase. Cell 1993; 72:269–278.
47. Fry MR, Ghosh SS, East JM, Franson RC. Role of human sperm phospholipase A2 in fertilization: effects of a novel inhibitor of phospholipase A2 activity on membrane perturbation and oocyte penetration. Biol Reprod 1992; 47:751–759.
48. Posada J, Cooper JA. Requirements for phosphorylation of MAP kinase during meiosis in *Xenopus* oocytes. Science 1992; 255:212–215.
49. Nebreda AR, Gannon JV, Hunt T. Newly synthesized protein(s) must associate with $p34^{cdc2}$ to activate MAP kinase and MPF during progesterone-induced maturation of *Xenopus* oocyte. EMBO J 1995; 14: 5597–5607.
50. Rosenberg MP, Aversa CR, Wallace R, Prost E. Expression of the v-mos oncogene in male meiotic germ cells of transgenic mice results in metaphase arrest. Cell Growth Differ 1995; 6:325–336.
51. Loveland KL, Hayes TM, Meinhardt A, Zlatic KS, Parvinen M, De Kretser DM, McFarlane JR. Microtubule-associated protein-2 in the rat testis: a novel site of expression. Biol Reprod 1996; 54:896–904.