Membrane hyperpolarization activates trout sperm without an increase in intracellular pH

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Summary
Sperm from trout, like other sperm, are immotile in the seminal tract and initiate motility upon dilution into an appropriate fertilizing environment. Trout sperm motility is inhibited by high extracellular [K+] and can be activated by dilution of extracellular [K+]. Activation of trout sperm by the dilution of extracellular [K+] suggests regulation by membrane potential. Using the membrane potential-sensitive fluorescent dye 3,3'-dipropylthiocarbocyanine iodide (diS-C3-(5)) we directly measured the K+ contribution to the membrane potential. Manipulating the membrane potential with Cs+ and the ionophore valinomycin can override K+ regulation. We show that trout sperm can also be activated in the presence of inhibitory [K+] by the addition of divalent cations. Activation by divalent cations is explained by the cations' ability to mask membrane surface potential and thus alter the potential sensed by membrane voltage sensors. Using the surface potential-sensitive dye, 1-anilino-8-naphthosulfonate (ANS), we directly measure the divalent cations' ability to mask surface potential. We propose a model where membrane hyperpolarization is the trigger that initiates the cascade of events leading to trout sperm activation.

An increase in intracellular pH has been suggested to be a conserved step in the activation of sperm motility. We show that increasing intracellular pH by procedures that activate sea urchin and mammalian sperm does not activate trout sperm. In contrast, there is a decrease in intracellular pH upon activation of trout sperm motility. Artificially decreasing intracellular pH is not sufficient for activation of motility in trout sperm in an inhibitory [K+]. Thus, unlike some other sperm, changes in intracellular pH do not regulate trout sperm motility.

Key words: activation of motility, intracellular pH, membrane potential, sperm, surface potential.

Introduction
Sperm cells are terminally differentiated cells with a limited number of cellular functions, which include initiation and regulation of motility. Most sperm cells are immotile in the seminal tract and become motile in a fertilization environment. In this study we investigated the mechanism by which trout sperm recognize the signal to activate and initiate motility. Trout sperm, like most teleost sperm, lack an acrosome, thereby eliminating interference from acrosomal functions in the assessment of motility-specific functions. Immediate and synchronous activation of trout sperm motility upon dilution into a fertilizing environment allows for quantitative assessment of activation-related functions correlated with motility. Thus, trout sperm is a good model for studying initiation and regulation of sperm motility.

Although it has been known since 1938 that K+ is primarily responsible for keeping trout sperm inactive in the seminal tract (Schlenk and Kahmann, 1938), it was not clear how K+ regulated initiation of movement. In most animal cells, including sea urchin sperm, the resting membrane potential is primarily set by K+ permeability (Schackmann et al. 1984). Recently it has been shown that K+ can contribute to the resting membrane potential in trout sperm under long-term incubation (Gattl et al. 1990).

Thus, it is possible that K+ regulation is mediated through changes in the membrane potential. Influx of Ca2+ (Tanimoto and Morisawa, 1988; Cossom et al. 1989) increases in intracellular [cyclic AMP] (Benau and Terner, 1980; Morisawa and Ishida, 1987), and changes in intracellular pH (Robitaille et al. 1987) have also been reported to contribute to the activation of trout sperm. In addition, an increase in intracellular pH has been suggested to be a conserved mechanism for activation of sperm motility (Babcock and Pfeiffer, 1987; Shapiro and Tombs, 1989). In this study, we investigated the initial steps in the activation of trout sperm motility, particularly the roles of membrane potential and intracellular pH.

Materials and methods
Solutions
All solutions were made from reagent grade chemicals obtained from Mallinkrodt, Sigma and Baker Chemical Companies. Carboxyfluorescein diacetate (CFDA), and 3,3'-dipropylthiocarbocyanine iodide (diS-C3-(5)) were obtained from Molecular Probes, Eugene, OR. Valinomycin, nigericin, digitonin and 1-anilino-8-naphthosulfonate (ANS) were obtained from Sigma Chemical Company.

All activation solutions (AS: 150 mM NaCl, 20 mM glycine, 10 mM Tris-HCl, pH 8.7) activate full movement of trout sperm,
while all non-activation solutions (NAS) do not activate trout sperm motility. The compositions of NAS differed slightly for different experimental purposes and are listed below.

In the divalent activation experiments, all motility tests solutions had 20 mM glycine, 10 mM Tris-HCl, pH 8.7, variable concentrations of KCl, and a total osmolality adjusted to 330 mosmol with NaCl. Solutions with divalent cations had, in addition, 2 mM EGTA. Calcium and magnesium were added from stock solutions as their chloride salts while cadmium was added as the acetate. [Free Ca\(^{2+}\)] and [free Mg\(^{2+}\)] were calculated using the algorithm described by Brokaw (1986) and association constants reported by Sillén and Martell (1964). [Free Ca\(^{2+}\)] was estimated by subtracting [total EGTA] from [total Cd\(^{2+}\)].

Solutions used to test the effect of external pH contained 2 mM EGTA and an appropriate buffer for each pH tested: piperazine-N\(^{-}\)-2-ethanesulfonic acid (Pipes), pH 6.5–7.0; N\(^{-}\)-2-hydroxyethylpiperazine-N\(^{-}\)-2-ethanesulfonic acid (Hepes), pH 7.0–8.0; tris(hydroxymethyl)amino-methane (Tris) pH 7.5–9.0; or 3-(N-Tris-(hydroxymethyl)methylamino)-propane sulfonic acid (Traps) for pH 8.5–9.5. Osmolality was kept constant in all solutions by the addition of NaCl (total osmolality = 332 mosmoles). AS contained no K\(^{+}\), NAS and divalent cation solutions contained 30 mM K\(^{+}\), Mg\(^{2+}\) solution contained, in addition, 18 mM free Mg\(^{2+}\), and Ca\(^{2+}\) solution contained 8 mM free Ca\(^{2+}\).

Seminal mimic solution (SM) contained the following: 127 mM NaCl, 1.5 mM MgCl\(_2\), 37 mM KCH\(_3\)CO\(_2\), 30 mM Hepes, and pH as stated in text. Cesium seminal mimic (CsSM) contained the following: 97 mM NaCl, 27 mM Na(CH\(_2\)CO\(_2\)), 10 mM K(CH\(_2\)CO\(_2\)), 37 mM CsCl, 1.5 mM MgCl\(_2\), 2.6 mM CaCl\(_2\), 30 mM Hepes, pH 7.2.

Extender solution contained the following: 32 mM KCl, 198 mM NaCl, 12 mM NaHCO\(_3\), 4 mM NaH\(_2\)PO\(_4\), 1.0 mM MgSO\(_4\), 1.0 mM CaCl\(_2\), 0.1% glucose, 1160 i.u. ml\(^{-1}\) streptomycin and 1250 i.u. ml\(^{-1}\) penicillin.

diS-C\(_3\)-(5) was stored in absolute ethanol at a concentration of 0.2 M.

ANS was stored in NAS at a concentration of 2 M.

Collection of sperm

Sperm were obtained from Oncorhynchus mykiss (Rainbow and Steelhead trout) maintained at the Washington State University Campus Fish Center. The trout were anesthetized with 0.2 M. Valinomycin, an ionophore that transports K\(^{+}\) across biological membranes (Pressman et al., 1967), does

**Results**

**The role of membrane potential in trout sperm activation**

TROUT sperm can be activated by lowering the external [K\(^{+}\)]. Once activated, the subsequent increase in external [K\(^{+}\)] to inhibitory levels within 1 s did not inhibit motility. In order to determine if K\(^{+}\) contributes to membrane potential in trout sperm on a time scale necessary for the rapid and irreversible activation, we directly monitored membrane potential with the fluorescent probe 3,3\'-dipropylthiocarbocyanine iodide (diS-C\(_3\)-(5)) (Fig. 1). The positively charged diS-C\(_3\)-(5) is redistributed across the lipid bilayer of the membrane, driven by the membrane potential (Cabrini and Verkman, 1986). Hyperpolarization of the membrane decreases diS-C\(_3\)-(5) fluorescence, while depolarization increases its fluorescence. Upon addition of external K\(^{+}\), the fluorescence of diS-C\(_3\)-(5) immediately increased, indicating a depolarization of the membrane. Valinomycin, an ionophore that transports K\(^{+}\) and Ca\(^{2+}\) across biological membranes (Pressman et al., 1967), does

**Surface potential measurements**

ANS in NAS at a concentration of 2 M was added to sperm in 2 ml of NAS (30 mM KCl, 70 mM NaCl, 20 mM glycine, 10 mM Tris–HCl, pH 8.7, at 4 × 10\(^6\) to 6 × 10\(^6\) cells ml\(^{-1}\)) to a final concentration of 16 μM ANS. K\(^{+}\), Ca\(^{2+}\) and Mg\(^{2+}\) were added from 1 M stocks of their chloride salts to the final concentrations listed in Fig. 1. diS-C\(_3\)-(5) fluorescence was monitored at \(\lambda\)ex 620 nm and \(\lambda\)em 670 nm with continuous stirring with a glass-coated stir bar at 10°C. diS-C\(_3\)-(5) loading alone did not significantly change the motility of the trout sperm.
not alter diS-C\(_3\)-(5) fluorescence to increased external [K\(^+\)] (Fig. 1A). Cs\(^+\) is not membrane permeant and does not contribute to the membrane potential as indicated by the lack of change in the diS-C\(_3\)-(5) fluorescence upon the addition of Cs\(^+\) to the external medium (Fig. 1B). However, with the addition of valinomycin, Cs\(^+\) becomes membrane permeant. In the presence of valinomycin the fluorescence of diS-C\(_3\)-(5) increased upon the addition of Cs\(^+\) (Fig. 1B), indicating that in the presence of valinomycin, Cs\(^+\) contributes to the membrane potential.

To investigate whether changes in membrane potential regulate trout sperm motility, we observed the behavior of sperm whose membrane potential was manipulated by the ionophore valinomycin and Cs\(^+\) (Fig. 2). Cs\(^+\) at the concentration used does not reduce the percentage motility of untreated sperm. The motility of sperm suspended in a solution that mimics the seminal plasma (SM) still retains significant motility in the presence of Cs\(^+\). The absence of inhibition of motility by Cs\(^+\) is expected, since Cs\(^+\) alone does not contribute to the membrane potential. In the presence of valinomycin (SM+Val; CsSM+Val), Cs\(^+\) dramatically reduces the percentage activation under conditions that normally fully activate trout sperm (0K\(^+\), 30Cs\(^+\)). This result is consistent with an inward Cs\(^+\) current, depolarizing the membrane and inhibiting activation. Sperm preincubated in CsSM with valinomycin are activated upon the dilution of external Cs\(^+\), even in the presence of a normally inhibitory [K\(^+\)] (15K\(^+\), 0Cs\(^+\)). Thus, an outward Cs\(^+\) current can overcome the inhibitory K\(^+\) gradient, hyperpolarize the membrane, and activate motility. These effects of Cs\(^+\) in the presence of valinomycin are consistent with a depolarization inhibiting and a hyperpolarization triggering activation.

In addition to activation by the dilution of external [K\(^+\)], trout sperm can be activated in the presence of normally inhibitory [K\(^+\)] by the addition of the divalent cations. In Fig. 3 we show activation of trout sperm in normally inhibitory [K\(^+\)] by the addition of the divalent cations Ca\(^{2+}\), Mg\(^{2+}\), and Cd\(^{2+}\). In addition, Mn\(^{2+}\) can also overcome K\(^+\) inhibition in a range of concentration similar to that of Mg\(^{2+}\) (data not shown). The concentration of divalent cations needed to activate motility is dependent upon the external [K\(^+\)]. The divalent cations' ability to overcome K\(^+\) inhibition can be interpreted as a masking of membrane surface potential to provide a hyperpolarizing environment to membrane voltage sensors (see Fig. 7, below). We can estimate the surface-charge masking effect of divalent cations from the plot of the concentration of divalent cations on a log scale required to overcome K\(^+\) inhibition, plotted as the Nernst Potential for K\(^+\) (Fig. 3). The interpretation of the effects of divalent cations on the membrane in terms of surface charge screening predicts a potential change of 27 mV per 10-fold change in divalent cation concentration (McLaughlin et al. 1971). The plot in Fig. 3 shows, as predicted, a roughly linear relationship between the calculated K\(^+\) equilibrium potential versus log of divalent cation concent-

**Fig. 1.** Membrane potential measured by diS-C\(_3\)-(5) fluorescence. Each addition of K\(^+\) or Ca\(^{2+}\), where indicated, increased their final concentrations by 25 mM. VAL indicates the addition of valinomycin to a final concentration of 1.25 μM.
Divalent ion concentration (mM)

Fig. 3. Divalent cation effect on K⁺ inhibition of activation. The logarithm of the concentration of divalent ions, Mg²⁺ (○), Ca²⁺ (●), Cd²⁺ (x), required to produce normal activation is plotted against the Nernst Potential for the inhibitory [K⁺], calculated using the value for intracellular [K⁺] of 120 mM (Babcock, 1983). Normal motility indicates that motility is indistinguishable from dilution into activation solution (AS), which had 150 mM NaCl and no KCl. Only two points are shown for Cd²⁺, due to the low solubility of Cd(CH₃CO₂)₂ at alkaline pH.

The role of pH in activation of trout sperm motility

In Fig. 5, we show that percentage motility of trout sperm in divalent cation-supplemented K⁺ solutions, as well as in K⁺-free solution, is pH dependent. The fact that trout sperm activation is inhibited at low external pH is consistent with the hypothesis that an increase in intracellular pH is necessary for activation. Thus, we tested to see if increasing intracellular pH can activate trout sperm motility. We increased the intracellular pH of trout sperm by two methods. The addition of 10 mM NH₄Cl increased the intracellular pH, but did not activate trout sperm in the presence of inhibitory [K⁺] (Fig. 6A). An alternative method of raising the intracellular pH, using the K⁺/H⁺ exchanger, nigericin, in the presence of high external [K⁺] at pH 8.7 (Babcock, 1983), also increased intracellular pH, but did not activate motility (Fig. 6B). Therefore, raising intracellular pH is not sufficient to activate trout sperm. Furthermore, upon activation there is an acidification of approximately 0.19 pH unit, rather than an alkalinization (Fig. 6C). A similar magnitude of acidification induced by weak acids did not activate trout sperm in the presence of inhibitory [K⁺] (Fig. 6D). The time course of acidification suggests that it is a result of, rather than the trigger for, activation. All of the above manipulations of intracellular pH were not deleterious to

Fig. 4. The membrane surface potential measured by ANS fluorescence. (A) Each addition of K⁺, Mg²⁺ or Cs⁺, where indicated, increased their final concentrations by 12.5 mM. The decrease in fluorescence upon addition of K⁺ or Cs⁺ is comparable to the decrease due to dilution alone. The increase in fluorescence due to the surface masking effect by Mg²⁺ is thus decreased by the same dilution effect. (B) The changes in ANS fluorescence due to Mg²⁺, adjusted for the decrease to dilution, is plotted against total [Mg²⁺].
pH of external solution

Fig. 5. pH dependence of activation. Percentage motility is plotted against pH for three different solutions that support trout sperm activation: AS (■), Mg²⁺-supplemented NAS (○), Ca²⁺-supplemented NAS (●). n=6 experiments, error bars indicate s.d.

the trout sperm, since the treated sperm were activated upon subsequent dilution into an activation solution.

Discussion

Our work shows the importance of membrane potential to the regulation of the activation of trout sperm. Our direct measurement of membrane potential demonstrates that K⁺ contributes to rapid changes in the membrane potential of trout sperm. By manipulating the membrane potential of trout sperm with Cs⁺ and the ionophore valinomycin we regulate sperm activation independently of [K⁺]. Thus, K⁺ regulation of trout sperm activation is mediated through its effect on the membrane potential.

Gatti and coworkers (1990) used slow lipophilic tracers to show that K⁺ contributes to changes in the membrane potential. However, measurement of membrane potential using lipophilic tracers is on a time scale of minutes. Since trout sperm are irreversibly activated in less than 1 s and cease to move within 1–2 min, the relevance of changes at these time scales to activation is unclear. Tanimoto and Morisawa (1988) used K⁺-channel blockers to inhibit activation in a K⁺-free activation solution. They concluded from this result that activation is dependent on a K⁺ efflux, and thus membrane potential changes. Our measurements show that K⁺ contributes to bulk potential changes on a time scale relevant to activation and that membrane potential changes can regulate activation independently of K⁺.

Divalent cation addition can also overcome K⁺ inhibition of trout sperm. Although previous authors have mentioned the antagonistic effects of Ca²⁺ on K⁺ inhibition of activation (Schlenk and Kuhmann, 1938; Baynes et al. 1981; Tanimoto and Morisawa, 1988; Cosson et al. 1989), no unifying mechanism has been proposed to explain other divalent cations’ abilities to overcome K⁺ inhibition. Tanimoto and Morisawa (1988), and Cosson and coworkers (1989) have proposed that calcium activation in the presence of inhibitory [K⁺] involves calcium influx. However, such a mechanism does not explain the ability of other divalent cations to overcome K⁺ inhibition.

We directly measured the intracellular pH of trout sperm at rest and upon activation. Trout sperm undergo an acidification upon activation. A similar magnitude of acidification induced by weak acids does not activate trout sperm in the presence of inhibitory [K⁺]. Robitaille and coworkers have previously measured changes in intracellular pH of trout sperm after activation using ³¹P NMR (Robitaille et al. 1987). The time scale of measurements using ³¹P NMR does not allow for measurement of changes in pH that are physiologically relevant to trout sperm activation. In other sperm, including those of sea urchin.
by membrane voltage sensors is the initial trigger for bull sperm are regulated by membrane potential (Babcock and Pfeiffer, 1987; Shapiro and Tombes, 1989). How an addition of divalent cations, produces a similar local hyperpolarization in the membrane. The local potential sensed by a membrane voltage sensor is depicted by the circle within the membrane. The potential profile is represented by the continuous line outside the membrane and the dotted line in the lipid bilayer of the membrane. The rightmost diagram indicates the activation of trout sperm caused by the dilution of extracellular K⁺. In this case, the bulk potential is −90 mV and the local potential is −60 mV. The leftmost diagram indicates high external K⁺ and inactive sperm. In this case, the bulk potential is −60 mV and the local potential is −30 mV. The middle diagram illustrates the activation by divalent cations in the presence of high external K⁺. In this case, although the bulk potential, primarily set by K⁺ permeability, is identical to the non-active case (−60 mV), the masking of surface potential by divalent cations (++ enclosed in oval) produces a local hyperpolarization sensed by the membrane voltage sensor (−60 mV). This local hyperpolarization is similar to the hyperpolarization in the case of the activation by the dilution of external K⁺. The absolute values of the membrane potential depicted are hypothetical.

We have shown that trout sperm can be activated by three different methods (by dilution of external K⁺, addition of divalent cations or hyperpolarization with Cs⁺ and valinomycin) that affect the sensed potential in the membrane and we suggest that hyperpolarization sensed by membrane voltage sensors is the initial trigger for activation. This hyperpolarization leads to activation of motility without mediation by intracellular pH. Thus, intracellular pH change cannot be a conserved step in the activation of sperm motility.

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References


Regulation of trout sperm motility 349