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High Water Permeability of Human Spermatozoa Is Mercury-Resistant and Not Mediated by CHIP28[1]

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ABSTRACT

A novel integral membrane protein with an apparent molecular mass of 28 kDa (CHIP28) was first isolated from human erythrocytes and is now recognized as a water channel protein. The expression of this protein has been found in several other cell types that all require high water permeability for their functions. Recent studies have shown that the water permeability (L_p) of human spermatozoa is among the highest reported for mammalian cells. Together with the low activation energy of human spermatozoa for L_p, this suggests that CHIP28 water channel may be present in the plasma membrane of human spermatozoa. However, our current studies do not support this hypothesis. Results from Western blot analysis on human sperm plasma membrane proteins, performed through use of an antibody against human erythrocyte CHIP28 protein, indicated that human spermatozoa do not express CHIP28 protein on their cell surface (n = 10). Consistent with the Western blot finding, mercuric chloride ($HgCl_2$), a known water channel blocker, failed to reduce the osmotic water permeability of human spermatozoa. The calculated L_p values were 1.30 ± 0.29 μm/min/atm (n = 16; mean ± SEM) for the control group and 1.31 ± 0.29 (n = 9; mean ± SEM), 1.04 ± 0.27 (n = 11; mean ± SEM), and 1.34 ± 0.19 (n = 6; mean ± SEM), respectively, for the 10 μM, 30 μM, and 50 μM $HgCl_2$-treated groups. These L_p values are not different ($p > 0.05$). In contrast, the same concentration of $HgCl_2$ significantly blocked the osmotic water transport across the membrane of human erythrocytes. These data strongly suggest that the high water permeability of human sperm plasma membranes is not due to CHIP but may be mediated by other water channel proteins that are mercury-resistant.

INTRODUCTION

In contrast to most ion movement across biological membranes, which occurs through membrane-spanning protein channels, carriers, or pumps, water transport in most cells is mediated by a membrane solubility-diffusion mechanism [1]. In general, for any biological membrane, two different water permeability coefficients can be measured: 1) the osmotic or filtrational permeability coefficient (L_p or P_f) generated when an osmotic gradient is applied across the cell membrane and 2) the diffusional permeability coefficient (P_d) measured when cells are suspended in an isotonic solution, for which water movement is mainly diffusional and depends entirely on the composition of the membrane [1]. In cells where the P_f has been found to be significantly greater than the corresponding P_d, the presence of specific water channels has been proposed [1]. The effort to identify and purify protein water channels has had a long history involving many investigators, but only recently, with the use of advanced cellular and molecular biological techniques, has this become possible [2–5]. Channel-forming integral membrane protein with a molecular mass of 28 kDa (CHIP28) was the first water channel identified [3]; it mediates 90% of water movement across the human erythrocyte membrane. The CHIP28 cDNA has recently been cloned and sequenced [6] and found to belong to a family of membrane transport proteins named MIP (major intrinsic protein) [7, 8]. Injection of the CHIP28 mRNA into *Xenopus* oocytes and insertion of the CHIP28 protein into biosynthetic liposomes produced a greatly increased osmotic water permeability that could be inhibited by mercuric chloride [3, 9]. Immunocytochemical studies by Nielsen et al. [10] demonstrated that the CHIP28 protein is expressed in the constitutively high water-permeable segment of the nephron including the proximal convoluted and straight tubules and descending thin limbs. Immunoreactive CHIP28, however, is not detected in water-impermeable ascending thin limbs, ascending thick limbs or distal tubules, or in collecting ducts whose permeability to water is regulated by antidiuretic hormone according to the physiological requirement. CHIP water channel proteins have also been demonstrated in mature rat hematopoietic tissues, red pulp of spleen, choroid plexus, ciliary and lens epithelia, hepatic bile duct, gall bladder epithelium, intestinal lacteals, and capillary bed [10–12]. These findings sug-

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gest that CHIP28 as a water channel is involved in osmoregulation, resorption, and secretion. With respect to the male reproductive tract, CHIP28 has been found to be present in the plasma membrane of nonciliated efferent ductal cells, epithelial cells in the ampulla of the vas deferens, seminal vesicles, and prostate. However, the seminiferous tubules, epididymis, and proximal parts of the vas deferens are negative for CHIP28 [13].

Recent studies have shown that the water permeability of human spermatozoa is among the highest reported for mammalian cells [14], exceeded only by that of human erythrocytes, in which over 90% of the water transport is mediated through CHIP28 water channels. The molecular basis underlying the high water permeability of human spermatozoa is unknown at present. In the study reported here, expression of the CHIP28 protein on human spermatozoa was investigated to ascertain whether or not the high water permeability of human spermatozoa is attributable to the presence of water channels as demonstrated in other mammalian cells.

MATERIALS AND METHODS

Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

Preparation of Cellular Protein

Human sperm samples were collected from normal healthy men, aged 20–40 yr, by masturbation. After a standard swim-up procedure [15], the motile, capacitated spermatozoa were suspended at a concentration of approximately 10^7 cells/ml in a lysing buffer composed of single-strength ice-cold PBS (Gibco, Grand Island, NY), 0.1% Triton X-100, 1 mM PMSF, and 10 μg/ml leupeptin. After 30–45 min lysation at 4°C with intermittent vortexing, the lysate was centrifuged (10 000 × *g*, 4°C) for 30 min. The supernatant was saved as Triton X-100-soluble protein. The pellet, consisting of nuclei and cytoskeletal elements, was subsequently solubilized in 2% SDS, since CHIP28 has been reported to associate with Triton X-100-insoluble cytoskeletal elements [2].

Human erythrocyte ghosts were prepared by directly lysing the cells in distilled water [16]. The membrane component was pelleted (10 000 × *g*, 4°C) for 10 min and then resuspended in ice-cold PBS.

Sample protein concentration was determined by measuring the light absorbance at 595-nm wavelength in a spectrophotometer after protein samples had been reacted with Bio-Rad protein assay dye (manufacturer's procedure; Bio-Rad Labs., Hercules, CA). BSA was used as a protein concentration standard. All protein samples were stored frozen at −20°C until needed.

Western Blot Analysis

Western blot analyses were performed according to Laemmli et al. [17] through use of a Bio-Rad miniblot system. Briefly, 20–50 μg of protein was denatured at 65°C in an SDS-reducing buffer containing 10 mM Tris (pH 6.8), 1.5% SDS, 6% glycerol, and 0.5 M dithiothreitol (DTT) [3]. Electrophoresis was carried out at 200 V for approximately 45 min in a discontinuous SDS-acrylamide gel composed of 4% stacking gel, pH 6.8, and 12% separating gel, pH 8.8. Proteins separated in this way were electrophoretically transferred to a nitrocellulose membrane at 0.25 amp for 1 h in a transfer buffer (pH 7.4) consisting of 0.4 M Tris, 0.2 M sodium acetate, 20 mM Na_2-EDTA, and 0.02% SDS. Following 1-h blocking with 5% nonfat milk in a blocking buffer containing 50 mM $NaPO_4$ (pH 7.4), 150 mM NaCl, and 0.1% Tween 20, the blot was immunoreacted with rabbit anti-human CHIP28 (purified from human erythrocyte membrane) antiserum at 1:500 dilution for 48–72 h [2]. After several washes (two for 5 min, two for 10 min) in the blocking buffer, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG for 1 h at room temperature. The membrane was similarly washed again. To reveal the signal, equal volumes of DETECT A and B (Amersham's chemiluminescence kit; Amersham Corp., Buckinghamshire, UK) was reacted with the membrane for 1 min. To drain off the excess detection reagent, the blot was held vertically and its edge was touched against tissue paper (washing is not necessary). The membrane was wrapped in plastic wrap and exposed to a hyperfilm-ECL (Amersham Corp.) for 15 sec to 5 min. Membrane proteins of human erythrocyte (20 μg) were always co-electrophoresed in each gel as a positive control for the CHIP protein. The molecular mass marker used was purchased from Amersham Corp. For nonspecific binding, preimmune rabbit serum, obtained from the same animal from which the anti-CHIP antibody was generated, was used (at the same dilution) instead of primary rabbit anti-human CHIP28 antiserum. The exposure time of the blot reacted with preimmune serum was sometimes extended purposely (2–10 min) to ensure that disappearance of the specific CHIP band was a true phenomenon.

Toxicity of $HgCl_2$ to Human Spermatozoa

The cytotoxicity of $HgCl_2$ on human spermatozoa was determined before the water permeability experiments were undertaken. Spermatozoa were exposed to various concentrations of $HgCl_2$ (5, 10, 15, 30, 50, and 100 μM) in isotonic HEPES-buffered Tyrode's lactate solution (TL-HEPES) [18] for 30, 40, and 50 min, respectively. The membrane integrity of the spermatozoa was measured by double-staining the cells with carboxyfluorescein acetate (CFDA) and propidium iodide (PI) [14]. The percentage of cells exhibiting CFDA (membrane-intact cells) or PI fluorescence (lysed cells) was measured by flow cytometry using a FACStar Plus Analyzer (Becton, Dickinson and Co., Rutherford, NJ). Concomitantly, motility of the $HgCl_2$-treated spermatozoa was measured via computer-assisted semen analysis [19–21].

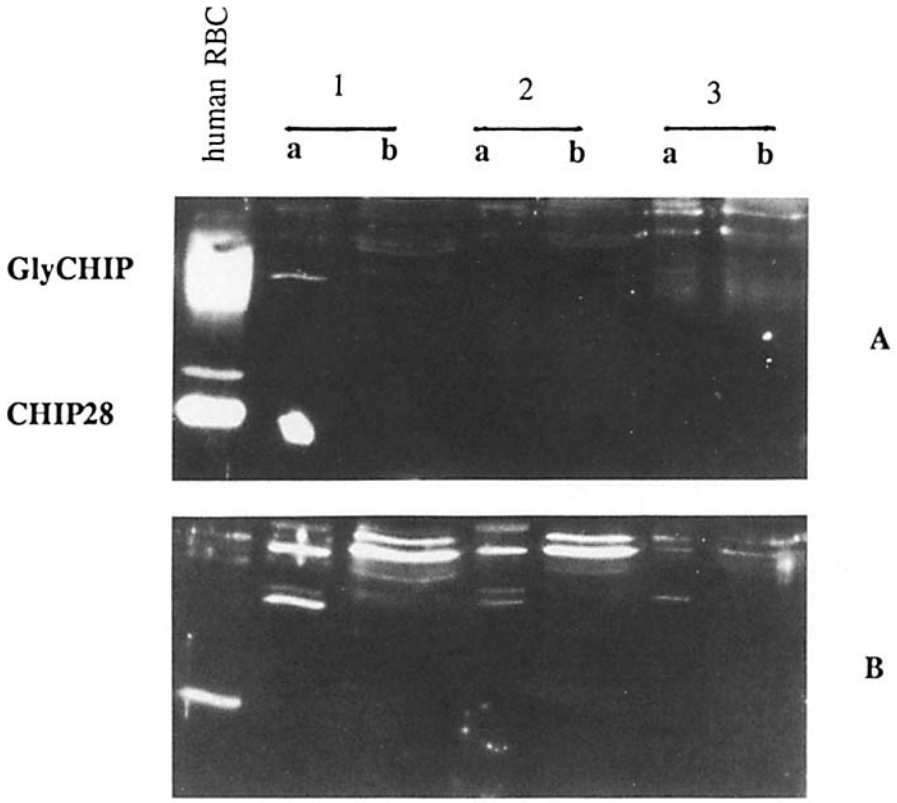

FIG. 1. Representative Western blot analysis showing typical glycosylated CHIP protein (glyCHIP) and CHIP28 protein in human erythrocytes. The CHIP protein is apparently negative for both Triton X-100-soluble (a) and -insoluble (b) sperm protein fractions (see *Materials and Methods*). **1, 2,** and **3**: Three different sperm samples. A) Immunoblotting with rabbit anti-human CHIP28 antiserum. B) Immunoblotting with normal rabbit serum. Molecular weight (MW) of migrated proteins were estimated according to co-electrophoresed Rainbow-colored protein MW marker from Amersham (not shown). A bright spot in (A) (< 280 kDa) is an artifact that may have resulted from the trapping of detection solution in a certain area of the blot and/or incomplete immersion of a certain area in the incubation/washing solutions.

Effect of $HgCl_2$ on L_p of Human Erythrocytes

The $HgCl_2$ toxicity study on human spermatozoa showed that these cells cannot tolerate $HgCl_2$ concentrations higher than 30 μM for more than 30 min. To determine whether this concentration would be sufficient for demonstrating a blocking effect by $HgCl_2$ and whether the kinetic volume measurements we intended to use were feasible for revealing a blocking effect, we chose human erythrocytes as reference cells, since these cells have been very well characterized both in their water permeability and their sensitivity to water channel blocking by mercurial compounds [22–24]. Osmotic water permeabilities of human erythrocytes, in the presence or absence of $HgCl_2$ (10, 30, 50, and 100 μM), were determined by measuring the kinetic changes in cell volume in response to aniso-osmotic solution in an electronic particle counter (Coulter counter model ZM; Coulter Electronic, Hialeah, FL) [25]. When a cell traverses the orifice (70 μm in diameter) of the electronic counter, it produces an electronic pulse with a magnitude proportional to the volume of the cell. These electronic signals were captured by a custom interface (The Great Canadian Computer Company, Spruce Grove, AB, Canada) and analyzed with a microcomputer [25]. To perform the experiments, 100 μl of the cell suspension (approximately $1–2 \times 10^7$ cells/ml) was abruptly injected with mixing into a vial containing 10 ml of a hypotonic solution (150 mOsm PBS). Values for L_p were calculated from the initial slopes of each swelling curve [26, 27].

Effect of $HgCl_2$ on L_p of Human Spermatozoa

Kinetic changes in the cell volume of human spermatozoa in a hypoosmotic (100 mOsm TL-HEPES) solution was measured in a manner similar to that described above. The calibration bead used for sizing human spermatozoa was a polystyrene bead 3.98 μm in diameter (Duke Scientific Corp., Palo Alto, CA). The osmotic water permeability (L_p) of human spermatozoa, in the presence or absence of $HgCl_2$, was determined by least squares curve-fitting of the experimental data to a differential equation describing osmotic water movement across the plasma membrane [28]. Despite the known fact that human spermatozoa undergo a curling of the tail in response to a hypoosmotic condition, this shape change does not affect the electronic particle counter measurement, which is primarily size-dependent but relatively

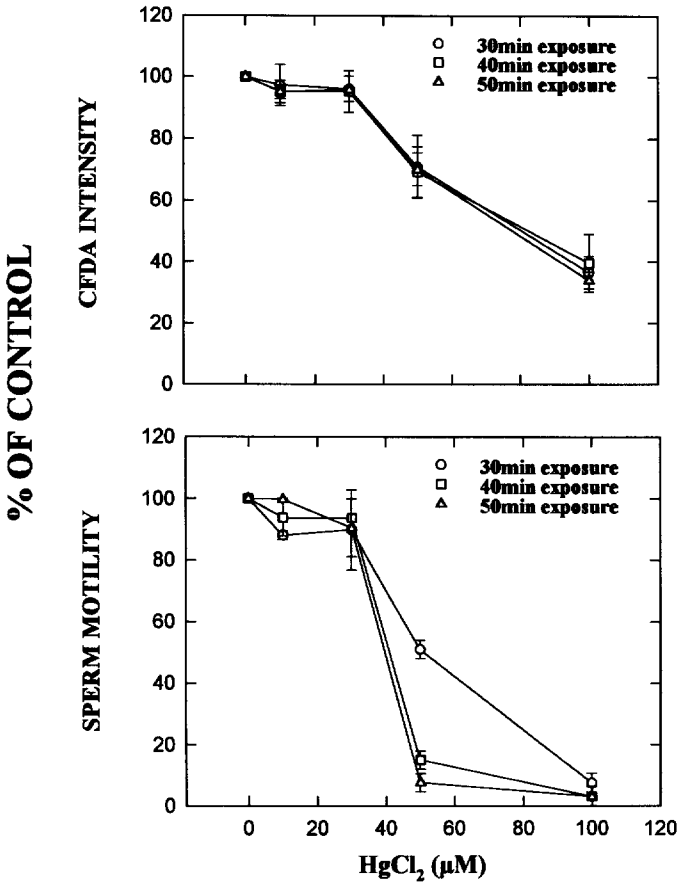

FIG. 2. Effect of $HgCl_2$ treatment on (top) membrane integrity (n = 4) and (bottom) motility of human spermatozoa (n = 3). Values are mean ± SD.

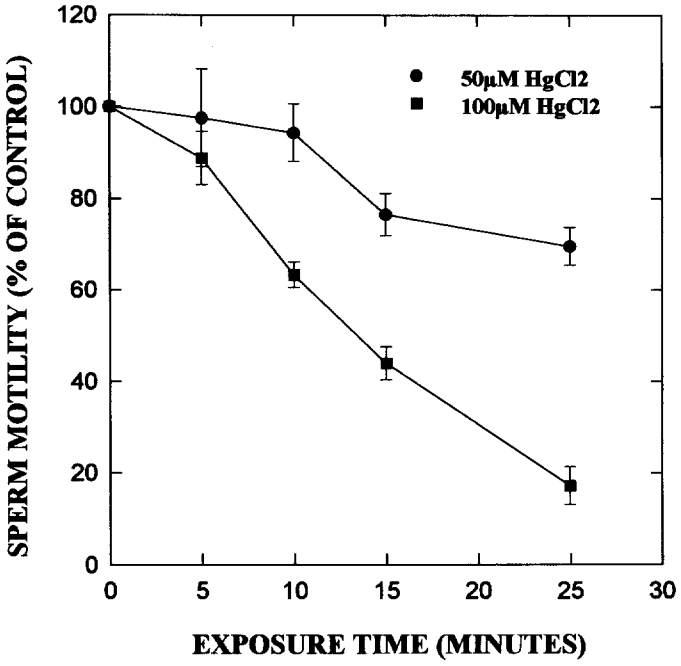

FIG. 3. Time-dependent toxicity of high concentrations of $HgCl_2$ on human spermatozoa (n = 3). Values are mean ± SD.

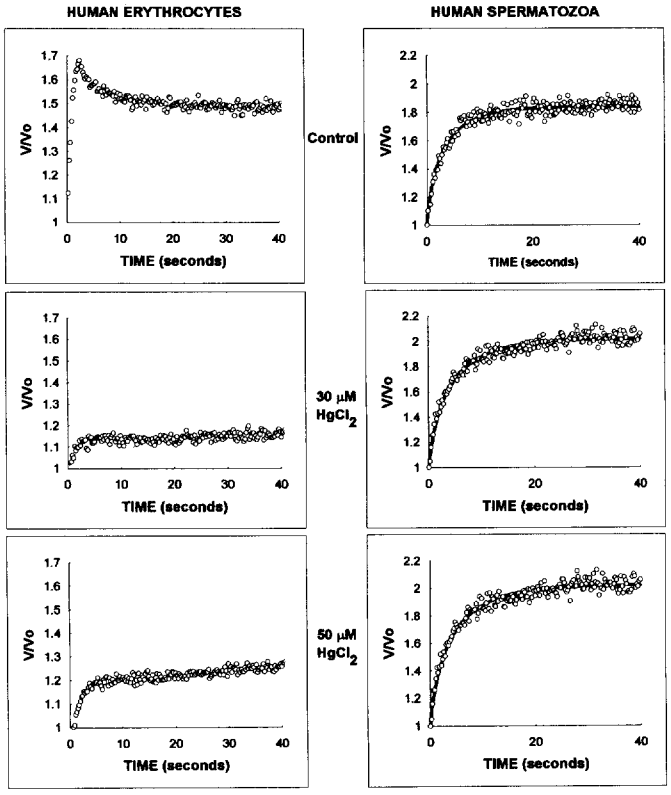

FIG. 4. Representative kinetic volume measurements showing slowed rates of hypoosmotic (150 mOsm) swelling of human erythrocytes and unchanged rates of hypotonic (150 mOsm) swelling of human spermatozoa after treatment with various doses of $HgCl_2$.

shape-independent. Data from this laboratory has been used to validate the use of an electronic particle counter to measure water permeability (L_p) for human spermatozoa (Gilmore JA, McGann LE, Gao DY, Kleinhans FW, Peter AT, Critser JK; unpublished results). The L_p values obtained on the basis of the volume change in different hypoosmotic solutions and the corresponding activation energy (E_a) calculated were essentially the same as those previously reported by Noiles et al. [14].

Time to spermolysis in a hypotonic solution, first described by Noiles et al. [14], was also determined for both control and $HgCl_2$-treated human spermatozoa. Briefly, aliquots (10 μl) of sperm samples (from the control or $HgCl_2$-treated group) were abruptly exposed to a 40 mOsm TL-HEPES solution (1 ml) with constant vortexing for 5, 10, 15, 25, and 30 sec, respectively. At the end of each time point, 100 μl of tonicity restoration solution (2640 mOsm NaCl) was added during vortexing to return the sperm cells to an isotonic condition. Membrane integrity was measured by CFDA/PI staining as described above.

Statistical Analysis

Data were analyzed by either Student's *t*-test or Wilcoxon rank sum test [29].

TABLE 1. Effect of $HgCl_2$ treatment on water permeabilities of human erythrocytes and spermatozoa.[a]

$HgCl_2$ (μM)	Erythrocyte L_p from Initial Slopes (μm/min/atm)	Spermatozoa L_p (μm/min/atm)
0	4.06 ± 0.57 (7)	1.30 ± 0.29 (16)
10	1.17 ± 0.51 (8)	1.31 ± 0.29 (9)
30	1.26 ± 0.53 (6)	1.04 ± 0.27 (11)
50[b]	1.05 ± 0.43 (9)	1.34 ± 0.19 (6)
100[b]	0.74 ± 0.05 (6)	1.10 ± 0.18 (4)

[a]Cells were treated with concentrations of $HgCl_2$ for 10–15 min. Values are expressed as mean ± SD. Numbers in parentheses represent the number of measurements.
[b]Cells were treated with these concentrations of $HgCl_2$ for only 5 min.

RESULTS

Western Blot Analysis

Western blot analyses on CHIP28 expression were performed on human sperm samples randomly selected from 10 normal healthy donors (Fig. 1). Membrane protein preparations of human erythrocytes were used as a positive control. The results showed that, as previously reported [8], the membrane preparations of human erythrocytes contained strong immunoreactive CHIP proteins that migrated in SDS-acrylamide gel as two major bands: a smear band known as glyCHIP (35–65 kDa), representing a collection of CHIP proteins with various degrees of glycosylation, and a single 28-kDa band corresponding to the native protein (Fig. 1A). A 30-kDa protein band revealed in the immunoblot was considered a nonspecific band, since it reappeared when the same blot was stripped and reprobed with preimmune rabbit serum (Fig. 1B). In the case of human spermatozoa, immunoreactive CHIP proteins were consistently negative in both Triton X-100-solubilized and SDS-solubilized protein fractions of human sperm cells (Fig. 1A).

Toxicity of $HgCl_2$ on Human Spermatozoa

$HgCl_2$ at concentrations higher than 30 μM is highly toxic to human spermatozoa, with regard to both membrane integrity and sperm motility (Fig. 2). Sperm motility is more sensitive to the toxic effect of $HgCl_2$ than is the membrane integrity. The toxicity of $HgCl_2$ is both dose- and time-dependent; 10-min exposure to 50 μM or 5-min exposure to 100 μM $HgCl_2$ did not significantly affect the motility of human spermatozoa (Fig. 3).

Effect of $HgCl_2$ on the L_p for Human Erythrocytes and Spermatozoa

Determinations of water permeability in human erythrocytes demonstrated that for the control group, cells swelled so rapidly in response to a hypoosmotic environment that the electronic counter could not track the volumetric changes kinetically. $HgCl_2$ treatment (10, 30, 50, and 100 μM) slowed the rate of cell swelling, generating clearly observable swelling curves in the electronic counter. Values for L_p, calculated from the initial slopes of the curves, were 4.06 μm/min/atm for the control group and 1.17, 1.26, 1.05, and 0.74, respectively, for groups treated with 10 μM, 30 μM, 50 μM, and 100 μM $HgCl_2$ (Table 1 and Fig. 4).

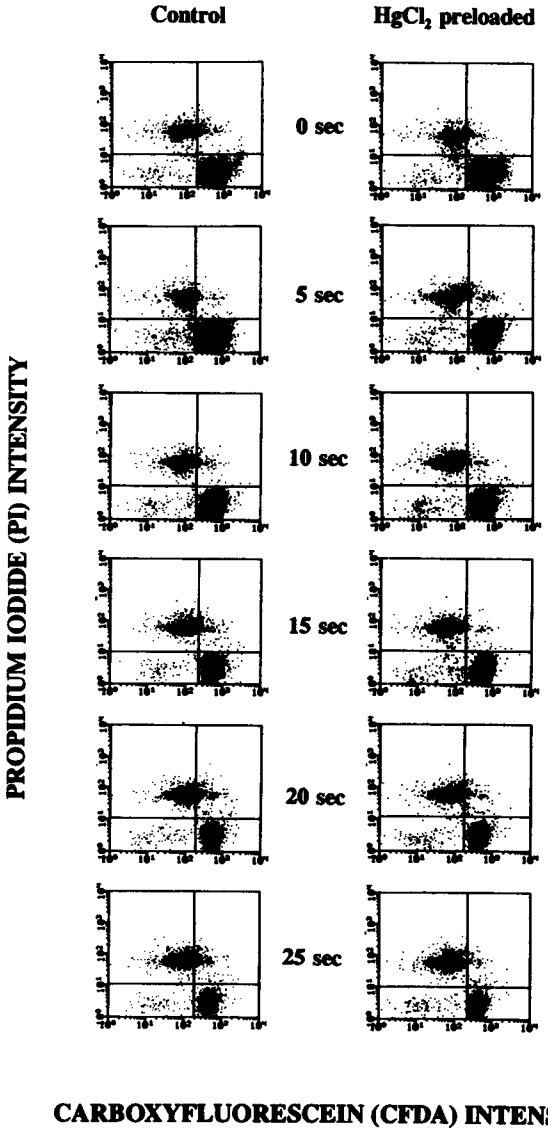

FIG. 5. Flow cytometric measurement to determine the time to 50% spermolysis in a 40 mOsm solution. Upper left quadrant, PI positive; upper right quadrant, PI and CFDA double positive; lower left quadrant, PI and CFDA double negative; lower right quadrant, CFDA positive.

In contrast to the blocking effect of $HgCl_2$ on the osmotic water permeability of the human erythrocytes, $HgCl_2$ treatment at the same concentrations failed to reduce L_p for human spermatozoa. Calculated L_p values were approximately 1.30 μm/min/atm for the control group and 1.31, 1.04, 1.34, and 1.10 μm/min/atm, respectively, for groups treated with 10, 30, 50, and 100 μM $HgCl_2$ (Table 1 and Fig. 4). No significant difference was obtained when the L_p values of any of the two groups were compared ($p > 0.05$). When high concentrations of $HgCl_2$ (50 and 100 μM) were used, cells

were treated for only 5 min before the water permeability was measured. During this period of time, no significantly toxic effect of $HgCl_2$ was observed (Fig. 3). A 5-min exposure to $HgCl_2$ has been shown to effectually reveal a blocking effect on the osmotic water permeability [3]. Consistent with the L_p determinations, there was no significant difference when the hypoosmotic spermolysis rate of the control group was compared to that of the group treated with 30 μM $HgCl_2$ (Fig. 5). Fifty percent of spermolysis in a 40 mOsm solution was achieved at 17 sec for the control group and at 16 sec for the $HgCl_2$-treated group (16.67 ± 1.41 and 15.75 ± 0.66, respectively; three replicates; $p > 0.05$). In addition, other mercurial sulfhydryl reagents, including *p*-chloromercuribenzene and fluorescein mercuric acetate, also failed to inhibit the osmotic water permeability of human spermatozoa (data not shown).

DISCUSSION

This study was conducted to investigate the molecular basis of the high water permeability of human spermatozoa. In general, most cells in an organism do not require high water permeability to perform their functions; their water permeability coefficients usually fall around 0.4 μm/min/atm (1×10^{-3} cm/sec) [30]. Variation in water permeabilities in these cells is accounted for simply by the differences in the bilayer structure of their plasma membranes. Plasma membranes of erythrocytes, renal tubules, and certain epithelial cells [1, 4, 10, 11], however, exhibit high water permeability that cannot be explained solely by membrane bilayer structure and has now been demonstrated to result from the presence of selective molecule water channels. A recent study has shown that the water permeability of human spermatozoa is unusually high, exceeded only by that of human erythrocytes [14]. These findings, together with the low activation energy (3.92 ± 0.59 kcal/mol) of human spermatozoa, suggest the strong likelihood that the membrane of human spermatozoa may contain water channels; this would constitute a molecular basis for the high water permeability of this cell type. Our current studies focused mainly on the expression of CHIP proteins because CHIP was the first water channel to be recognized, it is well characterized, and it is expressed in most cells with high water permeability [10–12]. However, our Western blot analysis failed to detect any CHIP signals on membrane preparations of human spermatozoa. Mercurial compounds, at concentrations that significantly blocked water transport across the membrane of human erythrocytes, had no significant effect on the osmotic water permeability of human spermatozoa. Thus, it appears unlikely that the high water permeability of human spermatozoa is mediated by a CHIP water channel. In spite of the negative expression of the CHIP water channel in human spermatozoa, we still argue for the possibility that a novel, mercury-resistant water channel (perhaps a CHIP homologue) is present in the plasma membrane of human spermatozoa. This argument is based on two previous findings. First, human spermatozoa have a high P_f (or L_p) and low E_a. It has been shown that human and adult rat erythrocytes that have high P_f and low E_a also have water channels on their cell membrane, whereas fetal rat erythrocytes, which have low P_f and a high E_a, do not have plasma membrane water channels [32]. Second, sensitivity of water channels to mercurial compounds is a common but not a necessary phenomenon, since it largely depends on where the cysteine residues reside. In this regard, Preston et al. [33] have shown that CHIP28, with a cysteine to serine mutation at residue 189 (only cysteine residue in the CHIP is sensitive to $HgCl_2$), can still function as a water channel but is Hg^{2+}-insensitive in nature. Furthermore, Hasegawa et al. [34] have recently cloned a 32-kDa mercury-insensitive water channel (MIWC) from a rat lung cDNA library. This MIWC has been shown to have a unique distribution in cells that do not express CHIP28.

These results do not exclude the possibility that the high water permeability of these cells may simply be due to the lipid composition of their membrane. It has been demonstrated that in the process of capacitation, spermatozoa lose much of their membrane cholesterol [35–39]. On the basis of studies of model membranes, such a loss of sperm membrane cholesterol would be expected to increase overall membrane permeability [40]. If the high water permeability of human spermatozoa is attributable solely to their membrane composition, this cell type would represent an exception to a common phenomenon: that high water permeability and an associated low activation energy are associated with the existence of transmembrane water channels.

Further investigation of the mechanism by which water moves across the sperm plasma membrane is needed. One specific approach would be to measure the ratio of the osmotic to the diffusional water permeability coefficient (P_f/P_d) using a nuclear magnetic resonance technique. If the P_f/P_d ratio is significantly higher than 1, there would be strong evidence suggesting the existence of channel-mediated water movement across the cell membrane, and human spermatozoa might be selected as a cell system for the future identification and cloning of a new water channel protein.

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