Effects of extracellular environment on the osmotic signal transduction involved in activation of motility of carp spermatozoa

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The mechanism by which a hypo-osmotic shock activates motility of carp spermatozoa was studied. The direct role of osmolality at the axoneme was investigated after demembration of spermatozoa with Triton X-100 and reactivation in various ionic or anionic solutions containing Mg-ATP: demembrated spermatozoa remain motile in solutions of osmolality up to 550 mOsm kg⁻¹ while non-demembrated spermatozoa are immotile when osmolality rises above 250 mOsm kg⁻¹ with the same salt solutions as well as in non-ionic solutions. Suspension in hypo-osmotic saline solutions triggered the swelling of native carp spermatozoa. No motility or swelling occurred above 200–300 mOsm kg⁻¹ and this osmolality is probably that of the cytosol. The swelling of carp spermatozoa is the result of an entrance of water but this was not affected by pCMBS, an inhibitor of the aquaporin CHIP28, or by various inhibitors of the co-transport of water with ions. Various pharmacological agents that affect the motility of different sperm species had no effect on carp sperm motility when used under similar conditions. However, prolonged exposure to a solution devoid of K⁺ or Cl⁻ affects the activation of motility in a reversible manner, suggesting that these ions have a role in the perception or transduction of the osmotic signal. Altering the concentration of intracellular second messengers such as Ca²⁺ and cAMP, and the pH did not affect the motility of carp spermatozoa. However, DMSO at 1–20% (400–3200 mOsm kg⁻¹) affects the motility of carp spermatozoa 3–4 min after mixing. These results show that the activation signal of carp sperm motility differs from that known for spermatozoa of other species of fish such as trout. Our results indicate that the activation mechanism may involve a co-transport of ions or specific 'stretch-activated channels' that are sensitive to osmotic pressure.

Introduction

Fish spermatozoa remain quiescent in the genital tract and in the seminal plasma in most species that have external fertilization. They become transiently motile at spawning when released into the surrounding water. Most of the studies on the regulation of activation of motility were carried out on trout spermatozoa. These cells become motile as a result of changes in the properties of the plasma membrane potential and its ionic conductance (Cosson *et al.*, 1989; Gatti *et al.*, 1990; Boitano and Omoto, 1991, 1992; Tanimoto *et al.*, 1994), due to a decrease in the concentration of extracellular K⁺ (Morisawa *et al.*, 1983a; Stoss, 1983; Tanimoto *et al.*, 1994). These membrane modifications induce intracellular changes in the

cascade of second messengers such as cAMP, calcium, in pH, or in protein phosphorylation (Morisawa and Ishida, 1987; Cosson *et al.*, 1989; Boitano and Omoto, 1991, 1992) which then lead to activation of axonemal movement.

Ionic effects at the plasma membrane are not the universal triggering agent of fish sperm motility. An alternative regulation mechanism involves osmotic variation in the environment. Spermatozoa of the marine puffer fish are quiescent in their seminal plasma (around 300 mOsm kg⁻¹) and become motile when there is an increase in the osmolality of the surrounding medium (1200 mOsm kg⁻¹) (Oda and Morisawa, 1993). This hyperosmotic shock could induce an increase in the intracellular K⁺ (Takai and Morisawa, 1995) and in the Ca²⁺ concentration, and an internal acidification (Oda and Morisawa, 1993). It has been proposed that these series of variations are the trigger for the activation of motility. Conversely, the motility of carp

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spermatozoa is inhibited by the high osmolality present in the seminal plasma (300 mOsm kg⁻¹; Morisawa *et al.*, 1983b; Redondo-Müller *et al.*, 1991; Perchec *et al.*, 1995); exposure to hypo-osmotic media with osmolality < 200 mOsm kg⁻¹, irrespective of the ionic composition, triggers sperm motility. This low osmolality also induces swelling in the sperm cells, leading to disruption of the plasma membrane in freshwater because the osmotic shock is too great (Billard, 1978; Morisawa *et al.*, 1983b; Perchec *et al.*, 1996). It was suggested that the osmotic shock modifies membrane permeability and organization of the lipid bilayer (Marian *et al.*, 1993). Other results suggest that an efflux of K⁺ ions through K⁺ channels is involved in the activation (Krasznai *et al.*, 1995) and the decrease in the intracellular K⁺ concentration may trigger the activation of spermatozoa of freshwater zebrafish (Takai and Morisawa, 1995).

Taken together, these observations indicate that the mechanism for activating carp spermatozoa is still poorly understood compared with the ionic mechanism described for trout spermatozoa. More information about the osmotic response is available on cellular types like erythrocytes (Lauf, 1982; Dickman and Goldstein, 1990), plant cells, bacteria (Csonka, 1989) and yeast (Hosono, 1992; Berner and Gervais, 1994). In response to hypo-osmotic stress, these cells swell as a result of the influx of water into the cytoplasm which is then followed, in contrast to sperm cells, by a biochemical readjustment that restores the initial cell volume by changing the concentration of intracellular electrolytes (such as K⁺, Na⁺, Ca²⁺, Cl⁻) or of nonelectrolyte (like amino acid, glycerol, sorbitol, inositol) solute concentration.

In the present study, the effect of osmotic shock on the initiation of motility of carp spermatozoa was tested directly at the axoneme using demembranated-reactivated sperm cells. The relationship between the changes in sperm volume and the activation of motility was also studied. In addition, the role of water and ions in the activation process and the intervention of an intracellular second messenger were investigated using some pharmacological agents.

Materials and Methods

Collection of spermatozoa

Male carp (*Cyprinus carpio*) weighing between 0.9 and 1.5 kg were obtained from INRA (Jouy en Josas), and from a private fish farm (M. de Courson, Champagne Ardennes). Fish were kept in 2 m³ tanks under natural photoperiod and temperature. Water was renewed twice a day (flow rate of 3 l min⁻¹) and the fish were fed with carp pellet food every other day.

Spermiation started 12–24 h after injecting 0.2 ml Ovaprim (Syndell Laboratories Ltd) kg⁻¹ body weight i.p. (4 µg Arg⁶ Pro⁹ NEt GnRH). Semen was collected daily for the next 3 or 4 days by applying abdominal pressure to males anaesthetized with 0.5 ml 2-phenoxyethanol l⁻¹. Milt contamination by water, urine or faeces was carefully avoided. Semen was collected into individual 5 ml tubes and kept on ice.

Analysis of motility

The movements of carp spermatozoa were recorded at 50 frames per second using a camcorder (canovision EX 1 Hi,

Canon) and a dark-field microscope (Olympus BH-L objective × 20) illuminated by a stroboscope light source (Chadwick Helmut Strobex). The focal plane was always positioned near the surface of the glass slide. Spermatozoa were visualized on a video monitor (Panasonic BT-M1420PY) at a final magnification of × 750 on the screen. Video records were analysed frame by frame. The slide (without coverslip) was coated with 1% poly-vinyl alcohol to prevent spermatozoa from sticking. Observations were made at room temperature.

The percentage of motile spermatozoa (with progressive forward motility) was calculated 30 s after activation by measurements on successive video frames.

The curvilinear velocity of spermatozoa was determined 30 s after activation in a medium that allowed sperm motility. After 30 s of motility, carp spermatozoa still have a high velocity in saline solution (Perchec *et al.*, 1995). The delay of 30 s was essential to ensure that at the time of the recording the focus was adjusted and that a large number of spermatozoa was in the field. For 1 s, the successive positions of the sperm heads of ten different spermatozoa were drawn on transparent paper placed on the video screen so that the tracking analysis of head trajectories could be analysed. Spermatozoa were randomly chosen from a homogeneous population.

Demembration and reactivation of spermatozoa

The reactivated spermatozoa were prepared after a two-step dilution procedure adapted from Cosson and Gagnon (1988). The plasma membrane was first removed by adding 10 µl of intact spermatozoa to 990 µl of a Triton demembrating solution. After 30 s, 1 µl of the permeabilized spermatozoa was placed directly under the stage of the microscope in 20 µl of the reactivating solution.

Four different demembrating and reactivating solutions were prepared, the major compounds of which were either ionic (KCl, NaCl or choline chloride) or non-ionic (glucose). The potassium demembrating solution comprised: 0.05–0.45 mol KCl l⁻¹, 20 mmol Tris-HCl l⁻¹, 0.5 mmol EDTA l⁻¹, 0.1 mmol EGTA l⁻¹, 1 mmol dithiothreitol l⁻¹ and 0.04% (w/v) Triton X-100, pH 8.2. For the other media, potassium was omitted and substituted with NaCl, choline chloride or glucose. The reactivation solution comprised 20 mmol Tris-HCl l⁻¹, 0.5 mmol EGTA l⁻¹, 1 mmol dithiothreitol l⁻¹, 2 mg BSA ml⁻¹, and 1 mmol Mg-ATP l⁻¹, pH 8.2, as well as 0.05 to 0.45 mol l⁻¹ KCl, NaCl, choline chloride or glucose. The osmolality of these media ranged from 100 to 880 mOsm kg⁻¹ according to the concentration of the major compound. In addition, a demembrating and a reactivating solution with osmolalities of 60 mOsm kg⁻¹ were prepared as described above but omitting glucose or the major salt (NaCl, KCl or choline chloride).

The influence of osmolality on intact spermatozoa was studied after the same two-step dilution procedure using the following media. The first solution had a high osmolality (300 mOsm kg⁻¹), to maintain the sperm quiescence. The ionic solution comprised 0.15 mol l⁻¹ KCl, NaCl or choline chloride, 20 mmol Tris-HCl l⁻¹, 0.5 mmol EDTA l⁻¹, 0.1 mmol EGTA l⁻¹ and 1 mmol dithiothreitol l⁻¹, pH 8.2. In media containing glucose, salts were omitted and substituted by 0.25 mol

glucose 1^{-1} . After 30 s, the second dilution was performed in the media described for demembrated spermatozoa.

Measurement of cell swelling

The change in the volume of carp spermatozoa in relation to the osmotic gradient was performed by a spermatocrit method. Semen from two or three males were pooled to increase the volume of the semen samples and was then centrifuged for 15 min at 600 *g* to reduce the seminal plasma volume by half. The concentrated semen was then diluted 1/10 in various saline (KCl, NaCl or choline chloride) or glucose solutions in which the concentration ranged from 0 to 0.45 mol l^{-1} (buffered with 30 mmol Tris-HCl l^{-1} , pH 8.0, osmolality from 40 to 800 mOsm kg^{-1}).

Spermatozoa were also diluted in a DMSO solution containing: 0–20% (v/v) dimethylsulfoxide (DMSO), 50 mmol KCl l^{-1} , 30 mmol Tris-HCl l^{-1} , and glucose to adjust the osmolality to between 400 and 3400 mOsm kg^{-1} .

The diluted semen was introduced into an haematocrit tube (diameter 1.1–1.2 mm, length 75 mm) and then centrifuged for 10 min at 1500 *g*. The percentage volume occupied by spermatozoa in each medium was calculated based on the volume of 10^{10} spermatozoa in a solution of 0.2 mol KCl l^{-1} which has routinely been used to maintain the quiescence of carp spermatozoa (Redondo-Müller *et al.*, 1991; Perchec *et al.*, 1993, 1995).

During centrifugation with the glucose solution, the plasma membrane of the spermatozoa was disrupted and so no values could be obtained.

Kinetics of head swelling in distilled water

For measuring the kinetics of head swelling, a zoom lens was fixed to the camera, allowing a final magnification of $\times 2100$ on the video monitor screen. Semen was first diluted (1/100) in a potassium saline solution (200 mmol KCl l^{-1} , 30 mmol Tris-HCl l^{-1} , pH 8.0; osmolality 390 mOsm kg^{-1}) in which they remain quiescent. Spermatozoa were activated directly under the microscope stage, and 1 μ l of the above sperm suspension was diluted with 19 μ l of cold distilled water (final osmolality 20 mOsm kg^{-1}) to delay the activation. The diameters of the heads of ten spermatozoa were measured using a calliper square, and averaged at different times after activation. Measurements were made every 3–5 s during the first 20 s of activation and then every 15 s for 2 min. After activation for 1 min, 5 μ l KCl (1 mol l^{-1}) was added, increasing the osmolality to 370 mOsm kg^{-1} . The head diameter was measured every 2 s for 10 s and then after 10 s.

Modification of the transmembrane ionic exchange

Carp spermatozoa were first diluted 1/100 in medium 1 that comprised 50 mmol l^{-1} KCl or NaCl, 30 mmol Tris-HCl l^{-1} , pH 8.0, and glucose to adjust the osmolality to 390 mOsm kg^{-1} , so that the cells were immotile. Pharmacological agents were present in this medium at the concentrations given in Tables 1–4 (see below). In case the motility was not triggered in these media, the capacity for motility was tested at various

times after mixing, in medium 2 (dilution rate 1/20) containing the same concentration of pharmacological agents in a solution composed of 50 mmol l^{-1} KCl or NaCl, 30 mmol Tris-HCl l^{-1} , pH 8.0, and glucose to adjust the osmolality to 170 mOsm kg^{-1} .

Different categories of pharmacological agents were used to target the transport of water across the plasma membrane alone or coupled with ions (Table 1), the transmembrane transport of ions (Table 2), and the role of major ions (Table 3) and of intracellular second messengers in the activation of motility (Table 4). These pharmacological agents were administered at concentrations used in previous studies.

The role of the major ions Na^{+} , K^{+} and Cl^{-} was investigated (Table 3) after a two-step dilution procedure. Carp spermatozoa were first diluted (1/100) in solutions of high osmolality (390 mOsm kg^{-1}) containing glucose (350 mmol glucose l^{-1} , 30 mmol Tris-HCl l^{-1} , pH 8.0), choline chloride (200 mmol choline chloride l^{-1} , 30 mmol Tris-HCl l^{-1} , pH 8.0) or sodium isethionate (200 mmol sodium isethionate l^{-1} , 30 mmol Hepes l^{-1} , pH 8.0). Motility was tested after a second dilution step (1/20) in a buffered solution of low osmolality (170 mOsm kg^{-1}) containing the homologous compound: glucose (140 mmol glucose l^{-1} , 30 mmol Tris-HCl l^{-1} , pH 8.0), choline chloride (50 mmol choline chloride l^{-1} , 30 mmol Tris-HCl l^{-1} , pH 8.0) or sodium isethionate (50 mmol sodium isethionate l^{-1} , 30 mmol Hepes l^{-1} , pH 8.0).

The role of NaCl and KCl in the activation mechanism was investigated using a different protocol. After incubation for 10 min in the presence of glucose or choline chloride media with high osmolality (390 mOsm kg^{-1}), NaCl or KCl (stock solution was 2 mol l^{-1}) was added separately up to a concentration of 100 mmol l^{-1} . The incubation was then prolonged for 10 min and motility was checked by a 1/20 dilution in a medium of low osmolality containing: 50 mmol KCl l^{-1} , 30 mmol Tris-HCl l^{-1} , pH 8.0, osmolality < 170 mOsm kg^{-1} , or in distilled water.

Pharmacological agents

The pharmacological agents were purchased from Sigma (Saint Quentin Fallavier) except for tetraphenylphosphonium (TPP, Merck, Nogent sur Marne) and were either diluted in DMSO:ethanol (1:1) or in distilled water. In these experiments, the control diluant which contained 0.25% (v/v) DMSO had no influence on motility.

Statistical analysis

Data are presented as means $\pm$ SEM and were treated by an analysis of variance (ANOVA) with a significance level of 5% confidence.

Results

Effect of osmolality on intact or demembrated spermatozoa

Intact spermatozoa. Carp spermatozoa were fully motile (95%) immediately after dilution in media with an osmolality

Fig. 1. (a) The percentage of motile carp spermatozoa and (b) their velocity as a function of osmolality and composition of the activating solution. Measurements were made 30 s after mixing with the activating solution containing KCl ($\square$), NaCl ($\blacksquare$), choline chloride ($\triangle$) or glucose ($\blacktriangle$). Each datum point represents the average of spermatozoa from three different males and ten spermatozoa per sample were observed (mean $\pm$ SEM).

between 60 and 140 mOsm kg⁻¹ (Fig. 1a) which corresponds to concentrations of 0–0.05 mol l⁻¹ of NaCl, KCl, choline chloride or 0–0.1 mol l⁻¹ of glucose-buffered solutions. Increasing the osmolality above 150 mOsm kg⁻¹ strongly decreased the percentage of motile spermatozoa and only a few were activated at 200–230 mOsm kg⁻¹, irrespective of the composition of the solutions (ions or glucose). When the osmolality reached 300 mOsm kg⁻¹ (in solutions containing 0.15 mol salt l⁻¹ or 0.25 mol glucose l⁻¹), all of the spermatozoa were immotile, but could be reversibly activated by decreasing the osmolality.

Spermatozoa displayed a forward motility up to 30 s after activation in all the ionic or non-ionic media tested with osmolalities between 60 and 140 mOsm kg⁻¹ (Fig. 1b). Their velocity significantly increased ($P < 0.05$) from $73 \pm 10 \mu\text{m s}^{-1}$ to $105 \pm 19 \mu\text{m s}^{-1}$ and was quite similar in all the media. The velocity progressively decreased in parallel with sperm movement when osmolality was increased to 300 mOsm kg⁻¹.

Demembrated spermatozoa. When permeabilized spermatozoa were transferred into a reactivating medium of low ionic concentration (0–0.05 mol l⁻¹) with an osmolality between 60 and 140 mOsm kg⁻¹, or into a medium of low glucose concentration (Fig. 2a), the nucleus burst, while the axonemes apparently remained intact. In media devoid of Na⁺ and K⁺ or containing only glucose, axonemal motility could be triggered only after addition of at least 25 mmol l⁻¹ KCl or NaCl (giving an osmolality of about 100 mOsm kg⁻¹). This result shows that a low concentration of ions is not deleterious to the axonemal machinery as it remains functional even at low osmolality.

In the presence of KCl, NaCl or choline chloride in the reactivating medium, 95% of demembrated spermatozoa were motile in media with an osmolality between 140 and 400 mOsm kg⁻¹ (0.05–0.20 mol salt l⁻¹) (Fig. 2a). The percentage of reactivated spermatozoa progressively decreases as the osmolality increases, and at 600–650 mOsm kg⁻¹

(0.30–0.35 mol salt l⁻¹), no axonemal movement was observed 30 s after dilution. At an osmolality of 480–550 mOsm kg⁻¹ (0.25–0.30 mol salt l⁻¹), 80–90% of spermatozoa were poorly motile 5 s after dilution; movement decreased rapidly and all were immotile 45–60 s later.

The velocity of spermatozoa measured 30 s after dilution in the saline reactivating media increased progressively in media of 60 mOsm kg⁻¹ up to a peak in media at 225 mOsm kg⁻¹ (0.1 mol salt l⁻¹) (Fig. 2b). The maximum velocity depended on the ionic composition of the reactivating medium; velocity in media containing KCl ($154 \pm 20 \mu\text{m s}^{-1}$) or NaCl ($135 \pm 33 \mu\text{m s}^{-1}$) was significantly higher ($P < 0.05$) than in choline chloride ($93 \pm 12 \mu\text{m s}^{-1}$). At higher osmolalities (600–650 mOsm kg⁻¹), the velocity decreases until complete immotility occurs.

Measurement of volume change of intact spermatozoa

Osmolality and swelling of spermatozoa. The volume of intact spermatozoa evaluated by a spermatocrit method 15 min after mixing with the different saline solutions decreased as the osmolality of the medium was increased, irrespective of the saline composition (Fig. 3). When spermatozoa are immotile, their volume remains identical to that of spermatozoa in the control buffered solution (200 mmol KCl l⁻¹). The volume of motile spermatozoa decreased with the extracellular osmolality. The volume of spermatozoa in a Tris-HCl solution at 45 mOsm kg⁻¹ showed a 3.5-fold increase without bursting. The threshold between activation and inhibition of motility (200–300 mOsm kg⁻¹) corresponded approximately to the transition between swelling and not swelling.

Kinetics of sperm head swelling. Spermatozoa were kept immotile in a buffered solution of 200 mmol KCl l⁻¹ and the head diameter was measured on the video screen. When motility was triggered with distilled water (final osmolality

Fig. 2. Variation in (a) the percentage of motile carp demembrated spermatozoa and (b) their velocity as a function of osmolality and composition of the reactivating solution. Measurements were made 30 s after dilution in media containing KCl (□), NaCl (■), choline chloride (△) or glucose (▲). Values are means $\pm$ SEM for three experiments performed and ten demembrated spermatozoa per sample were observed.

Fig. 3. Percentage change in volume of carp spermatozoa performed by a spermatocrit method. Spermatozoa were suspended in media of different osmolalities containing KCl (□), NaCl (■) or choline chloride (△). The straight line represents the barrier between activation of motility or quiescence. Each datum point represent the average of two samples, each of which was obtained by pooling two different milt. At least two measurements were made per sample. Values are means $\pm$ SD.

Fig. 4. The diameters of the heads of carp spermatozoa as a function of time after activation with cold distilled water (□). After 60 s, motility was stopped by adding 5 μl KCl (1 mol l⁻¹) which increased the osmolality to 370 mOsm kg⁻¹, and the shrinking of the head was measured (■). Each datum point corresponds to the mean of measurements taken from spermatozoa of three different males, and an average of ten spermatozoa per sample was measured. Values are means $\pm$ SEM.

20 mOsm kg⁻¹), the head progressively increased in diameter during 60 s and then reached a plateau (Fig. 4). Increasing the osmolality of the medium, by adding KCl to a final osmolality of 370 mOsm kg⁻¹ stopped all sperm movement and triggered a shrinking of the sperm head, which recovered its initial volume within the next 3–4 s. This change can be reversed and motility of carp spermatozoa can be re-induced by decreasing the osmolality of the medium.

Transmembrane exchanges occurring at the activation step

The transmembrane ionic or osmotic equilibrium that maintains the immotility of carp spermatozoa was manipulated to analyse the effect on sperm motility in media with high osmolality (> 300 mOsm kg⁻¹; medium 1) or low osmolality solution (< 170 mOsm kg⁻¹; medium 2).

We first investigated the implication of water transport alone or water co-transported with ions or glucose across the plasma membrane in the activation process (Table 1). A specific inhibitor of the water channel CHIP28 (28 kDa channel-forming integral protein), *para*-chloromercuribenzenesulfonate (pCMBS), which has been tested on the bovine red blood cell (Lahajnar *et al.*, 1995), neither activates carp spermatozoa in a medium of high osmolality nor interferes with the subsequent activation in a medium of low osmolality. The use of the mammalian antidiuretic hormone arginine vasopressin (AVP), which increases water permeability in the renal collecting duct (Nielsen *et al.*, 1993), does not trigger the motility of carp spermatozoa incubated in a solution of 390 mOsm kg⁻¹; nevertheless these spermatozoa can be further activated in a medium of 170 mOsm kg⁻¹. The pharmacological agents bumetanide and furosemide that inhibit the co-transport of water and KCl across the epithelium (for review see Zeuthen and Stein, 1994) had no effect on carp spermatozoa either in a high or in a low osmolality medium. The inhibitors of anion–water co-transport, 4,4'-diisothiocyanato-stilbene-2,2'-disulfonic acid (DIDS), which was tested in human erythrocytes (Blank *et al.*, 1994) and 4-acetamido-4-isothiocyanato-stilbene-2,2'-disulfonic acid (SITS) in human neutrophils (Korchak *et al.*, 1980) have no effect on carp sperm activation.

For investigating the possible role of the glucose transporter (GLUT) as a water channel, which has been demonstrated in *Xenopus* oocytes and in mammalian spermatozoa (Fischbarg *et al.*, 1990; Curry *et al.*, 1995), cytochalasin B and phloretin were used: they have no effect on carp spermatozoa in conditions of high and low osmolality.

The effect of modifying the ionic transmembrane equilibrium on motility of carp spermatozoa are shown (Table 2). The various ionophores that were used are known to increase selectively the permeability to K⁺ (valinomycin) or to catalyse the Ca²⁺–H⁺ (A23187) and the Na⁺–H⁺ exchange (monensin). These compounds did not stimulate the activation of carp spermatozoa under conditions of high osmolality or inhibit the activation in medium 2. Reagents that affect K⁺ transport through channels, such as 4-aminopyridine, glybenclamide (Han *et al.*, 1993) and MgCl₂ (Lauf *et al.*, 1992) did not interfere with the motility of spermatozoa in medium 1 and subsequent activation in medium 2. However, after incubation for 30 min in medium 1 with 4-aminopyridine, the period of motility in medium 2 containing the drug strongly decreased to less than 30 s compared with 10 min in the control. Ouabain, an inhibitor of the Na/K-ATPase pump, did not interfere with motility. Two extracellular calcium chelators EGTA and 1,2-bis(2-aminophenoxy)ethane-*N,N,N'*-*N'*-tetraacetic acid (BAPTA) did not activate the motility of spermatozoa in medium 1 or the subsequent initiation of motility in medium 2 containing these agents. Similar results were obtained with gadolinium, which inhibits the stretch-activated channels (Yang and Sachs, 1989). Moreover, modifying the plasma membrane potential by incubating the spermatozoa with TPP (Gatti *et al.*, 1990) did not trigger the motility or inhibit the sperm activation under conditions of low osmolality.

Incubation for 10 min in media lacking Na⁺ or K⁺ (i.e. glucose or choline chloride solutions with osmolalities of 390 mOsm kg⁻¹) strongly decreases the percentage of motile

spermatozoa when tested by transfer to a medium of low osmolality (170 mOsm kg⁻¹) from 85% to 0–20% (Fig. 5 and Table 3). The same result was obtained using sodium isethionate. However, when such spermatozoa were transferred in distilled water (which has lower osmolality), the activation was triggered in 80–90% of the sperm population. This effect can be counteracted by an additional incubation for 10 min in glucose or choline chloride media supplemented with 100 mmol l⁻¹ KCl or NaCl: under these conditions 20–30% of spermatozoa were activated when tested in a medium with an osmolality of about 170 mOsm kg⁻¹.

The effects of various agents that have been shown to have intracellular targets are shown (Table 4). Various concentrations of NH₄Cl were used in an attempt to increase the intracellular pH (Gatti and Christen, 1985; Boitano and Omoto, 1991); similarly, lactate was added, as it has been shown to decrease the intracellular pH of bovine spermatozoa (Babcock *et al.*, 1983): neither NH₄Cl nor lactate trigger or affect carp spermatozoa motility. Two other compounds, KHCO₃ and isobutyl methyl xanthine (IBMX) that raise the intracellular cAMP concentration by increasing the adenylate cyclase activity and inhibiting the phosphodiesterase, respectively (Schoff and First, 1995) had no effect on motility of carp spermatozoa.

However, DMSO (from 1 to 20% v/v) triggered motility after incubation for 3–4 min in media with an osmolality of 400–3200 mOsm kg⁻¹. In contrast, 0–1% DMSO did not trigger sperm motility under the same conditions. Motile spermatozoa in DMSO solutions swell and progressively showed a curling in the distal portion of the flagellum. The increase in volume of the motile spermatozoa remains the same (200%) irrespective of the DMSO concentration in the range 1–20% (Fig. 6).

Discussion

Intact carp spermatozoa are maintained immotile when the extracellular environment has an osmolality higher than 250–300 mOsm kg⁻¹: this corresponds to solutions containing 0.15 mol salt l⁻¹ or 0.25 mol glucose l⁻¹ and to the osmolality of the seminal plasma (286 mOsm kg⁻¹; Plouidy and Billard, 1982; 300 mOsm kg⁻¹; Morisawa *et al.*, 1983b). Whatever the external medium composition, motility is triggered in more than 95% of spermatozoa when the osmolality is less than 200 mOsm kg⁻¹. We observed that the value of the sperm velocity is quite similar in all the solutions, irrespective of the presence of ions. These results contrast with a higher score of both motility and velocity of intact spermatozoa in a KCl medium reported by Morisawa *et al.* (1983b).

Carp spermatozoa can be demembrated by Triton X-100, which allows the axonemal machinery to come directly into contact with the external environment (Cosson and Gagnon, 1988). The highest percentage of motile spermatozoa and the highest velocity occurred when the reactivating solution contained 0.05 to 0.20 mol salt l⁻¹ (140 to 400 mOsm kg⁻¹). With respect to these two parameters, the better reactivation is observed at 0.10 mol l⁻¹ (about 220 mOsm kg⁻¹). Intact spermatozoa are optimally activated at osmolalities of 60 to 140 mOsm kg⁻¹. The present results contrast with the recent

Table 1. Impact of pharmacological agents affecting water transport alone or the co-transport of water with ions on the activation of motility of carp spermatozoa

Compound added to media 1 and 2	Principal target	Conditions of immotility			Conditions of motility	
		Medium 1		Activation	Medium 2	
		(390 mOsm kg ⁻¹)	Time (min)		(170 mOsm kg ⁻¹)	Activation
pCMBS (2 mmol l ⁻¹)	Inhibitor of water channels	KCl (50 mmol l ⁻¹)	90	No	KCl (50 mmol l ⁻¹)	Yes
AVP or AVT (10 ⁻² , 10 ⁻⁴ mmol l ⁻¹)	Increase water permeability	KCl (50 mmol l ⁻¹)	150	No	KCl (50 mmol l ⁻¹)	Yes
DIDS (10 ⁻⁴ –1 mmol l ⁻¹)	Anion transport inhibitor and water transport activator	NaCl (50 mmol l ⁻¹)	60	No	NaCl (50 mmol l ⁻¹)	Yes
SITS (10 ⁻⁴ –1 mmol l ⁻¹)	Anion and water transport inhibitor	NaCl (50 mmol l ⁻¹)	60	No	NaCl (50 mmol l ⁻¹)	Yes
Furosemide 10 ⁻⁴ , 10 ⁻⁵ mmol l ⁻¹)	KCl and water transport inhibitor	NaCl or KCl (50 mmol l ⁻¹)	60	No	NaCl or KCl (50 mmol l ⁻¹)	Yes
Bumetanide 10 ⁻⁴ , 10 ⁻⁵ mmol l ⁻¹)	KCl and water transport inhibitor	NaCl or KCl (50 mmol l ⁻¹)	60	No	NaCl or KCl (50 mmol l ⁻¹)	Yes
Cytochalasin B (10, 25, 50 mmol l ⁻¹)	Glucose as water channel inhibitor	NaCl (50 mmol l ⁻¹)	60	No	NaCl (50 mmol l ⁻¹)	Yes
Phloretin (0.1 or 1 mmol l ⁻¹)	Glucose as water channel inhibitor	KCl (50 mmol l ⁻¹)	Up to 150	No	KCl (50 mmol l ⁻¹)	Yes

AVP, arginine vasopressin; AVT, arginine vasotocin; DIDS, 4,4'-diisothiocyanato-stilbene-2,2'-disulfonic acid; pCMBS, *para*-chloromercuribenzenesulfonate; SITS, 4-acetamido-4-isothiocyanato-stilbene-2,2'-disulfonic acid.

The time reported represents the length of time spermatozoa were incubated in medium 1.

In case the motility was not triggered in medium 1, the capacity for motility in medium 2 was tested.

Table 2. Impact of pharmacological agents affecting the ionic transmembrane equilibrium on the activation of motility of carp spermatozoa

Compound added to media 1 and 2	Conditions of immotility			Conditions of motility		
	Principal target	Medium 1 (390 mOsm kg ⁻¹)	Time (min)	Activation	Medium 2 (170 mOsm kg ⁻¹)	Activation
Valinomycin (ionophore) (25 µmol l ⁻¹) 4-Aminopyridine (5 mmol l ⁻¹)	K ⁺ and Cs ⁺ transport Voltage-gated K ⁺ channel	KCl or CsCl (0–200 mmol l ⁻¹) KCl or NaCl (50 mmol l ⁻¹) or glucose (350 mmol l ⁻¹)	15 s 30 min	No No	KCl or CsCl (0–50 mmol l ⁻¹) Buffered distilled water	Yes Yes
MgCl ₂ (5, 10, 20 mmol l ⁻¹) Glybenclamide (50–500 mmol l ⁻¹) Monsensin (25 µmol l ⁻¹) Ouabain (10 ⁻⁴ –1 mmol l ⁻¹)	K ⁺ –Cl ⁻ co-transport K _{ATP} channel inhibitor Na ⁺ –H ⁺ exchange Na ⁺ /K ⁺ -ATPase inhibition	KCl (50 mmol l ⁻¹) NaCl (50 mmol l ⁻¹) NaCl (0–1 mmol l ⁻¹) KCl or NaCl (50 mmol l ⁻²) or glucose (350 mmol l ⁻¹)	Until 180 min 60 min 15 s Until 180 min	No No No No	KCl (50 mmol l ⁻¹) NaCl (50 mmol l ⁻¹) NaCl (0–50 mmol l ⁻¹) KCl or NaCl (50 mmol l ⁻¹) or glucose (100 mmol l ⁻¹) + Ca ²⁺	Yes Yes Yes Yes
A23187 (10 µmol l ⁻¹)	Ca ²⁺ –H ⁺ exchange	KCl (50 mmol l ⁻¹) + Ca ²⁺ (0–20 mmol l ⁻¹)	15 s	No	KCl (50 mmol l ⁻¹)	Yes
BAPTA or EGTA (5, 10, 20 mmol l ⁻¹) Gadolinium (0.1 to 1 mmol l ⁻¹)	Ca ²⁺ chelator Inhibition of stretch-activated channels	KCl (50 mmol l ⁻¹) KCl (50 mmol l ⁻¹)	Until 330 min 15 min	No No	KCl (50 mmol l ⁻¹) KCl (50 mmol l ⁻¹)	Yes Yes
TPP (1.5 mmol l ⁻¹)	Membrane potential depolarization	KCl (50 mmol l ⁻¹)	Until 90 min	No	KCl (50 mmol l ⁻¹)	Yes

BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N'-N''-tetraacetic acid; EGTA, ethylene-glycol-bis(β-amino-ethyl ether)-N,N-tetraacetic acid; TPP, tetraphenyl phosphonium. The time reported represents the length of time spermatozoa were incubated in medium 1. In case the motility was not triggered in medium 1, the capacity for motility in medium 2 was tested.

Fig. 5. Variation in the percentage of motile carp spermatozoa as a function of time after suspension in glucose (▲) or choline chloride (△) medium with high osmolality (390 mOsm kg^{-1}). Spermatozoa were activated in media containing the same compounds but with low osmolality (170 mOsm kg^{-1}). Values are means $\pm$ SEM; $n = 3$ males).

observation of Morisawa (1994) that demembrated axonemes in the spermatozoa of the freshwater zebrafish are resistant to reactivation at low osmolality ($0.05 \text{ mol KCl l}^{-1}$) and are fully reactivated at high osmolality ($0.15 \text{ mol KCl l}^{-1}$); this tends to show that direct control of axonemal movement by osmolality is not a general rule among different fish species. These results on freshwater fish are slightly different from those reported for sea urchin spermatozoa (Gibbons and Gibbons, 1972), which are reactivated most readily in solutions containing 0.15 to $0.25 \text{ mol salt l}^{-1}$.

It appears that the ionic composition of the medium is more important for motility in reactivated than in intact spermatozoa. While intact spermatozoa showed similar motility whatever the ionic composition of the external medium of low osmolality, the velocity of demembrated spermatozoa depends on the type of cation included in the medium. At the axoneme, K^+ and Na^+ have a more crucial role than choline, perhaps due to their small size. Moreover, in contrast to intact spermatozoa, the very low ionic strength of a glucose medium maintains the immotility of demembrated spermatozoa. Ionic interactions are needed to maintain the cohesion between the axonemal compounds and the dynein ATPase responsible for the flagellar beating. These results confirm those already reported by Gibbons and Gibbons (1972) for sea urchin spermatozoa. On the other hand, increasing the salt concentration slightly increases the viscosity of the medium, which may in turn significantly decrease the velocity of reactivated spermatozoa. Moreover, a medium with a higher ionic strength decreases the frequency of axonemal movement due to a partial extraction of the outer dynein arm (Gibbons and Gibbons, 1973). The immotile axoneme in solutions of low or high ionic strength can be rendered motile by the addition or dilution of ions, respectively (data not shown). These results suggest that variations in the extracellular osmolality is not directly detected by the axoneme because the plasma membrane acts as

a barrier. However, the mechanisms by which the external osmotic signal is transduced into a biochemical intracellular signal that in turn activates the dynein ATPases remains to be elucidated.

In response to an external osmotic signal, carp spermatozoa change their internal volume (this also occurs in erythrocytes (Dickman and Golstein, 1990), yeast (Berner and Gervais, 1994) and mammalian spermatozoa (Crichton *et al.*, 1994)). The final volume reached by carp spermatozoa depends on the extracellular osmolality and is independent of the major ion present in the surrounding solution. It appears that in carp, sperm swelling parallels the activation of motility: the threshold of osmolality between motility or swelling and the activation or inhibition of motility is $200\text{--}300 \text{ mOsm kg}^{-1}$ and probably reflects an equilibrium; it suggests that intracellular osmolality of carp spermatozoa is $200\text{--}300 \text{ mOsm kg}^{-1}$. It is worth noting that this value does not inhibit the flagellar beating after demembration, suggesting that the intracellular osmolality is higher than the extracellular osmolality.

The hypo-osmotic stress induces a gradient of concentration between the intracellular and extracellular medium and the sperm cell attempts to balance the osmolality on both sides of the plasma membrane by an influx of water. The subsequent increase in carp sperm volume (at about 60 s) is slower than that measured for human spermatozoa which occurs after 30 s (Noiles *et al.*, 1993), or of erythrocytes which occurs after 3–4 s; (Liu *et al.*, 1995) in hypo-osmotic medium. A water channel protein (aquaporin) named CHIP28 has been isolated and characterized in erythrocyte membranes (reviewed by Agre *et al.*, 1993). This water channel does not seem to occur in carp spermatozoa because pCMBS, a specific inhibitor of CHIP28 (Lahajnar *et al.*, 1995), did not interfere with their motility. Moreover, phloretin and cytochalasin B, two potent inhibitors of the glucose transporter (GLUT), which acts as a water channel in *Xenopus* oocytes (Fischbarg *et al.*, 1990), and perhaps in mammalian spermatozoa (Curry *et al.*, 1995), does not interfere with the activation process of carp spermatozoa. The use of arginine vasopressin (AVP) or arginine vasotocin (AVT), which increases the water permeability of inner medullary collecting ducts (Nielsen *et al.*, 1993), does not trigger the activation of carp spermatozoa. However, the action of AVP or AVT needs a specific plasma membrane receptor, which may not occur in carp spermatozoa. Inhibition of water and ion co-transport in epithelium by furosemide or bumetanide (Zeuthen and Stein, 1994) has no effect on carp sperm motility. Similarly, the inhibition of co-transport of water and anions using DIDS (Tanimoto and Morisawa, 1988; Blank *et al.*, 1994) or SITS (Korchak *et al.*, 1980) does not trigger or inhibit the activation of motility of carp spermatozoa. Thus, the passive diffusion of water across the plasma membrane alone could explain the slow entrance of water, as is the case for human spermatozoa (Curry *et al.*, 1995; Liu *et al.*, 1995). Moreover, in contrast to erythrocytes (Lauf, 1982) or to bacteria (Csonka, 1989) both of which can readjust their internal volume by changing the intracellular electrolyte or non-electrolyte solute concentration, the swelling of carp spermatozoa is not regulated and sperm cells shrink only if the external osmolality increases. These results suggest that a water channel or water and ion co-transport do not have a major role in the activation process. Moreover, because the swelling of carp spermatozoa is

Table 3. Media affecting the activation of motility of carp spermatozoa

Principal compound of media 1	Principal characteristic	Conditions of immotility			Conditions of motility	
		Media 1 (390 mOsm kg ⁻¹)	Time (min)	Activation	Media 2 (170 mOsm kg ⁻¹)	Activation
Glucose (350 mmol l ⁻¹)	Non-ionic medium	Tris-HCl (30 mmol l ⁻¹)	10	No	KCl (50 mmol l ⁻¹)	No
Choline chloride (200 mmol l ⁻¹)	Medium without major cation	Tris-HCl (30 mmol l ⁻¹)	10	No	Tris-HCl (30 mmol l ⁻¹)	Yes
Sodium isethionate (200 mmol l ⁻¹)	Medium without chloride	Hepes (30 mmol l ⁻¹)	20	No	KCl (50 mmol l ⁻¹)	No
					Tris-HCl (30 mmol l ⁻¹)	Yes
					Sodium isethionate (50 mmol l ⁻¹)	No
DMSO 1–20%	Diluant/membrane interaction/intracellular water	KCl (50 mmol l ⁻¹)	3	Yes	Hepes (30 mmol l ⁻¹)	Yes

DMSO, dimethylsulfoxide.

The time reported represents the length of time spermatozoa were incubated in medium 1.

In case the motility was not triggered in medium 1, the capacity for motility in medium 2 was tested.

Table 4. Effect of pharmacological agents, which are known to have intracellular targets, on the motility of carp spermatozoa

Compound added to media 1 and 2	Principal target	Conditions of immotility			Conditions of motility	
		Medium 1 (390 mOsm kg ⁻¹)	Time (min)	Activation	Medium 2 (170 mOsm kg ⁻¹)	Activation
NH ₄ Cl (5, 10, 20 mmol l ⁻¹)	Increase pH _i	KCl (50 mmol l ⁻¹)	Until 180	No	KCl (50 mmol l ⁻¹)	Yes
Lactate (0.06–0.6 mmol l ⁻¹)	Decrease pH _i	KCl (50 mmol l ⁻¹)	60	No	KCl (50 mmol l ⁻¹)	Yes
IBMX (1, 2 mmol l ⁻¹)	Phosphodiesterase inhibitor	KCl (50 mmol l ⁻¹)	90	No	KCl (50 mmol l ⁻¹)	Yes
KHCO ₃ (5, 10, 20 mmol l ⁻¹)	Adenylate cyclase activation	KCl (50 mmol l ⁻¹)	Until 180	No	KCl (50 mmol l ⁻¹)	Yes

IBMX, isobutyl methyl xanthine, pH_i, intracellular pH.

The time reported represents the length of time spermatozoa were incubated in medium 1.

In case the motility was not triggered in medium 1, the capacity for motility in medium 2 was tested.

a progressive phenomenon that occurs much later than initiation of movement, the entrance of water is neither the first nor the major event triggering motility.

The time taken for spermatozoa to shrink in a medium of high osmolality is rapid (3–4 s) compared with the time required for the swelling in distilled water: this suggests a difference in membrane permeability on both sides of the membrane. The change in size of the flagellum and midpiece in media of various osmolality could not be measured but it is assumed that it undergoes the same decrease or increase as the size of the head. A decrease in the sperm volume probably limits the intracellular space located between the axonemal machinery and the plasma membrane. This change could be involved in the inactivation process by inducing some mechanical constraints and/or by limiting the exchange between shuttle molecules (like phosphocreatine in trout spermatozoa; Robitaille *et al.*, 1987) and local energy.

When the ionic permeability was modified using ionophores or when the ionic environment of the plasma membrane was modified, no marked effect on carp sperm motility was

observed. Among the ionophores previously shown to affect the initiation of motility in sea urchin spermatozoa through the Na⁺–H⁺ exchange (monensin; Shackmann and Chock, 1986) or that of trout spermatozoa through K⁺ transport (valinomycin; Boitano and Omoto, 1991), neither have any impact on carp sperm motility. It is worth noting that sea urchin and trout spermatozoa are both activated through an ionic exchange across the plasma membrane (for review, see Gatti and Dacheux, 1995). By manipulating the intracellular Ca²⁺ concentration with the ionophore A23187, spermatozoa of the marine puffer fish can be activated (Oda and Morisawa, 1993). Similar treatment does not activate carp sperm motility when the extracellular concentration is decreased by the addition of the calcium chelator BAPTA or EGTA. Manipulating the transport of K⁺ through channels with glybenclamide (Wangemann *et al.*, 1992; Han *et al.*, 1993) or 4-aminopyridine (Krasznai *et al.*, 1995) does not trigger the motility of carp spermatozoa in media of high osmolality. However, as 4-aminopyridine decreases the intracellular ATP content (data not shown), it decreases the duration of motility which suggests a direct

Fig. 6. Percentage change in the volume of carp spermatozoa determined by a spermatocrit method. Spermatozoa were suspended in media containing various concentrations of dimethylsulfoxide (DMSO). The medium containing a very low percentage of DMSO contained glucose to adjust the osmolality to 400 mOsm kg^{-1} . The straight line represents the barrier between activation of motility or quiescence. Each datum point represents the average of two samples, each of which was obtained by pooling two different milts. At least two measurements were made per sample. Values are means $\pm$ SD.

action at the mitochondria. Inhibition of the co-transport of Na^+ interferes with carp sperm motility as is the case for sea urchin spermatozoa (Nelson, 1982; Gatti and Christen, 1985). Manipulating the membrane potential with TPP has no effect on carp sperm motility. However, ions are necessary for the signal transduction since a prolonged exposure to a medium devoid of major ions (i.e. glucose, choline chloride or sodium isethionate) decreases the osmotic threshold of activation: after such treatment spermatozoa became motile only in buffered distilled water. Nevertheless, the addition of NaCl or KCl (100 mmol l^{-1}) for an additional period of 10 min restores the motility in media 2 for 20–30% of spermatozoa, suggesting that these ions are involved in the transduction of the osmotic signal. Moreover, incubation of carp spermatozoa with a buffered medium containing $200 \text{ mmol KCl l}^{-1}$ (osmolality: 390 mOsm kg^{-1}) for a few hours progressively leads to the activation of spermatozoa in medium with increasing osmolality (up to 250 mOsm kg^{-1}) by modulating the threshold of perception of the osmotic signal (Redondo-Müller, 1990; G. Perchec Poupard, personal observations). A simple K^+ transport across the plasma membrane is not the factor responsible for the activation process as shown by the results obtained with valinomycin; however, K^+ and Cl^- (see results with sodium isethionate) seem to be involved in the perception of the osmotic signal by the membrane.

Although altering the cellular second messengers (via pH or cAMP) in sea urchin, trout, puffer fish and mammalian spermatozoa interferes with motility, it has no impact on carp sperm motility. Activation of carp spermatozoa appears to be independent of the intracellular pH since the addition of NH_4Cl does not trigger sperm motility as occurs in sea urchin

spermatozoa (Christen *et al.*, 1982). Moreover, carp sperm motility can be initiated in a similar way in media of pH 6.0–9.0 (Redondo-Müller *et al.*, 1991). Similarly, it has been shown that changing the external pH value induces a change in the intracellular pH of boar, ram and trout spermatozoa (Gatti *et al.*, 1990, 1993). The results reported here are in agreement with those of Krasznai *et al.* (1995), who observed that the intracellular alkalinization (by 0.15 pH units) that accompanied the initiation of motility of carp spermatozoa does not play a regulatory role in triggering axonemal movement. Similar results have been shown for the regulation of motility of trout spermatozoa (Gatti *et al.*, 1990; Boitano and Omoto, 1991). The addition of IBMX, a phosphodiesterase inhibitor, does not stimulate carp sperm motility, in contrast to its effect on bovine spermatozoa (Schoff and First, 1995). As for intracellular pH, cAMP does not seem to be a second messenger able to trigger carp sperm motility, in contrast to its role in trout spermatozoa (Morisawa and Okuno, 1982). Other experiments support this finding since the reactivation of demembrated carp spermatozoa using Mg-ATP does not require the addition of cAMP (Cosson and Gagnon, 1988). Moreover, it has been shown that intracellular concentrations of cAMP and Ca^{2+} increase in intact trout spermatozoa after activation; both compounds are necessary in the demembration–reactivation experiment with trout spermatozoa which is not the case for carp spermatozoa (Morisawa and Ishida, 1987; Cosson *et al.*, 1989; Boitano and Omoto, 1991).

Surprisingly, DMSO can trigger carp sperm motility in some conditions that are not permissive for motility in its absence. Nevertheless, it has previously been used extensively as a cryopreservative: the use of DMSO to cryopreserve carp spermatozoa could explain why they have a poor capacity for motility initiation after such treatment (Cognie *et al.*, 1989). DMSO relieves the inhibition of movement due to the high osmolality ($300\text{--}400 \text{ mOsm kg}^{-1}$) of medium 1. In the same way, DMSO has been shown to enhance the motility of sea urchin spermatozoa (Steinbach, 1966). Because carp spermatozoa swell following the addition of DMSO, it has been suggested that an influx of water occurs. However, the extent of this swelling is limited and a threshold is reached and sustained over a large range of concentration of DMSO. In contrast to the results presented here, DMSO induces a reduction of the intracellular water content of fowl spermatozoa, and a cell shrinking is observed using the same range of concentration as that used in the present study (Terada *et al.*, 1988). However, the mechanism by which DMSO triggers carp sperm motility is quite difficult to explain because the impact of DMSO on a cell is not fully understood. DMSO has a high membrane permeability and it is generally accepted that its entry into the cell is fast. The delayed response observed in this study for the activation of motility by DMSO (3–4 min after mixing) suggests that changes other than the influx of DMSO into spermatozoa are involved. Various effects of DMSO have been reported. In cells in general, this cryoprotectant has water-binding properties and prevents ice crystal formation. DMSO also acts on mitogenic signal transduction during the early developmental stages of mouse embryos (Ganapathy and Shanmugam, 1995). Moreover, DMSO can interact with phospholipid membranes as well as with isolated proteins (Anchordoguy *et al.*, 1991). It is possible that DMSO alters the

lipid bilayer structure of the sperm membrane; in this respect Marian *et al.* (1993) propose that a reorganization of the lipid bilayer structure of the plasma membrane of the carp spermatozoa is essential in transmembrane signalling.

In conclusion, our experiments show that the osmotic signal transduction that activates carp spermatozoa is totally different from the mechanisms previously described for the spermatozoa of other species such as sea urchin or trout. The extracellular osmotic signal could act through a change in intracellular osmolality as the factor triggering motility. Nevertheless, the implication of an intracellular effector(s) dilution seems to be ruled out since the entrance of water is a progressive phenomenon that occurs some time after motility is triggered. For the same reason, the influx of water alone does not trigger motility but probably follows a previous ionic transport across the plasma membrane. All the ions were studied in turn; a co-transport or a coupling between different ions has not been excluded. The measurement of the ionic flux should give more information. As carp spermatozoa are sensitive to variation in osmotic pressure, stretch-activated (Sachs and Sokabe, 1990) or mechanically activated channels (Opsahl and Webb, 1994), which are highly sensitive to membrane tension or pressure and which modify the activity of certain membrane proteins (Vandorpe *et al.*, 1994), could be involved in the activation. Gadolinium, which has no effect on motility and which inhibits stretch-activated channels, is not totally specific for these channels (Sachs and Sokabe, 1990), and the use of patch clamps on carp spermatozoa, as described for sea urchin spermatozoa (Darzon *et al.*, 1987) should give more information. Finally, studies on the way in which DMSO can bypass the extracellular osmotic barrier and trigger the motility of carp spermatozoa will give more insight into this mechanism.

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