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Role of ion channels and membrane potential in the initiation of carp sperm motility

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Abstract

The exposure of freshly spawned, immotile carp sperm to hypoosmotic media triggers the initiation of calcium-dependent flagellar motility. Intracellular calcium concentration has been thought to be the critical component in motility initiation, possibly acting through a novel signalling pathway. The sensitivity of sperm cells to changes of osmolality of the environment raises the question whether a mechanoregulated osmosensitive calcium pathway is involved in the activation mechanism of carp sperm motility. The sperm cells are in a depolarized state in the seminal plasma (Ψ = –2.6 ± 3 mV) and they hyperpolarize upon hypoosmosis-induced activation of motility (Ψ = –29 ± 4 mV). The intracellular sodium $[Na^+]_i$, potassium $[K^+]_i$ and calcium $[Ca^{2+}]_i$ ion concentrations were determined in quiescent cells, and at 20, 60 and 300 s after activation. The $[Na^+]_i$ and $[K^+]_i$ of the quiescent cells were similar to the $[Na^+]_e$ and $[K^+]_e$ of the seminal plasma. Following hypoosmotic shock-induced motility, both $[Na^+]_i$ and $[K^+]_i$ decreased to one-fourth of the initial concentration. The $[Ca^{2+}]_i$ doubled at initiation of the motility of the sperm cells and remained unchanged for 5 min. Bepridil (50–250 μM), a blocker of the Na^+/Ca^{2+} exchanger, blocked carp sperm motility reversibly. Gadolinium, a blocker of stretch-activated channels (10–20 μM), inhibited sperm motility in a dose-dependent manner and its effect was reversible. Hypoosmotic shock fluidized the membrane and gadolinium treatment made it more rigid in both quiescent cells and hypotonic shock treated but immotile sperm cells. Based on these observations, it is suggested that, besides the well-known function of potassium and calcium channels, stretch-induced conformational changes of membrane proteins are also involved in the sperm activation mechanism of common carp.

Résumé

Rôle des canaux ioniques et du potentiel membranaire dans l'initiation de la motilité des spermatozoïdes de carpes. L'exposition du sperme de carpe, fraîchement émis et non motile, à des milieux hypo-osmotiques déclenche l'initiation de la motilité des flagelles, cette motilité étant sensible à la concentration de calcium. La concentration de calcium intracellulaire est connue pour être un composant déterminant dans l'initiation de la motilité, agissant probablement au travers d'une signalisation calcique nouvelle. La sensibilité des spermatozoïdes aux modifications d'osmolalité de l'environnement soulève la question de savoir si le canal calcique osmosensitif et mécano-régulé est impliqué dans le mécanisme de l'activation de la motilité des spermatozoïdes de carpe. Dans le plasma séminal, la membrane des spermatozoïdes est soit dans un état de dépolarisation (Ψ = –2.6 ± 3 mV) soit d'hyperpolarisation lors de l'induction de la motilité par hypo-osmose de l'activation de la motilité (Ψ = –29 ± 4 mV). Les concentrations ioniques de sodium $[Na^+]_i$, de potassium $[K^+]_i$ et de calcium $[Ca^{2+}]_i$ intracellulaires ont été déterminées dans les cellules en repos, ainsi qu'à 20, 60 and 300 s après activation. Les concentrations de $[Na^+]_i$ et de $[K^+]_i$ des cellules quiescentes étaient similaires à celles de $[Na^+]_e$ et $[K^+]_e$, c'est-à-dire celles du plasma séminal. Suite à la motilité induite par le choc hypo-osmotique, les concentrations en $[Na^+]_i$ et $[K^+]_i$ diminuent pour atteindre 25% de la concentration initiale, alors que le $[Ca^{2+}]_i$ double lors de l'initiation de la motilité des cellules du sperme, mais reste inchangée pendant les 5 min suivantes. Le Bepridil (50–250 μM), un agent bloquant de l'échangeur ionique Na^+/Ca^{2+}, bloque la motilité du sperme de carpe de façon réversible. Le

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Gadolinium (10–20 μM), un agent bloquant des canaux activés par des contraintes mécaniques, inhibe la motilité du sperme, et son effet est réversible. Il est postulé que le choc hypo-osmotique rend plus fluide la membrane du spermatozoïde et que le traitement au Gadolinium la rend plus rigide, aussi bien chez les cellules quiescentes que chez les cellules soumises à un choc hypotonique mais non motiles. D'après ces observations, nous pouvons suggérer qu'à côté de la fonction bien connue des canaux ioniques potassium et calcium, les modifications conformationnelles, induites par des contraintes mécaniques, des protéines membranaires sont aussi impliquées dans le mécanisme d'activation des spermatozoïdes de la carpe.

Keywords: Sperm motility; Membrane; Excitability; Ion channel; Carp

1. Introduction

The spermatozoa of teleost fish are immotile within the testes and spawned semen. These sperm cells are also immotile when diluted into isoosmotic solutions of electrolytes and nonelectrolytes. However, when exposed to hypoosmotic media, carp spermatozoa immediately become motile (Morisawa and Suzuki, 1980; Morisawa and Okuno, 1992; Morisawa et al., 1983; Morisawa, 1994; Billard et al., 1995). Hypoosmotic induction of motility is accompanied by a reorganization of the membrane structure (Márián et al., 1993) and hyperpolarization of the membrane. The initiation of sperm motility is blocked by K^+ and Ca^{2+} channel blockers (Krasznai et al., 1995, 2000). It was hypothesized by Perchec et al. (1997) that co-transport of ions or stretch-activated channels might also be involved in the activation mechanism of carp sperm. This paper explores the effect of K^+, Ca^{2+} and mechanosensitive channels on motility initiation of spermatozoa of the common carp (*Cyprinus carpio*).

2. Materials and methods

Semen like solution (SLS) contained 75 mM NaCl, 75 mM KCl, 1 mM $CaCl_2$, 10 mM HEPES, pH 8. Fish physiological solution (FPS) consisted of 140 mM NaCl, 10 mM KCl, 1 mM $CaCl_2$, 10 mM HEPES, pH 8.5. Calcium free FPS (NoCaFPS) contained 140 mM NaCl, 10 mM KCl, and 5 mM EGTA, 10 mM HEPES, pH 8.5 while activating solution (AS) was composed of 25 mM KCl, 25 mM NaCl and 1 mM $CaCl_2$, 10 mM HEPES, pH 8.5. Calcium and potassium channel blockers, MCD peptide and the intracellular Ca^{2+} mobilizer, thapsigargin, were obtained from Alomone Laboratories Ltd. (Jerusalem, Israel). All other reagents including carbonyl cyanide *m*–chlorophenylhydrazone (CCCP) were purchased from Sigma Chemical Co. (St. Louis, MO) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.1. Sperm motility measurement

Twenty microlitres semen of the common carp was diluted into 1 ml physiological solution (FSP) and centrifuged for 5 min at 700 × *g*. The pellet was resuspended in 40 μl physiological solution. One microlitre from this washed sperm was diluted into 100 μl physiological solution containing the tested drug in the given concentration. After the desired incubation time, 1 μl sperm suspension was taken and dropped on a glass slide and activated with AS that also contained the tested drug. Images of tracks of sperm were taken through a high-sensitivity video camera (Hamamatsu 2400–07) mounted on a phase contrast microscope (Nicon-Optiphot) with an inverted contrast objective lens (Olympus Splan NH).

2.2. Membrane fluidity assessment

A stock solution of 1×10^{-4} M trimethylammoniodiphenyl-hexatriene (TMA-DPH) dissolved in dimethylformamide was made. The carp sperm was washed in FPS, centrifuged for 5 min at 700 × *g* and resuspended in FPS. Sperm concentration was adjusted to 6×10^6 ml^{-1}. Ten microlitres of TMA-DPH stock solution was added into 1 ml sperm suspension (final concentration of TMA-DPH 10^{-6} M) and incubated for 10 min in the presence or absence of 20 μM $GdCl_3$ in a quartz cuvette. Two microlitres of FPS (sperm remained immotile) or 2 ml distilled water (sperm initiated motility) was added into the cuvettes and the anisotropy was measured. The added FPS or distilled water contained the same concentrations of TMA-DPH and $GdCl_3$ as the incubation solution. Steady state anisotropy was determined using a Perkin Elmer spectrofluorimeter by measuring the vertically (I_V) and horizontally (I_H) polarized intensity components. The sample was excited with vertically polarized light (340 nm) and emission was measured above 430 nm. Fluorescence intensities were recorded after careful temperature equilibration to 25 °C. The anisotropy r_f value was computed as:

$$r_f = I_{VV} - GI_{VH}/I_{VV} + 2\,GI_{VH} \quad (1)$$

where VV and VH indices refer to parallel and perpendicular positions of the excitation and emission polarisers, respectively. The *G* value is an instrumental correction factor for the imperfectness of polarisers (and monochromators) and it is given as: $G = I_{HV} / I_{HH}$.

Anisotropy refers to the rotational motion of the TMA-DPH (located at the polar headgroup region of the outer leaflet of the phospholipid bilayer) and is inversely proportional to membrane fluidity. The lower r_f values refer to higher membrane fluidity.

2.3. Flow cytometry

A modified Becton Dickinson FACStar flow cytometer equipped with an argon ion laser was used. Small angle forward scattered light was used for the electronic gating of data collection, allowing dead cell exclusion from the analysis.

2.4. Determination of intracellular potassium and sodium concentrations, and membrane potential on the basis of intracellular pH measurement

2.4.1. Loading the cells with pH indicator dye

A stock solution of 1 mM 2,7-*bis*-carboxyethyl-5(6)-carboxyfluorescein acetoxymethyl-ester (BCECF-AM), in dimethyl sulphoxide (DMSO) was kept at –20 °C. Cells ((10–30) × 10^6 ml^{-1} in SLS) were loaded with 5–7 μM BCECF-AM at 37 °C. After 30 min incubation, the cells were pelleted and resuspended in SLS followed by further incubation at 37 °C for another 30 min. After this step, loaded cells were kept at room temperature and analysed within 1.5–2 h.

The intracellular free potassium concentration $[K^+]_i$ was determined by measuring the intracellular pH (pH_i) in BCECF-stained cells in the presence of 3 μM nigericin as described by Balkay et al. (1997). The nigericin ionophore sets $[H^+]_i$ / $[H^+]_e$ = $[K^+]_i$ / $[K^+]_e$ without influencing K_i, allowing the latter concentration to be calculated by determining the intracellular pH.

The intracellular sodium concentration $[Na^+]_i$ was measured in a similar way using monensin (Márián et al., 2000) in a final concentration of 5 μM maintaining:

$$[H^+]_i / [H^+]_e = [Na^+]_i / [Na^+]_e \quad (2)$$

in equilibrium without affecting the intracellular Na^+ concentration. Known $[H^+]_e$ and $[Na^+]_e$, and measured pH_i parameters allowed for the determination of $[Na^+]_i$ by analogy with the calculation of $[K^+]_i$.

The membrane potential was measured in a similar way using CCCP (Babcock, 1983). Ten micromoles of CCCP adjusted the intracellular pH_i to the value of the extracellular pH_e on the basis of membrane potential (Ψ):

$$\Psi = RT / ZF \times \log [H^+]_i / [H^+]_e = RT / ZF \times (pH_i - pH_e) \quad (3)$$

2.4.2. Intracellular calcium measurement

Flow cytometric measurement of intracellular Ca^{2+} was carried out using Calcium Green fluorescent dye (Krasznai et al., 2000). Cell loading was done as described above. Semen was diluted in 2 ml (10^6 cells ml^{-1}) of experimental medium in a test tube. A-23187 Ca^{2+} ionophore in 1 μM concentration was added to the solutions for the calibration of the intracellular Ca^{2+} concentration. Fluorescence was excited with the 488 nm line at 200–400 mW power with a Becton Dickinson FACS III flow cytometer using an argon ion laser. The output optics contained a combination of a 529 nm long pass filter and 540 nm band filter. Data of dead sperm cells were excluded from the analysis.

3. Data analyses

Every experiment was repeated at least three times and the mean and standard deviation (S.D.) were calculated. The significance was calculated by Student's *t* test (two tailed) ; $P = 0.01$ was considered significant.

4. Results

The intracellular $[Ca^{2+}]_i$, $[K^+]_i$, $[Na^+]_i$ and pH_i of quiescent and hypoosmotic shock-activated motile sperm cells were measured (Table 1). The quiescent sperm cells exhibited similar $[K^+]_i$ and $[Na^+]_i$ concentrations to those of the seminal plasma reported by Emri et al. (1998). Both $[K^+]_i$ and $[Na^+]_i$ decreased in time after initiating sperm motility. Five minutes after the hypoosmotic shock-induced motility started, $[K^+]_i$ and $[Na^+]_i$ decreased to around one-fourth of their values in the quiescent cells. Conversely, $[Ca^{2+}]_i$ doubled upon activation and remained at this value for 5 min. The quiescent cells were in a depolarized state in the seminal plasma (Ψ = –2.6 ± 3 mV). Immediately after dilution to hypotonic solution, the membrane hyperpolarized (Ψ = –29 ± 4 mV) and it remained in a hyperpolarized state for about 30 s. One minute after initiating sperm motility, the membrane potential decreased to half of its highest value and 5 min after the motility was activated, the membrane potential depolarized close to the value of the quiescent cells. The intracellular pH_i increased by about 0.3 pH units upon initiating sperm motility. The artificial increase of pH_i using nigericin (3 μM) and $[Ca^{2+}]_i$ by the Ca^{2+} ionophore A-23187 (1 μM) and/or the intracellular Ca^{2+} mobilizer thapsigargin (50 μM) did not initiate sperm motility. Membrane hyperpolarization and the hypoosmotic shock-induced initiation of sperm motility could be eliminated by voltage gated potassium channel blockers 4-AP (5 mM) and MCD-peptide

Table 1
Intracellular ion concentrations and membrane potential of quiescent and motile carp sperm. The intracellular parameters of quiescent cells were measured in SLS. After activation of the sperm cells with AS, the time dependent changes of the intracellular parameters were monitored with flow cytometer as described in Section 2

	Na^+_i (mM)	K^+_i (mM)	Ca^{++}_I (nM)	pH_i	Ψ (mV)	Membrane structure
Quiescent cells in SLS	78 ± 5	63 ± 6	43.5 ± 4.5	7.15 ± 0.17	–2.6 ± 3	Intact
20 s after activation	42 ± 7	37 ± 8	79 ± 8	7.38 ± 0.14	–29 ± 4	Swollen
60 s after activation	25 ± 5	22 ± 4	72 ± 6	7.43 ± 0.09	–15 ± 5	Swollen
300 s after activation	18 ± 7	20 ± 5	73 ± 9	7.31 ± 0.12	–4 ± 3	Swollen

(1 μM). Remaining motile fraction of spermatozoa after 5 min incubation time was 2.8 ± 1.2% using 4-AP and 3.5 ± 2% using MCD peptide. Ca^{2+}-activated K^+ channel blockers (PenitremA (50 μM) and rIberiotoxin (50 μM)) did not influence sperm motility. The increase of $[Ca^{2+}]_i$ and the initiation of sperm motility could also be eliminated by applying Ca^{2+} channel blockers (e.g. verapamil (100 μM), flunarizine (100 μM) and the ω-conotoxin family (2 μM). The remaining motile fraction of spermatozoa after 5 min incubation time was 0%, 2.7 ± 1.6% and 6.4 ± 3.5%. Upon activation, the membrane structure also changed. The cell was swollen and the membrane proteins became more flexible. The anisotropy value of the quiescent cells was $r_f = 0.26 \pm 0.01$. Hypoosmolality resulted in a more fluid membrane $r_f = 0.20 \pm 0.02$. Twenty micromoles of gadolinium (a blocker of the stretch-activated channels) made the membrane of both quiescent and hypotonic shock treated cells more rigid, $r_f = 0.30 \pm 0.01$ and $r_f = 0.24 \pm 0.01$, respectively. Gadolinium, a mechanosensitive channel blocker inhibited the motility of carp sperm in a dose-dependent manner (Fig. 1). Twenty micromoles of gadolinium chloride blocked carp sperm motility to 5% within 2 min. The effect of gadolinium was reversible. Bepridil, a blocker of the Na^+/Ca^{2+} exchanger, blocked carp sperm motility reversibly (Fig. 2). Following 5 min incubation in SLS containing 50 μM bepridil, the motility of carp spermatozoa decreased to 15 ± 5%. After the washout of bepridil with SLS and activating the spermatozoa with AS, motility recovered to 65 ± 7%.

5. Discussion

The intracellular K^+ concentration (63 ± 6 mM) of the quiescent carp spermatozoa determined in our experiment is close to the values published by Emri et al. (1998) (60 ± 7 mM). These K^+_i concentrations are very similar to the K^+ of the seminal plasma reported by Morisawa et al. (1983) and Emri et al. (1998) (75 and 72 mM, respectively). These

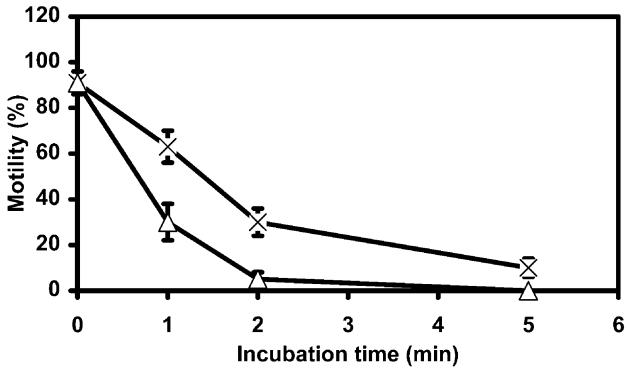

Fig. 1. Effect of gadolinium on the motility of carp sperm. Common carp sperm was washed and incubated in FPS containing 10 μM (X) and 20 μM (open triangle) $GdCl_3$ and the time-dependent effect of the drug on the motility % was measured. The AS contained similar concentration of $GdCl_3$ to that of the incubating medium. Bar represents mean ± S.D. of three independent experiments.

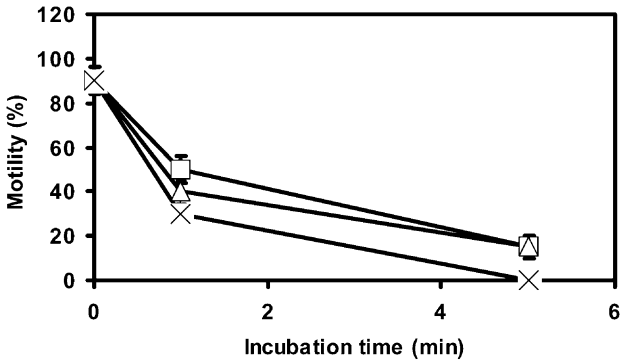

Fig. 2. Effect of bepridil on the motility of carp sperm. Common carp sperm was washed and incubated in FPS containing 50 μM (open square), 100 μM (open triangle) and 250 μM (X) bepridil and the time-dependent effect of the drug on the motility % was measured. The AS contained similar concentration of bepridil to that of the incubating medium. Bar represents mean ± S.D. of three independent experiments.

data suggest that the spermatozoa are in a depolarized state in the seminal plasma. This assumption has been fully confirmed by our membrane potential measurements, the membrane potential of the quiescent cells was $\Psi = -2.6 \pm 3$ mV. On initiating motility by hypotonic shock, the extracellular potassium and sodium concentrations drop dramatically and the membrane potential hyperpolarizes very rapidly ($\Psi = -29 \pm 4$ mV). This is in accordance with the results of Krasznai et al. (2000) claiming that membrane hyperpolarization is the first signal that induces recovery of calcium channels from inactivation leading to Ca^{2+} influx and subsequent motility of the common carp sperm. The membrane potential is basically determined by the distribution of the potassium ions across the membrane and the open probability of the voltage gated potassium channels. The observation that voltage gated potassium and calcium channel blockers (4-AP and MCD peptide) block the motility of carp spermatozoa supports this assumption. Calcium operated potassium channels are not involved in the process, since PenitremA and rIberiotoxin do not influence sperm motility. Interestingly bepridil, a blocker of the Na^+/Ca^{2+} exchanger, also blocks sperm motility, indicating that the exchanger has a significant role in carp sperm motility. Recent publications (Su and Vacquier, 2002; Vines et al., 2002) report that the Na^+/Ca^{2+} exchanger has a regulatory role in the motility of sea urchin and herring spermatozoa. During the active motile phase of the spermatozoa, the pH_i increases by about 0.3 units. This observation is in accordance with the observations of Emri et al. (1998), Babcock et al. (1992), Florman et al. (1992) and Boitano and Omoto (1991). However, ionophore or thapsigargin-induced increase of $[Ca^{2+}]_i$ and the increased pH_i are not enough to initiate the motility under isotonic conditions as change in external osmolality is also required. Márián et al. (1993) reported on the reorganization of the membrane structure at the hypoosmotic activation of sperm motility. Perchec et al. (1997) demonstrated the effects of extracellular environment on the osmolality induced signal transduction, involved in the initiation of the motility of common carp spermatozoa. They came to the conclusion that

the activation mechanism might involve co-transport or specific stretch-activated channels, sensitive to osmotic pressure. The successful block of the initiation of sperm motility by a stretch-activated channel blocker, gadolinium, indicates that osmo-regulated mechanosensitive channels may also be involved in the initiation of carp sperm motility. Most of the signal transduction processes depend upon the physical properties of the cytoplasmic membrane. Gadolinium binds to the lipid component of the membrane (Ermakov et al., 2001) and alters its physical properties. These changes may affect the dynamics of the conformational changes of transmembrane proteins, e.g. ion channels, receptors, etc. (Cantor, 1999). Gadolinium significantly decreased the fluidity of the membrane of the common carp sperm and also abolished the fluidizing effect of the hypotonic treatment (Table 1). Based on the observations that gadolinium causes immotility of the carp spermatozoa and it changes membrane fluidity, we suggest that mechanosensitive membrane proteins are also involved in the activation mechanism of the common carp spermatozoa.

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