The presence of water channel proteins in ram and human sperm membranes

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Ram and human spermatozoa have a high coefficient of osmotic water permeability \( (P_f) \) with a low activation energy \( (E_a) \), suggesting the presence of water channels within their plasma membranes. Sperm membranes were examined for the presence of two known water channel proteins, CHIP28 and glucose transporters belonging to the GLUT family of proteins. The water permeability of ram spermatozoa was not inhibited by mercuric chloride to which the CHIP28 channel is sensitive. The CHIP28 protein was not located in western blots of ram sperm membrane preparations that used an anti-CHIP28 antibody. The water permeability of ram and human spermatozoa was inhibited in the presence of phloretin, an inhibitor of glucose transport. Rabbit spermatozoa, which have a low \( P_f \) and a high \( E_a \) value, suggesting a non-porous membrane, were unaffected by phloretin. These results indicate that the erythrocyte and proximal tubule water channel, CHIP28, is not present in sperm membranes but that sperm membrane glucose transporters may have a secondary water channel function.

Introduction

Some degree of permeability to water is a universal property of cell membranes, but how this permeability is mediated at the molecular level remains poorly understood. What is clear is that water crosses cell membranes by two distinct routes (Finkelstein, 1987). In all cells, water crosses the plasma membrane by simple diffusion through the lipid bilayer. Water molecules enter small, mobile free-volumes created within the bilayer by 'kinks' in the ordered structure of the lipid hydrocarbon chains, and are carried across the bilayer by the 'kinks' (Trauble, 1971). The coefficient of diffusional permeability \( (P_d) \) of cell membranes varies with tissues but is generally small \( (< 1 \mu \text{m} \text{ min}^{-1} \text{ atm}^{-1}) \). Dependence on the membrane lipid content means that the diffusional permeability is also characterized by a high Arrhenius activation energy \( (E_a > 10 \text{kcal mol}^{-1}) \), i.e. permeability increases as lipid mobility increases with temperature (Fetipole and Haydon, 1980). Although diffusional permeability is relatively slow, it appears to be sufficient for most cell types. However, cells with more specialized water functions or requirements have a much higher water permeability than can be accounted for by passive diffusion across the lipid bilayer; in these cells there are water channels within the membrane, enabling water movement by facilitated diffusion. Transport of water across a pure lipid membrane is solely by simple passive diffusion, giving a ratio between the coefficients of osmotic water permeability \( (P_f) \) and diffusional water permeability \( (P_d) \) of unity. In contrast, where water channels are present, the osmotic water permeability coefficient greatly exceeds the diffusional coefficient so that \( P_f/P_d > 1 \). However, sensitivity to temperature is low since water movement is reliant on protein channels and not the lipid portion of the bilayer, resulting in a low Arrhenius activation energy.

Although the existence of membrane water channel proteins has been postulated for some time on the basis of membrane permeability measurements, it is only recently that such a protein has been isolated and characterized (Agre et al., 1993a). The CHIP28 (28 kDa Channel-forming Integral Protein) protein has been identified as the water channel protein of the erythrocyte and renal collecting duct. CHIP28 increases water permeability when expressed in Xenopus oocytes and has been immunodetected in a wide range of secretory and absorptive tissues (Agre et al., 1993b). It is also the site of mercurial inhibition of erythrocyte water permeability (Preston et al., 1993). Two other channel proteins increase water permeability when expressed in the Xenopus oocyte system; these are cystic fibrosis transmembrane regulator (CFTR), which appears to allow water transport only when active in the presence of cAMP (Hasegawa et al., 1992), and the glucose transporter family of proteins (GLUT 1–5) (Fischbarg et al., 1989, 1990).

Work on the fundamental cryobiology of spermatozoa has entailed measurements of water permeability of the sperm plasma membrane in an attempt to model optimal cooling protocols (Curry et al., 1994). Studies of the water permeability of sperm membranes of a number of mammals show that the osmotic water permeability coefficient is very high and that the associated activation energy is low (Table 1), which suggests a porous membrane. One exception is the rabbit sperm membrane, in which osmotic permeability coefficient is low and the activation energy is high (Curry et al., 1995), which suggests a

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Table 1. Osmotic water permeability coefficients \((P_f)\) and activation energies \((E_a)\) for spermatozoa from a range of species

<table>
<thead>
<tr>
<th>Species</th>
<th>Osmotic water permeability coefficient, (P_f) ((\text{µm min}^{-1} \text{atm}^{-1}))</th>
<th>Activation energy, (E_a) ((\text{kcal mol}^{-1}))</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ram</td>
<td>8.47</td>
<td>0.01</td>
<td>(Curry et al., 1994)</td>
</tr>
<tr>
<td>Human</td>
<td>2.89</td>
<td>1.93</td>
<td>(Curry et al., 1994)</td>
</tr>
<tr>
<td>Bull</td>
<td>2.4</td>
<td>3.92</td>
<td>(Nielson et al., 1993)</td>
</tr>
<tr>
<td>Fowl</td>
<td>10.8</td>
<td>3.0</td>
<td>(Watson et al., 1992)</td>
</tr>
<tr>
<td>Rabbit</td>
<td>10.5</td>
<td>—</td>
<td>(Drevius, 1971)</td>
</tr>
<tr>
<td></td>
<td>2.1</td>
<td>4.4</td>
<td>(Watson et al., 1992)</td>
</tr>
<tr>
<td></td>
<td>1.94</td>
<td>5.36</td>
<td>(Ravie and Lake, 1982)</td>
</tr>
<tr>
<td></td>
<td>0.63</td>
<td>17.8</td>
<td>(Curry et al., 1995)</td>
</tr>
</tbody>
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nonporous membrane. In this study, the sperm plasma membrane was investigated for the presence of the CHIP28 protein and for evidence that glucose transporters may be acting as water channels.

**Materials and Methods**

**Materials**

Polyclonal affinity-purified rabbit anti-CHIP antibody, specific for the 4 kDa COOH-terminal cytoplasmic domain of human red cell CHIP (Smith and Agre, 1991; Nielsen et al., 1993) was kindly donated by P. Agre, Johns Hopkins School of Medicine, Baltimore.

**Semen collection and processing**

Ram semen was obtained by artificial vagina from Friesland rams. Only ejaculates with high wave motion and high percentage motility were used. Human semen was obtained by masturbation from healthy donors; all samples were normospermic by World Health Organization standards (WHO, 1987). Rabbit semen was obtained by artificial vagina from New Zealand White rabbits. Only ejaculates with a high percentage motility (> 70%) were used.

Ram semen was diluted 1:2 with PBS \((160 \text{ mmol NaCl} \ L^{-1}; 8 \text{ mmol Na}_2\text{HPO}_4 \ L^{-1}; 2 \text{ mmol NaH}_2\text{PO}_4; 2 \text{H}_2\text{O} \ L^{-1}; \text{pH} 7.4, 300 \text{mmol} \ L^{-1})\). Human semen was washed using a discontinuous Percoll gradient (Mortimer, 1990) and resuspended in PBS to a final concentration of approximately \(10^8 \text{ mL}^{-1}\). Rabbit semen was washed in a modified Tyrode’s medium, using a discontinuous Percoll gradient (Mortimer, 1990) and resuspended to a volume of 300 µl in modified Tyrode’s medium.

**Stock solutions and sperm labelling**

Stock solutions of carboxyfluorescein diacetate (5-CFDA; Sigma Chemical Co., Poole) in dimethyl sulfoxide (0.5 mg ml\(^{-1}\)) and propidium iodide (PI; Sigma Chemical Co.) in distilled water (0.5 mg ml\(^{-1}\)) were prepared and stored frozen in 100 µl aliquots. Spermatozoa were preloaded with CFDA by adding stock solution to a final concentration of 10 µg ml\(^{-1}\) and allowing to stand at room temperature for 15 min. Stock PI was added before flow cytometry (see below) to a final concentration of 5 µg ml\(^{-1}\).

**Assessment of cell viability**

Cell survival was assessed on the basis of plasma membrane integrity, using a flow cytometer (EPICS® Profile Analyser Coulter Electronics Ltd, Luton). Cells retaining their CFDA staining were judged to be membrane intact, while cells having lost CFDA staining and having red PI-stained nuclei were scored as membrane lysed (Watson et al., 1992). A minimum of \(10^4\) spermatozoa was scored for each sample.

**Toxicity of mercuric compounds**

CFDA-loaded ram spermatozoa were incubated at room temperature in the presence or absence of various concentrations of mercuric chloride \((\text{HgCl}_2)\) or 
chloromercuriphenylsulfonic acid \((\text{p-CMPS})\) in PBS. At fixed time intervals, PI was added and the percentage of membrane-intact cells assessed.

**Inhibition of membrane water permeability by mercuric chloride**

Water permeability was indicated by the 50% cell lysis time, the time taken for 50% of cells to lyse on exposure to hyposmotic (30 mosmol L\(^{-1}\)) conditions (Curry et al., 1994). CFDA-loaded ram spermatozoa \((10 \mu l)\) were added to 30 mosmol PBS L\(^{-1}\) \((900 \mu l)\) on a vortex mixer in the presence or absence of 10 µmol HgCl\(_2\) L\(^{-1}\) and, at a variable time interval, 100 µl 10 x PBS was added to return the sperm rapidly to isosmotic conditions. Cells that had not swollen beyond their maximum volume:surface area ratio (critical volume) at the time of return to isosmotic conditions remained viable, while cells that had already exceeded their critical volume were lysed. For time zero controls, 10 x PBS was added before adding the sperm suspension. Stock PI \((5 \text{ mg ml}^{-1})\) was added and cell membrane integrity assessed. \(P_f\) values were calculated using equation 4 of Leibo (Leibo, 1980).
\[
L_p = \frac{V_i(V_1 - V_2) + V_r^2 \ln \left( \frac{V_1}{V_2} \right)}{V_1 - V_2}
\]

as described by Curry et al. (1994).

Detection of CHIP28

Ram sperm plasma membranes were isolated by nitrogen cavitation. Briefly, 4 ml semen (approximately \(20 \times 10^9\) spermatozoa) were washed using a discontinuous Percoll gradient (35%;50%), resuspended to a volume of 20 ml and subjected to nitrogen cavitation (4639 Cell Disruption Bomb; Parr Instrument Co., Moline, IL) at 4485 kPa for 10 min. Spermatozoa and membrane fragments were collected into a Tris-sucrose solution (5 mmol Tris \(1^{-1}\), 200 mmol sucrose \(1^{-1}\), 1 mmol EGTA \(1^{-1}\), 0.2 mmol phenylmethane sulfonyl fluoride \(1^{-1}\)), pH 5.0, and centrifuged at 1000 g for 10 min, to remove sperm heads and fragments. Centrifugation was repeated three times saving the supernatants each time. Combined supernatants were centrifuged at 3000 g for 30 min to remove nuclear fragments and tails, and the supernatant again retained. The supernatant was centrifuged at 160 000 g for 2 h at 4°C to sediment the membrane fragments.

Human erythrocyte membranes were used as positive controls for the presence of CHIP28. Whole blood was collected from healthy donors into acid citrate dextrose anticoagulant (Bennett, 1983). Erythrocyte membranes were prepared by the method of Bennett (1983), involving hypotonic lysis, spectrin-actin elution, and the preparation of 1 mol KI \(1^{-1}\) extracted stripped inside-out vesicles.

All samples were dissolved in sample buffer containing 1.5% (w/v) SDS and 4% (v/v) mercaptoethanol and heated to 60°C for 10 min before electrophoresis into a 12% (w/v) SDS-polyacrylamide gel, using the buffer system of Laemmli (Laemmli, 1970). Separated proteins were transferred to nitrocellulose, and blots incubated with anti-CHIP antibody (500-fold dilution) at room temperature for 1 h, and then washed and incubated with a peroxidase-labelled anti-rabbit second antibody (1000-fold dilution. Amersham Corp., Amersham) at room temperature for 1 h. Labelled bands were visualized using an enhanced chemiluminescence kit (Amersham Corp.).

Inhibition of membrane water permeability by phloretin

Ram and human sperm lysis time experiments were performed as described, in the presence and absence of 100 µmol phloretin \(1^{-1}\) in dimethyl sulfoxide (DMSO); control samples contained an equal volume of DMSO carrier. Values for \(P_t\) were calculated as before. Lysis time experiments with rabbit spermatozoa were performed using distilled water in place of the 30 mosmol \(1^{-1}\) solution.

Results

Toxicity

Mercuric chloride was toxic to ram spermatozoa even at the lowest concentration used (1 µmol \(1^{-1}\)). Cell survival declined from 77% after 5 min to 64% after 60 min. At the highest concentration (100 µmol \(1^{-1}\)), all the cells were non-viable within 20 min (Fig. 1). The other mercuric inhibitor tested, p-CMPS, was less toxic than was HgCl\(_2\); at 10 µmol \(1^{-1}\), cell survival was unaffected after 60 min incubation while, at 100 µmol \(1^{-1}\), cell survival fell from 77% at 5 min to 59% after 60 min. At 1 mmol \(1^{-1}\) all cells were non-viable at 60 min (Fig. 2).

Inhibition by mercuric chloride

Lysis time experiments were performed in the presence and absence of 0.01 mmol HgCl\(_2\) \(1^{-1}\). Cells were incubated for 5 min in the presence of HgCl\(_2\) and lysis time experiments were performed and cell survival was assessed within a further 5 min, so that spermatozoa were exposed to the HgCl\(_2\) for less than 10 min in total. There was no difference in lysis times between control spermatozoa and those in the presence of HgCl\(_2\) (Fig. 3).

Immunolocalization of CHIP28

Western blots probed with the anti-CHIP28 antibody and detected with the enhanced chemiluminescence kit clearly showed bands corresponding to the CHIP28 protein present in the human erythrocyte membrane preparation. Glycosylated CHIP28 bands were less strongly labelled. No equivalent bands were detected in the two sperm membrane preparations (Fig. 4), each consisting of membranes pooled from two rams.

Inhibition with phloretin

Lysis time experiments were performed on ram and human spermatozoa in the presence and absence of 0.1 mmol phloretin.
inhibited cells, 50% lysis time was 10.5 s (Fig. 5). Human spermatozoa did not show the same initial rapid decline in cell survival (Curry and Watson, 1994) and, in this case, the mean survival curves differed significantly from 4 s. Lysis time was, therefore, measured as the time taken to reduce the surviving population to 50% of the initial cell population at time 0. For the control cells, 50% lysis time was 5 s while, in the phloretin-inhibited cells, 50% lysis time was 9 s (Fig. 6). Rabbit spermatozoa showed much longer lysis times than did either ram or human spermatozoa (Fig. 7), even though they were exposed to a steeper osmotic gradient, which is consistent with the lower permeability coefficient of rabbit spermatozoa noted by Curry et al. (1995). The presence of phloretin had no significant effect on the lysis times (Fig. 7).

**Discussion**

The water permeability of erythrocytes and of renal proximal tubule epithelia is reversibly inhibited by low concentrations of mercurial sulfhydryl reagents (Macey, 1984). Mercuric inhibition of CHIP28 has been shown to occur specifically via the SH group of cysteine 189 of the CHIP protein (Preston et al., 1993). However, there are problems in using mercurial compounds to reduce water permeability of spermatozoa as metallo-compounds such as p-CMPS and phenyl mercuric acid have a spermicidal effect in vitro (Mann and Lutwak-Mann, 1981). Liu et al. (1995) reported significant inhibition of human erythrocyte P_f at 10 μmol HgCl_2 l^−1 with approaching maximal inhibition at 100 μmol l^−1 demonstrating that HgCl_2 can act as an effective water channel inhibitor in the concentration range used in these experiments. However, 20 μmol p-CMPS l^−1 causes only minimal inhibition of P_f in rabbit erythrocytes and maximal inhibition at a concentration of 1 mmol l^−1 is achieved only after incubation for 60 min (Tsai et al., 1991). The results presented here show that ram spermatozoa are even more sensitive to HgCl_2 than are human spermatozoa but, because of its greater efficacy as an inhibitor and the fact that it does not require prolonged incubation times, HgCl_2 was used in preference to p-CMBS. At 10 μmol l^−1, HgCl_2 did not inhibit water permeability of ram spermatozoa. This is consistent with the finding of Liu et al. (1995) that the P_f of human spermatozoa is not inhibited by HgCl_2 at concentrations up to 100 μmol l^−1.

The highly spermicidal nature of mercurial compounds leaves a question over their usefulness as water channel inhibitors. The absence of CHIP28 was confirmed by using anti-CHIP28 to attempt to demonstrate the protein in the ram sperm plasma membrane. Although the protein was clearly present in human erythrocyte membrane preparations, it was absent from ram sperm membranes. The anti-CHIP28 antibody is specific for the cytoplasmic 4 kDa carboxyl terminus of the human erythrocyte CHIP28 but, although the carboxyl terminus is generally well conserved among mammals, it is possible that the antibody is failing to recognize a putative ram CHIP28 protein. However, Liu et al. (1995) could not locate the protein in preparations of human spermatozoa.

The results of the inhibition studies and immunoblotting strongly suggest that CHIP28 is not the ram sperm water channel protein. However, CHIP28 is one of a larger family of
MIP-like channel proteins, termed aquaporins (Agre et al., 1993a) and it is possible that a sperm water channel may be a member of this protein family. The other aquaporins identified so far, CD-WCH (Fushimi et al., 1993) and γ-TIP (Maurel et al., 1993), are both, like CHIP28, sensitive to mercuric inhibition. Mercury-insensitive mutations of CHIP28 that retain their channel function fully have been produced (Preston et al., 1993) and such a variant, if naturally occurring, could be present in the sperm membrane, undetected by the inhibition studies.

Facilitative glucose transport occurs via a family of structurally related proteins, GLUT 1–5 (Pessin and Bell, 1992). The five isoforms identified so far have different tissue distributions and affinities for glucose transport. Of the five isoforms, GLUT3 (Haber et al., 1993) and GLUT5 (Burant et al., 1992) have been identified in spermatozoa, but other isoforms may also be present.

Several lines of evidence suggest a secondary water channel function for glucose carriers. Infra-red spectroscopy of reconstituted glucose carriers suggests that a large proportion of the protein is accessible to deuterium for exchange (Jung et al., 1986; Alvarez et al., 1987), which is consistent with a pore capable of conducting water. Measurements of osmotic water flow in 1774 macrophages demonstrated that inhibitors of glucose transport, such as cytochalasin B, tometin and

Fig. 4. (a) Western blot of ram sperm plasma membrane (lanes 3 and 4) and human erythrocyte membranes (lanes 1, 2 and 5) stained with Coomassie blue to show numerous protein bands in the sperm membrane preparations. The CHIP28 protein does not stain with Coomassie blue. (b) Immunoblot probed with anti-CHIP28 antibody and visualized with enhanced chemiluminescence, showing the presence of the CHIP28 protein and of glycosylated CHIP28 (GlycCHIP28) protein in the erythrocyte membranes (lanes 1, 2 and 5) but not in the sperm membrane preparations (lanes 3 and 4).

Fig. 5. Time to lysis of ram spermatozoa exposed to 30 mosmol \(1^{-1}\) solution in the presence (●) or absence (◇) of 100 μmol phloretin \(1^{-1}\). Points represent means (±SEM) of replicates from different ejaculates from four individuals (n = 8).

Fig. 6. Time to lysis of human spermatozoa exposed to 30 mosmol \(1^{-1}\) solution in the presence (●) or absence (◇) of 100 μmol phloretin \(1^{-1}\). Points represent means (±SEM) of replicates from different individuals (n = 4).
phloretin, reduce water flow in line with glucose flux (Fischbarg et al., 1989). Injection of mRNA encoding glucose transporter into Xenopus oocytes results in increased water permeability (Fischbarg et al., 1990).

Phloretin (100 µmol l⁻¹) reduced the Pᵣ of ram spermatozoa by approximately 40% and that of human spermatozoa by approximately 55%. This does not appear to be complete inhibition of all channel-mediated permeability, which would reduce Pᵣ to a value equal to Pₑ which for lipid bilayers would be expected to be < 1 µm min⁻¹ atm⁻¹. It is possible that not all glucose carriers are inhibited and that higher concentrations of phloretin would achieve the greater than 90% inhibition seen with HgCl₂ in erythrocytes. Preliminary experiments (data not shown) suggest that higher concentrations of phloretin may further increase the 50% lysis time of ram spermatozoa but, as phloretin concentrations are increased, a much greater proportion of the spermatozoa undergo prior swelling rapid lysis, making it difficult to estimate the true 50% lysis time. Alternatively, more than one type of water channel may be contributing to the total permeability rate.

Phloretin did not inhibit rabbit spermatozoa, indicating that glucose transporters are not acting as water channels. This result is consistent with the low Pᵣ and high Eₑ values recorded for rabbit spermatozoa (Curry et al., 1995), which imply a lipid membrane without water channels. It is not clear to what extent this difference in water permeability may reflect differences in the extent or nature of glucose transport between the species.

Cells with a high water permeability, and particularly those in which water channels have been identified, have particular water regulatory functions, either osmotic, secretory or absorptive. However, spermatozoa do not have any obvious physiological need for high water permeability but do require efficient glucose uptake. It is possible that the high Pᵣ values recorded for spermatozoa are a secondary consequence of glucose metabolism.

Fig. 7. Time to lysis of rabbit spermatozoa exposed to distilled water in the presence (▲) or absence (□) of 100 µmol phloretin l⁻¹. Points represent means (± SEM) of replicates from different individuals (n = 5).

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