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Initiation of motility by steelhead (*Oncorhynchus mykiss*) sperm: Membrane ion exchangers and pH sensitivity

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ABSTRACT

Initiation of motility in salmonid sperm is sensitive to the pH of the extracellular medium, however, the basis of this sensitivity is not clear. Sperm incubated in an immobilization buffer (SI) at low pH (~7.1–7.2) become motile when diluted with activating medium (AM) at high (~8.5) but not low pH. Based on this observation, various agents were tested to determine whether the onset of steelhead sperm motility upon activation with high pH AM, following incubation with low pH SI, could be blocked by inhibiting membrane exchangers postulated to be important in intracellular pH (pHi) regulation. Amiloride (inhibitor of proton:sodium exchange), SITS and DIDS (inhibitors of anion exchange) and bafilomycin A 1 (inhibitor of H^+-ATPase activity) were not effective in this experimental design. However, regardless of SI pH, DIDS was effective in blocking motility as was replacing chloride with thiocyanate or including the chloride channel blocker, niflumic acid, in SI suggesting that chloride efflux plays a key role in motility initiation. Nonetheless, the results of this study suggest that the rapid onset of sperm motility with activation at high pH following incubation at low pH is probably not based on rapid adjustment of pHi via membrane exchangers/transporters but rather due to an effect of pH on motility-associated processes at the extracellular surface of the sperm.

1. Introduction

Morisawa and Morisawa (1986, 1988) demonstrated that salmonid sperm taken directly from the testis generally lack the potential to become motile upon dilution into freshwater, while most sperm taken from the sperm duct do become motile upon dilution. The basis for this difference in capacity for motility appears to be a hormonally controlled, secretion of bicarbonate into the sperm duct, which results in an increase in semen pH (Morisawa and Morisawa, 1988; Miura et al., 1992). The pH values of the semen within the testis and sperm duct of the rainbow trout (*Oncorhynchus mykiss*) are 7.3 and 7.8, respectively and 7.5 and 8.2, respectively, for the chum salmon (*Oncorhynchus keta*) (Morisawa and Morisawa, 1988). A similar phenomenon can be demonstrated in vitro. Salmonid sperm diluted into freshwater demonstrate low and high fractions of motile sperm after sperm are maintained in immobilization solutions of pH ≤ 7.4 and ≥ 8.0, respectively (Bencic et al., 2000, 2001; Ingermann et al., 2002).

Intracellular pH (pHi) of steelhead sperm is less than that of the external medium and it changes approximately in parallel with changes in extracellular pH (pHe) (Woolsey and Ingermann, 2003). This latter finding indicates that an elevated pHi, associated with elevated pHe, is permissive of motility while low pHi, associated with low pHe, is not.

The mechanism by which low pHi prevents onset of sperm motility is not clear but is likely to be due to multiple mechanisms which probably include the pH sensitivities of sperm cAMP levels (Miura et al., 1992), membrane potential (Gatti et al., 1990), and dynein ATPase activity (Woolsey and Ingermann, 2003). Direct inhibitory actions of low pH via extracellular effects also remain possibilities.

We have recently noted that few steelhead sperm incubated in an immobilization solution at pH 7.1 initiate motility when activated with a buffered saline at pH 7.5 but most initiate motility upon dilution in that saline titrated to pH 8.5 (Woolsey et al., 2006). Since this motility occurred essentially immediately upon dilution, this observation suggests that there is a rapid intracellular alkalinization at activation with a high (pH ~ 8.5) but not low pH (~7.1–7.2) medium. This experimental result also suggests a means for exploring the mechanism of pHi regulation: can inhibitors of specific transport mechanisms associated with acid/base balance reduce the high fraction of motile sperm seen when sperm are incubated at low pH but activated at high pH? We predicted that an inhibitor of an important mechanism underlying acid/base balance would block the onset of sperm motility, but it would have no such effect if the mechanisms it inhibited were

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unimportant, when sperm are activated at high pH following low pH incubation. We also predicted that an inhibitor of an important mechanism in acid/base balance should have no effect on motility if sperm were both incubated and activated at high pH since the presumed alkalinization of the cytoplasm would not be necessary. Therefore, a difference in response to inhibitors between sperm incubated at low or high pH when activated in high pH would suggest a specific and rapid pHi regulatory mechanism. Such experiments were conducted to probe for a possible mechanism(s) of pHi regulation. Within this context, the effects of inhibitors of the sodium:proton exchanger, as well as sodium replacement in the activating solution, were examined. To investigate the possible involvement of anion exchange, the effects of inhibitors of the bicarbonate:chloride exchanger, inhibitors of carbonic anhydrase and, roles of bicarbonate and chloride were examined. Carbonic anhydrase catalyzes the reversible hydration of carbon dioxide in the reaction: $CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$. H^+-ATPase activity exists in the trout gill (Lin and Randall, 1993) and it is conceivable that this enzyme system is involved in pHi regulation. This possibility was examined with the use of bafilomycin A1 as well as more general inhibitors of this enzymatic activity (Lin and Randall, 1993; Grosell and Wood, 2002). Finally, as Krasznai et al. (2003) have suggested that mechano-sensitive membrane channels are involved in the initiation of motility of osmolality-sensitive sperm and since osmotic concentration influences salmonid sperm, we also examined the effects of gadolinium, a mechano-sensitive channel inhibitor.

2. Materials and methods

2.1. Collection and handling of biological samples

Fresh semen samples were collected by hand stripping from mature steelhead, *O. mykiss* (Salmonidae), between early February and early May at the Dworshak National Fish Hatchery in Ahsahka, ID, USA. Samples were stored in 3.8 L ZipLock plastic bags (S.C. Johnson & Son, Inc., Racine, WI, USA) under humidified, mechanically pumped air, and maintained on wet ice for up to 2 h prior to subsequent experimentation or processing.

2.2. General maintenance and analyses

One volume of semen was diluted in 9 volumes of sperm immobilizing solution (SI; in mM: 80 NaCl, 40 KCl, 0.1 $CaCl_2$, 30 Tris [hydroxymethyl]aminomethane [Tris] and, titrated to either pH 6.5 or 8.5 with HCl). (After such dilution, samples had pH values of 7.1 ± 0.1 and 8.6 ± 0.1, $n = 3$, respectively). Samples were mixed gently and stored on wet ice for 2 h prior to subsequent motility estimates and final pH measurements. Motility was usually initiated by diluting approximately 1 μL semen suspension with approximately 100 μL ice-cold activation medium (AM; in mM: 125 NaCl, 0.1 $CaCl_2$, 30 Tris titrated to pH 8.5, unless otherwise indicated, with HCl.) on a microscope slide. Sperm motility was estimated visually using a light microscope at 400× magnification (Terner, 1986; Moccia and Munkittrick, 1987; Munkittrick and Moccia, 1987; Bencic et al., 2000). The evaluator of sperm motility was unaware of incubation conditions or the composition of the various dilution media. All undiluted semen samples initially demonstrated fraction motile sperm of approximately 0.5 or greater upon dilution in pH 8.5 AM. Solution pH values were determined with a model 815 MP Accumet pH meter with an Accu pHast electrode (Fisher Scientific) calibrated at 10 °C with phosphate buffer standards (pHydrion buffer; Micro Essential Lab, Brooklyn, NY, USA).

2.3. Inhibitors of membrane transport

One volume of semen was diluted with 9 volumes of SI (pH 6.5 or 8.5). Inhibitors were then added dissolved in DMSO (0.5% final concentration, v/v) to give the following final concentrations: amiloride, 0.5 mM; 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS), 1 mM; 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), 1 mM; niflumic acid, 0.2 mM; dicyclohexylcarbodiimide (DCCD), 1 mM; N-ethylmaleimide (NEM), 1 mM; diethylstilbestrol (DES), 25 μM and bafilomycin A1, 2 μM. Gadolinium chloride was dissolved in water and diluted to yield a final concentration of 40 μM; DMSO was also added to make the treatment comparable to the control. After 2 h on wet ice, samples were activated with AM and motility assessed.

2.4. Ion elimination or replacement

The role of calcium was evaluated by eliminating it from and adding 2 mM ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetracetic acid (EGTA) to 1) low pH SI and 2) both low pH SI and high pH AM.

The importance of sodium was assessed by replacing sodium chloride with choline chloride in low and high pH SI and/or high pH AM.

To evaluate the importance of bicarbonate in the activating solution, one volume of semen was diluted in 9 volumes of SI (pH

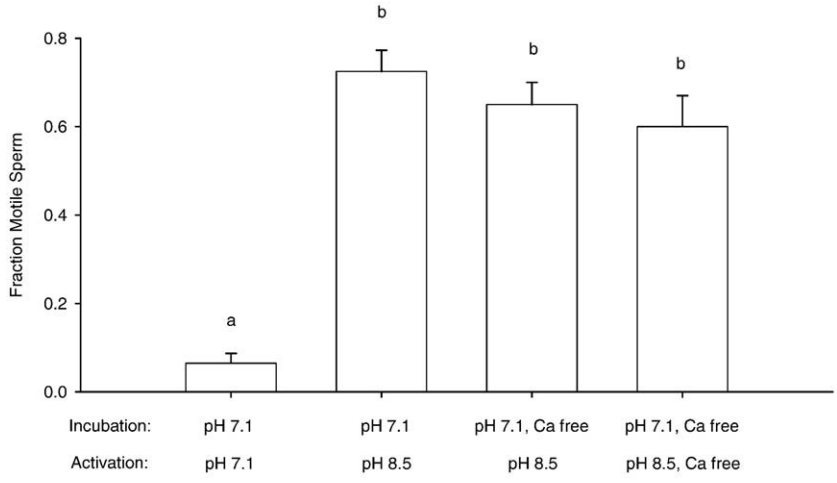

Fig. 1. Effect of SI and AM variants on fraction of motile sperm at activation. Sperm were incubated in low pH SI containing either calcium or no calcium and 2 mM EGTA then activated with AM at low or high pH, containing either calcium or no calcium and 2 mM EGTA. Different letters indicate significant differences ($P = 0.03$).