Regulation of hamster sperm hyperactivation by extracellular Na⁺

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Abstract

Mammalian sperm motility has to be hyperactivated to be fertilization-competent. Hyperactivation is regulated by extracellular environment. Osmolality of mammalian semen is higher than that in female reproductive tract; however, the effect of them on hyperactivation has not been investigated. So we investigated the effect of osmotic environment on hyperactivation using hamster spermatozoa at first. Increase in the osmolality of the media (~370 mOsm) by increasing the concentration of NaCl (~150 mmol/L) caused the delay of the expression of hyperactivation. When NaCl concentration varied in the same range (75–150 mmol/L) whereas the osmolality was fixed at 370 mOsm by adding mannitol, the delay of hyperactivation occurred dependent on NaCl concentration. Increase in NaCl concentration also caused suppression of curvilinear velocity, bend angle, and sliding velocity of the flagellum at the onset of incubation, suggesting that NaCl concentration affect both activation and hyperactivation in hamster spermatozoa. Hamster sperm intracellular Ca²⁺ concentration decreased as extracellular NaCl concentration increased, whereas membrane potential and intracellular pH were unaffected by extracellular NaCl concentration. SN-6 and SEA0400, inhibitors of Na⁺-Ca²⁺ exchanger (NCX), increased intracellular Ca²⁺ and accelerated hyperactivation in the presence of 150 mmol/L NaCl. Tyrosine phosphorylation on fibrous sheath proteins was unaffected by extracellular NaCl concentration. These results suggest that extracellular Na⁺ suppresses hamster sperm hyperactivation by reducing intracellular Ca²⁺ via an action of NCX in a tyrosine phosphorylation-independent manner. It seems that the removal of suppression by extracellular Na⁺ leads to the expression of hyperactivated motility.

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Introduction

Mammalian spermatozoa have to undergo physiological qualitative change named “capacitation” to be able to fertilize with ovum (Yanagimachi 1994). Capacitation consists of several physiological events such as acrosome reaction and hyperactivation. The acrosome reaction is an exocytosis of acrosomal content, whereas the hyperactivation is a specialized flagellar motility characterized by increased flagellar bending with asymmetric waveform. Both the acrosome reaction and the hyperactivation are reported to be necessary for the success of fertilization *in vivo* and *in vitro* (Yanagimachi 1994, Quill *et al.* 2003, Ho *et al.* 2009, Alasmari *et al.* 2013). Various factors and events are reported to be associated with the regulation of capacitation (Yanagimachi 1994, Visconti & Kopf 1998, Visconti *et al.* 1998, Fujinoki 2009, Fujinoki *et al.* 2015).

For example, capacitation/hyperactivation of mammalian spermatozoa is regulated by various ligands and hormones. In human spermatozoa, progesterone and melatonin regulate hyperactivation (du Plessis *et al.* 2010, Armon & Eisenbach 2011) and acrosome reaction (Baldi

et al. 2009). Such an effect of progesterone is suppressed by estrogen (Baldi *et al.* 2009). Gamma-aminobutyric acid (GABA) seems to increase hyperactivation in ram and human spermatozoa (Calogero *et al.* 1996, de las Heras *et al.* 1997, Ritta *et al.* 1998).

In hamster spermatozoa, hyperactivation is also regulated by various extracellular materials (Fujinoki 2009, Fujinoki *et al.* 2015). Sperm hyperactivation is enhanced by serotonin (Fujinoki 2011), melatonin (Fujinoki 2008), and progesterone (Noguchi *et al.* 2008), whereas enhancement of hyperactivation by progesterone is suppressed by estrogen (Fujinoki 2010) or GABA (Kon *et al.* 2014). The enhancement of hyperactivation by melatonin is also suppressed by estrogen (Fujinoki & Takei 2015). These hormones are known to change during female estrous cycle (Louzan *et al.* 1986, Libersky & Boatman 1995, Schillo 2009), and hyperactivation/capacitation is suggested to be regulated by monitoring the changing environment of the oviduct (Fujinoki 2008, Coy *et al.* 2012). In other words, the balance between facilitative factors and suppressive factors regulates the timing of hyperactivation/capacitation to occur precisely at the time when ovum reach to the ampulla after ovulation

(Fujinoki 2010, 2014, Kon *et al.* 2014, Fujinoki & Takei 2015). As mentioned above, abundant knowledge about the enhancing or facilitating factors of hyperactivation/capacitation already exists. However, although there are already some known factors that suppress the enhancing effect on hyperactivation, factors that suppress hyperactivation *per se* have not reported so far.

Meanwhile, spermatozoa of the species that undergo external fertilization, such as teleosts, activate flagellar motility in response to the drastic change of extracellular environment such as osmotic pressure and concentration of the K^+ (Alavi & Cosson 2006, Takei *et al.* 2012, 2015). The osmolality of the fluids from mammalian male genitalia is generally higher than that of other fluids (Yeung *et al.* 2006). Therefore, mammalian spermatozoa also experience osmotic shock upon ejaculation under physiological condition. The cellular volume regulatory mechanisms upon ejaculation-related osmotic shock have been shown to be essential to accomplish fertilization (Yeung *et al.* 2006, Chen & Duan 2011, Chen *et al.* 2011). However, the impact of osmotic shock on other aspects, such as motility and capacitation, has not yet been elucidated.

In this study, we first aimed to elucidate the impact of physiological osmotic shock on hamster sperm motility and hyperactivation. Then, we unexpectedly found that higher concentration of extracellular Na^+ ion suppressed motility and delayed hyperactivation of hamster spermatozoa. It is likely that Na^+-Ca^{2+} exchanger (NCX) is involved in the regulation of hamster hyperactivation by Na^+ .

Materials and methods

Reagents and solutions

$DisC_3$ (5) (3,3'-dipropylthiadicarbocyanine iodide) was from Invitrogen. Hepes, BCECF (2,7-biscarboxyethyl-5(6)-carboxyfluorescein)-AM, and fluo-4 AM were from Dojindo (Kumamoto, Japan). Bovine serum albumin (BSA) fraction V was purchased from Merk KGaA (Darmstadt, Germany). Na-gluconate was from Sigma-Aldrich. All other chemicals were purchased from Wako pure chemical.

Modified Tyrode's albumin lactate pyruvate medium (mTALP) consisted of 101 mmol/L NaCl, 2.68 mmol/L KCl, 0.36 mmol/L NaH_2PO_4 , 1.8 mmol/L $CaCl_2$, 0.49 mmol/L $MgCl_2$, 35.7 mmol/L $NaHCO_3$, 4.5 mmol/L glucose, 1 mmol/L sodium pyruvate, 9.0 mmol/L lactic acid, 0.5 mmol/L hypotaurine, 0.05 mmol/L (-)epinephrine, 0.2 mmol/L sodium taurocholate, 5.26 μ mol/L sodium metasulfate, 0.1 mmol/L EDTA, 0.05% (w/v) penicillin G, 0.05% (w/v) streptomycin sulfate, and 15 mg/mL BSA (Maleszewski *et al.* 1995). Osmotic pressure of mTALP was adjusted by changing the concentration of NaCl in a range of 75–150 mmol/L, or by adding 50–150 mmol/L mannitol. Tyrode's lactate pyruvate medium (TLP) solution was prepared by eliminating BSA from mTALP with 150 mmol/L NaCl. Sucrose- Ca^{2+} solution consisted of 300 mmol/L sucrose, 2.65 mmol/L $CaCl_2$, and 10 mmol/L Hepes-NaOH, pH 7.4.

Animals

Spermatozoa and fluids of the male and female genitalia were collected from sexually matured golden hamsters (*Mesocricetus auratus*). Hamsters were killed by overdose of isoflurane and the male genitalia were excised by dissection. The excised organs were used as described below. All experimental animals were kept and used in accordance with the guidelines of the Dokkyo Medical University, Mibu, Tochigi, Japan, and this study was approved by the Animal Care and Use Committee of Dokkyo Medical University (Experimental permission no.: 0107).

Collection of fluids and measurements of osmolality and concentration of elements

The fluid of the seminal vesicle was directly collected by syringe and 23-gauge needle. The fluid of prostate was collected as follows: the prostate was isolated by dissection, put into 1.5 mL tube, and minced. After centrifugation at 5000 g, 4°C for 10 min, supernatant was used as prostatic fluid. The epididymal fluid was collected in a similar way: epididymis was isolated by dissection and punched with needles, and the epididymal content was squeezed out and collected into 0.6 mL tube. After centrifugation at 6340 g, 4°C for 10 min, supernatant was used as epididymal fluid.

Oviductal fluid was collected from the females which were induced superovulation by intraperitoneal injection of 30 IU pregnant mare serum gonadotropin (PMSG) and 30 IU human chorionic gonadotropin (hCG), and was collected 3–5 h after administration of hCG. The collection of oviductal fluid was performed as reported previously (Libersky & Boatman 1995). Briefly, oviducts of anesthetized hamsters were cannulated according to a surgical procedure where the infundibulum of the oviduct is reflected out of the bursal sac. The cannula (Eppendorf GELoader; Eppendorf, Hamburg, Germany) was inserted through the fimbria until the tip reaches just inside the ampulla and then clamped with a nylon suture (Natsume Seisakusho, Tokyo, Japan). Both sides of oviducts were cannulated for 1 h, and the oviductal fluid filled in the cannula was collected and used for the measurements.

Osmotic pressure of the fluids and media was determined by osmometer using depression of freezing point technique (Osmomat 030-D; Gonotec, Berlin, Germany). The concentrations of Na, K, and Ca (including nonionic Na, K, and Ca) in the collected fluids were determined by atomic absorption spectrophotometer (Z-5300; Hitachi).

Preparation and analysis of hyperactivation

Spermatozoa were obtained from cauda epididymis. Hyperactivated spermatozoa were prepared and analyzed by the methods described previously with some modifications (Fujinoki *et al.* 2006). Hamsters were killed by overdose of isoflurane, and the caudal epididymis was excised. The excised cauda epididymis was punched with needle, and the epididymal spermatozoa were gently squeezed out from the epididymis. A drop of squeezed epididymal spermatozoa were put in 3.5-cm dish, diluted with mTALP, and incubated at 37°C in humid CO_2 incubator (5% CO_2 and 95% air) until observation for hyperactivation to occur.

Motility of the spermatozoa was observed by CCD camera (Progressive 3CCD; Sony, Tokyo, Japan) mounted on inverted phase-contrast microscopy (IX70; Olympus) equipped with a small CO₂ incubator (MI-IBC; Olympus) and recorded on videotapes by video recorder (NV-SX10; Panasonic, Osaka, Japan). Recordings were done every 30 min up to 3–4 h.

Inhibitors were dissolved in ethanol or dimethyl sulfoxide (DMSO) and added to the incubating medium at the start of the incubation. The concentration of the vehicle was fixed at 0.1% in all experiments.

Sperm motility was analyzed by manually counting the total spermatozoa, motile spermatozoa, and hyperactivated spermatozoa on slow-motion playback of the video recordings. Hyperactivation was defined as sperm motility with asymmetric and whiplash flagellar motion and a circular and/or octagonal swimming locus (Fujinoki *et al.* 2015). Curvilinear velocity (VCL) was determined manually by tracing the locus of the sperm head from video recordings onto the transparent plastic sheet. Beat frequency, bend angle, and sliding velocity were determined from the flagellar waveform traced manually onto transparent plastic sheet, as reported previously (Takei *et al.* 2014). Beat frequency was calculated from the number of video fields required to complete one beat cycle. The bend angle of the flagellum was determined by measuring the angle between the tangents at two adjacent points of inflection and then the flagellar bends reach the center of the flagellum. The angles were measured for both principal (pro-hook) and reverse (anti-hook) bends, and data were expressed as the sum of them. Sliding velocity was calculated as the product of beat frequency and bend angle (Takei *et al.* 2014). All the analyses were performed in the blinded way.

Measurement of membrane potential

Measurement of sperm membrane potential was performed as reported previously with slight modifications (Espinosa & Darszon 1995, Zeng *et al.* 1995, Izumi *et al.* 1999). Briefly, caudal epididymal spermatozoa were diluted directly with mTALP to a concentration of 10⁶–10⁷ cells/mL in quartz cuvette. Subsequently, 1 µmol/L CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) (final) was added to cuvette to cancel mitochondrial potential, and 1 µmol/L DisC₃(5) (final) was added to cuvette and incubated for 1 min at room temperature. After incubation, the fluorescence at 620 nm excitation/670 nm emission was measured by fluorescence spectrophotometer (F-4010; Hitachi). Subsequently, calibration of membrane potential was performed as described previously (Demarco *et al.* 2003) by adding 1 µmol/L valinomycin and then sequentially adding 2.66, 2, 4, and 8 µL 2 M KCl (corresponding to 2.68, 8, 12, 20, and 36 mmol/L K⁺ respectively). Intracellular K⁺ concentration used for the determination of membrane potential was estimated to be 90 mmol/L as reported previously (Zeng *et al.* 1995). The measured sperm membrane potentials correspond to those at the onset of incubation.

Measurement of intracellular pH

Measurement of sperm intracellular pH ([pH]_i) was determined as reported previously using fluorescent dye,

BCECF (Negulescu & Machen 1990, Takai & Morisawa 1995). In short, spermatozoa from cauda epididymis were suspended in sucrose–Ca²⁺ solution containing 4 µmol/L BCECF-AM and incubated at 37°C, 5% CO₂ for 30 min to load fluorescent dye. After incubation, spermatozoa were washed by brief centrifugation and resuspended in fresh sucrose–Ca²⁺ solution. Washed spermatozoa were diluted ten-fold by mTALP supplemented with 20 mmol/L Hepes–NaOH (pH 7.4) containing different concentration of NaCl, and the fluorescence was measured at both 440 nm and 490 nm excitation/single 530 nm emission by fluorescence spectrophotometer (F-4010; Hitachi). The [pH]_i value was calibrated by *in situ* calibration reported previously with slight modifications (Negulescu & Machen 1990, Takai & Morisawa 1995). Briefly, dye-loaded spermatozoa were suspended ten-fold in calibration solution containing 70 mmol/L K-gluconate, 20 mmol/L KCl, 10 mmol/L NaCl, 1 mmol/L MgCl₂, 10 mmol/L glucose, and 30 mmol/L Hepes–KOH at several values of pH (pH 6.5–7.5) with 10 µmol/L nigericin. The spermatozoa suspended in calibration solution were incubated for additional 30 min at 37°C, and the fluorescence was measured as described above. The calibration curve was drawn by plotting pH of the calibration solution against the fluorescence emission ratio of 490/440 nm. The [pH]_i values of the each experiment were determined from the fluorescence ratio of 490 to 440 nm using the calibration curve. The measured sperm [pH]_i values correspond to those at the onset of incubation.

As albumin in mTALP interfered with the measurement excitation at 440 nm, albumin in mTALP was substituted with 1 mg/mL polyvinyl alcohol (PVA) to support capacitation (Uto & Yamahama 1996).

Measurement of intracellular calcium

Concentration of intracellular Ca²⁺ ([Ca²⁺]_i) was measured with fluorescent dye, fluo-4. Caudal epididymal spermatozoa were suspended in TLP solution or sucrose–Ca²⁺ solution containing 4 µmol/L fluo-4, 2.5 mmol/L probenecid, and 0.1% Powerload and incubated for 30 min at 37°C, 5% CO₂ for loading the dye in hamster spermatozoa. After incubation, spermatozoa were washed by brief centrifugation and resuspended in fresh TLP or sucrose–Ca²⁺ solution. Washed spermatozoa were diluted 20-fold by mTALP with different concentration of NaCl, and fluorescence was measured at 490 nm excitation/515 nm emission by fluorescence spectrophotometer (F-4010; Hitachi).

The [Ca²⁺]_i was determined with the following equation:

$$K_d \frac{[F - F_{\min}]}{[F_{\max} - F]},$$

where *F* represents the measured fluorescence of the each experimental condition. *F*_{max} was the maximal fluorescence determined by addition of 0.15% triton X-100, and *F*_{min} was the minimum value of fluorescence subsequently determined by adding 9 mmol/L EGTA. The *K*_d value of 0.345 was used according to manufacturer's data sheet. The measured sperm [Ca²⁺]_i values correspond to those at the onset of incubation.

Fluorescence microscopy

Fluorescent dye-loaded hamster spermatozoa were observed as follows. Fluorescent dyes (BCECF and fluo-4) were loaded to hamster spermatozoa as mentioned above. After loading, spermatozoa were washed as described above and diluted 100-fold in 75–150 mmol/L NaCl mTALP. An aliquot of sperm suspension was placed onto glass slide, covered with coverslip, and observed and recorded by EMCCD camera (Luca-R; Andor Technology, Belfast, UK) attached to fluorescence microscopy (IX 70; Olympus). The observation was done with 460–480 nm excitation, 490 nm dichroic, and 495–540 nm emission.

Preparation of sperm fibrous sheath proteins

Protein samples of hamster sperm fibrous sheath were prepared as follows (Fujinoki *et al.* 2006). Hamster spermatozoa were suspended in mTALP and incubated for an indicated time at 37°C. After incubation, sperm suspension was centrifuged at 15,000 *g* for 10 min at 4°C. Pelleted spermatozoa were suspended in urea solution consisted of 7 M urea and 10% β -mercaptoethanol and incubated for 10 min on ice. Then sperm suspension was centrifuged again at 15,000 *g* for 10 min, 4°C. The pelleted samples were resuspended in urea-thiourea solution consisted of 5 M urea, 1 M thiourea, 10% β -mercaptoethanol, 10 mmol/L sodium pyrophosphate, 2% NP-40, and incubated for 10 min on ice. The suspension was centrifuged at 15,000 *g* for 10 min at 4°C, and the supernatant was used as the fibrous sheath proteins.

SDS-PAGE and western blotting

SDS-PAGE and western blotting were performed as reported previously with slight modifications (Laemmli 1970, Fujinoki *et al.* 2001). In short, fibrous sheath proteins prepared as mentioned above were mixed with sample buffer consisted of (final) 2.5% sodium dodecyl sulfate, 0.1% β -mercaptoethanol, and 78 mmol/L Tris-HCl pH 6.8 and boiled for 10 min at 100°C. Then boiled sample was electrophoresed in 3% polyacrylamide stacking gel and 10% polyacrylamide separating gel (w/v) and blotted to polyvinylidene fluoride membrane (Immobilon-P; Millipore). The blotted membrane was blocked by 5% (w/v) skim milk in Tris-buffered saline (TBS) consisted of 150 mmol/L NaCl and 20 mmol/L Tris-HCl pH 7.5 for 1 h at room temperature, washed four times with TBS, and then incubated with anti-phosphotyrosine mouse monoclonal antibody (clone PT-66, 1:1000 dilution in TBS supplemented with 20 mg/mL BSA; Sigma) for 1 h at room temperature. After washing four times with TBS, membrane was incubated with anti-mouse IgG antibody conjugated with peroxidase (1:10,000 dilution in TBS supplemented with 20 mg/mL BSA) for 1 h at room temperature. After washing

with TBS, immunoreactive proteins were detected using ECL Prime Western Blotting Detection Reagent (GE Healthcare) and Ez-capture MG (ATTO, Tokyo, Japan). Densitometry analyses of the bands were done by computer software CS Analyzer 3 (ATTO; Tokyo, Japan).

Statistical analysis

Statistical analysis was done with one-way ANOVA and Tukey–Kramer *post hoc* test. A *P*-value <0.05 was considered statistically significant.

Results

The osmolality and ionic composition of the fluids

First of all, the osmolality of the fluids of the hamster male genitalia (seminal vesicle, prostate, and cauda epididymis) and female genitalia (vaginal fluid and oviductal fluid) was investigated. As summarized in Table 1, the osmolality of the fluids of seminal vesicle, prostate, and cauda epididymis was 382.2 ± 9.7 , 358.4 ± 8.5 , and 371.4 ± 11.5 mOsm, respectively, and was higher than that of the blood plasma (327.2 ± 1.7 mOsm). The significant difference between seminal vesicle fluid and blood plasma and between epididymal fluid and blood plasma was observed. The osmolality of oviductal fluid (347.1 ± 18.6 mOsm) was also lower than that of the fluids from male genitalia, although there were no significant differences. These results indicate that the osmolality of semen is higher than that in the other fluids such as blood plasma and oviductal fluid, as reported in other mammals. By contrast, osmolality of the vaginal fluid (374.0 ± 13.9 mOsm) was almost the same as that of the fluids from male genitalia.

Then, the concentration of Na, K, and Ca of those fluids was measured (Table 2). The concentration of Na, K, and Ca was quite different among fluids. Na concentrations in the fluids of male genitalia (seminal vesicle, prostate, and cauda epididymis) were 51.73 ± 1.0 , 50.33 ± 2.70 , and 31.17 ± 1.64 mmol/L, respectively, and were significantly lower than that of blood plasma (149.4 ± 1.0 mmol/L). Na concentration in oviductal fluid was higher (158.79 ± 5.76 mmol/L) than that of blood plasma, although there were no significant differences between Na concentration of blood plasma and oviductal fluid.

In contrast to Na concentration, K concentration in seminal vesicle, prostate, and cauda epididymis (60.79 ± 1.60 , 46.30 ± 3.58 , and 24.85 ± 1.47 mmol/L respectively) was significantly higher than that of blood plasma (6.31 ± 0.32 mmol/L). In addition, K concentration of oviductal fluid (20.95 ± 3.74 mmol/L) was also significantly higher than in blood plasma.

Table 1 The osmolality of fluids from male and female genitalia.

	Blood plasma	Seminal vesicle	Prostate	Cauda epididymis	Oviduct	Vagina
Osmolality (mOsm)	327.2 ± 1.7^a	382.2 ± 9.7^b	$358.4 \pm 8.5^{a,b}$	$371.4 \pm 11.5^{a,b}$	$347.1 \pm 18.6^{a,b}$	$374.0 \pm 13.9^{a,b}$

All values are expressed in mOsm. Data are presented as mean $\pm$ s.e.m., *n* = 10 except for oviductal fluid. The osmolality value of oviductal fluid was the mean of four measurements and 7–20 hamsters were used per one measurement of oviductal fluid. The different superscript letters represent significant differences (*P* < 0.05).

Table 2 The Na⁺, K⁺ and Ca²⁺ concentrations of fluids of male and female genitalia were measured.

	Blood plasma	Seminal vesicle	Prostate	Cauda epididymis	Oviduct
Na (mmol/L)	149.40±0.95 ^a	51.73±1.05 ^b	50.33±2.70 ^b	31.17±1.64 ^c	158.79±5.76 ^a
K (mmol/L)	6.31±0.32 ^a	60.79±1.60 ^d	46.30±3.58 ^c	24.85±1.47 ^b	20.95±1.87 ^b
Ca (mmol/L)	2.36±0.09 ^b	0.39±0.06 ^d	3.08±0.20 ^a	0.21±0.01 ^d	1.42±0.16 ^c

All ionic species are expressed in mmol/L. Data are presented as mean±s.e.m., $n=10$ except for oviductal fluid. The ionic concentration value of oviductal fluid was the mean of four measurements and 7–20 hamsters were used per one measurement of oviductal fluid. Different superscript letters represent significant differences ($P<0.05$).

Ca concentration of caudal epididymal fluid (0.21 ± 0.01 mmol/L) and seminal vesicle fluid (0.39 ± 0.06 mmol/L) was significantly lower than that of blood plasma (2.36 ± 0.09 mmol/L), prostatic fluid (3.08 ± 0.20 mmol/L), and oviductal fluid (1.42 ± 0.16 mmol/L).

Hyperactivation was delayed when the osmolality of the media was increased by NaCl

To investigate the effect of seminal high osmotic pressure on hamster sperm motility and hyperactivation, we used mTALP of which osmotic pressures were adjusted to 230–370 mOsm by changing the NaCl concentration within 75–150 mmol/L. As shown in Fig. 1A, the percentages of motile spermatozoa were not affected when the osmotic pressure of mTALP was changed by NaCl. By contrast, VCL of hamster spermatozoa at 0 h was significantly decreased as the osmotic pressure increased by NaCl (Fig. 1B). After incubation for 0.5 h, VCL in 370 mOsm mTALP was increased, but still significantly lower than those in 230–330 mOsm mTALP. The significant differences of VCL were disappeared when the incubation time progressed to 1 and 1.5 h (Fig. 1B). The appearance of hyperactivation was delayed when osmotic pressure of mTALP was increased by NaCl (Fig. 1C). At 1 h, hyperactivation in 230 mOsm (75 mmol/L NaCl) mTALP was significantly higher than the other mTALP. At 1.5 h, almost 50% spermatozoa in 230 mOsm (75 mmol/L NaCl) mTALP showed hyperactivated motility, whereas nearly 0% spermatozoa showed hyperactivated motility in 370 mOsm (150 mmol/L NaCl) mTALP. The hyperactivation in 280 mOsm (101 mmol/L NaCl) and 330 mOsm (125 mmol/L NaCl) showed halfway value between 230 mOsm and 370 mOsm. At 2 h incubation, spermatozoa in 230 mOsm (75 mmol/L NaCl) mTALP reached a plateau at 80%, significantly higher than those in 330 mOsm (125 mmol/L NaCl) and 370 mOsm (150 mmol/L NaCl) mTALP. At 2.5 h incubation, hyperactivation in 370 mOsm (150 mmol/L NaCl) mTALP was still lower than the other mTALPs. At 3 h incubation, there were no differences in hyperactivation among the various osmotic pressures.

To examine whether extracellular NaCl affects motility parameters other than VCL and hyperactivation, we measured the time–course change of flagellar movement (beat frequency, bend angle, sliding velocity, and waveform). The results are shown in Fig. 2. Overall, beat frequency

was slightly increased at 0.5 h of incubation and gradually decreased as incubation time progressed (Fig. 2A). When osmotic pressure was changed by NaCl, beat frequency was increased as the osmotic pressure increased by NaCl, highest in 330 mOsm (125 mmol/L NaCl) mTALP and lowest in 230 mOsm (75 mmol/L NaCl) mTALP (Fig. 2A). The beat frequency remained significantly higher at 1 and 1.5 h incubation in the 330 and 370 mOsm (125 and 150 mmol/L NaCl) mTALP. At 2 and 2.5 h of incubation, beat frequency was decreased to approximately 9 Hz and almost unchanged among all conditions.

Bend angle of the flagellum was lower at the onset of incubation and gradually increased as incubation time progressed, as a whole (Fig. 2B). When osmotic pressure was changed by NaCl, bend angle was decreased as osmotic pressure of the mTALP was increased by NaCl, i.e., largest in 230 mOsm (75 mmol/L NaCl) mTALP and smallest in 370 mOsm (150 mmol/L NaCl) mTALP (Fig. 2B). The difference of bend angle was most prominent at the onset of incubation and gradually reduced as incubation time progressed. Finally, no difference in bend angle was observed among all conditions at 2 and 2.5 h of incubation (Fig. 2B). When we looked at principal bend and reverse bend separately, both principal and reverse bend increased dependent on time (Fig. 2C and D). Higher osmotic pressure by NaCl suppressed both principal and reverse bend at the onset of incubation. The difference of bend angle was gradually reduced as incubation time progressed (at 0.5–1.5 h of incubation). At 2 h of incubation, no difference in principal bend was observed among 230–370 mOsm (75–150 mmol/L NaCl) mTALP, whereas reverse bend was still significantly lower in 370 mOsm (150 mmol/L NaCl) mTALP when compared with 230 and 280 mOsm (75 and 101 mmol/L NaCl) mTALP (Fig. 2C and D). There was no difference in reverse bend at 2.5 h of incubation among all conditions (Fig. 2D).

As a whole, sliding velocity was lower at the onset of incubation and gradually increased as incubation time progressed (Fig. 2E). When osmotic pressure was changed by NaCl, sliding velocity was suppressed as osmotic pressure increased by NaCl at 0 h, highest in 230 mOsm (75 mmol/L NaCl) mTALP, and lowest in 370 mOsm (150 mmol/L NaCl) mTALP (Fig. 2E). The suppression of sliding velocity by osmotic pressure was reduced as incubation time progressed at 0.5–1 h, and significant difference was disappeared at 1.5 h of incubation and thereafter (Fig. 2E).

Figure 1 The effect of the difference of osmolality adjusted by NaCl on hyperactivation and motility of hamster spermatozoa. The effect of osmotic pressure of media on percentage of motile sperm (A), curvilinear velocity (VCL; B), and hyperactivation (C) was examined using mTALP whose osmotic pressure was adjusted to 230–370 mOsm by 75–150 mmol/L NaCl. Data are expressed as mean $\pm$ S.E.M., $n=4$ (A) and (C). (B) VCL value was determined as the mean of 24 sperm cells from four males. The different superscript letters represent significant differences among them ($P < 0.05$).

Figure 2 The effect of the difference of osmolality adjusted by NaCl on flagellar movement of hamster spermatozoa. The effect of osmotic pressure of media on beat frequency (A), bend angle (B), bend angle of principal bend (C), reversed bend (D), sliding velocity (E), and flagellar waveform (F) was examined in the same condition as in Fig. 1. (A, B, C, D and E) Values were determined as the mean of 15 sperm cells from three males. The different superscript letters represent significant differences among them ($P < 0.05$). (F) Flagellar waveforms in 230 mOsm (75 mmol/L NaCl) (a, e), 280 mOsm (101 mmol/L NaCl) (b, f), 330 mOsm (125 mmol/L NaCl) (c, g), and 370 mOsm (150 mmol/L NaCl) (d, h) mTALP at 0 h (a–d; activated) and at 2.5 h (e–h; hyperactivated) were traced for three beat cycle.

Waveform of hamster spermatozoa at 0 h (activated) and 2.5 h (hyperactivated) was shown in Fig. 2F. At 0 h of incubation, activated spermatozoa showed considerable change in beat amplitude (Fig. 2F a–d). In 230 mOsm

Figure 3 The effect of the difference of osmolality changed by NaCl and mannitol on hyperactivation of hamster spermatozoa. The effect of osmolality and NaCl concentration on the percentage of motile spermatozoa (A) and hyperactivation (B, C, D and E) is shown. (B) Time-course change of hyperactivation and (C, D and E) percentage of hyperactivation at 1, 1.5, and 2 h respectively. Data are expressed as mean $\pm$ S.E.M., $n = 4$. The different superscript letters represent significant differences among them.

(75 mmol/L NaCl) mTALP, spermatozoa showed fairly large amplitude (Fig. 2F a). Increase in osmotic pressure by NaCl caused narrowing of the wave amplitude, and narrowest in 370 mOsm (150 mmol/L NaCl) mTALP (Fig. 2F b–d). When hyperactivated, hamster spermatozoa exhibited almost the same waveforms in all NaCl concentrations (Fig. 2F e–h).

The delay of hyperactivation was caused by the increase in extracellular NaCl

We next investigated the effect of NaCl concentrations and osmotic pressure on hyperactivation by using mTALP of which NaCl concentrations varied from 75 to 150 mmol/L, whereas osmotic pressure was fixed at 330 or 370 mOsm by mannitol.

The results are shown in Fig. 3. The percentages of motile spermatozoa were unaffected when the concentration of NaCl and mannitol was changed (Fig. 3A). Hyperactivation of hamster spermatozoa

was delayed as NaCl concentration of the media was increased (Fig. 3B), as is shown in Fig. 1. When the osmotic pressure was set to 370 mOsm by adding mannitol, however, hamster sperm hyperactivation was considerably differed depending on the concentration of NaCl (Fig. 3B, C, D and E). At 1 and 1.5 h of incubation, hyperactivation was significantly higher in mTALP containing 75 mmol/L NaCl (75 mmol/L NaCl, 75 mmol/L NaCl + 50 mmol/L mannitol, 75 mmol/L NaCl + 100 mmol/L mannitol, and 75 mmol/L NaCl + 150 mmol/L mannitol) than those in mTALP containing higher concentrations of NaCl, regardless of the osmotic pressure of the mTALP (Fig. 3C and D). No significant differences were observed among mTALPs containing 75 mM NaCl (Fig. 3C and D). At 2 h of incubation, hyperactivation in mTALP containing 75 mmol/L NaCl was higher than that in mTALP containing 101–150 mM NaCl, although significant differences were not observed in some cases (Fig. 3E). In addition, there was no significant difference in hyperactivation among mTALP containing 101 mmol/L NaCl with different concentrations of mannitol at 1, 1.5, and 2 h of incubation (101 mmol/L NaCl, 101 mmol/L NaCl + 50 mmol/L mannitol, and 101 mmol/L NaCl + 100 mmol/L mannitol; Fig. 3C, D and E). These results suggest that delay of hamster sperm hyperactivation was caused dependently on NaCl concentration, but not osmolality of the media.

To examine the effect of Cl[−] concentration on hamster sperm hyperactivation, we used mTALP in which Cl[−] concentrations varied, whereas Na⁺ concentration was fixed by a combination of Na-gluconate and NaCl (Supplementary Figure 1, see section on supplementary data given at the end of the article). The results showed that the decrease of Cl[−] by substituting it with gluconate[−] caused further delay of the appearance of hyperactivation than 150 mmol/L NaCl mTALP (Supplementary Figure 1B). We also tested mTALP where Na⁺ concentrations varied whereas Cl[−] concentration was fixed by a combination of NaCl and choline chloride or NaCl and *N*-methyl-D-glucamin (NMDG)–Cl (Supplementary Figure 2). However, substitution of Na⁺ with choline⁺ significantly suppressed hyperactivation (Supplementary Figure 2B). In addition, the replacement of Na⁺ with NMDG⁺ caused significant alteration of the percentage of motile spermatozoa (Supplementary Figure 2C).

Extracellular Na⁺ did not alter the membrane potential or intracellular pH of hamster spermatozoa

The effect of extracellular Na⁺ on hamster sperm membrane potentials and intracellular pH (pH_i) was investigated using fluorescent dye, DiSC₃(5) and BCECF (Fig. 4). The membrane potential of hamster spermatozoa was -75.4 ± 2.1 mV in standard mTALP, which contains 101 mmol/L NaCl (Fig. 4A). The alteration of NaCl concentration in mTALP did not cause significant change in hamster sperm membrane potential (Fig. 4A). Hamster

Figure 4 The effect of NaCl on hamster sperm membrane potential and pH_i was examined by fluorescent dye DisC₃ (5) and BCECF respectively. The determined value of membrane potentials (A), pH_i (B), and fluorescent picture of BCECF-loaded spermatozoa in mTALP with various concentrations of NaCl (C) was shown. (C) a, 75 mmol/L NaCl; b, 101 mmol/L NaCl; c, 125 mmol/L NaCl; and d, 150 mmol/L NaCl. Left panels are fluorescent images and right panels are bright-field images. There was no significant difference among all the conditions tested. Data are expressed as mean $\pm$ S.E.M., $n=6$ in membrane potential and $n=4$ in pH_i .

sperm pH_i was 6.91 ± 0.06 in standard mTALP (101 mmol/L NaCl), whereas BSA was substituted with 1 mg/mL PVA (Fig. 4B). The alteration of NaCl concentration in mTALP did not cause significant change in hamster sperm pH_i (Fig. 4B). When BCECF-loaded spermatozoa were observed by fluorescence microscopy, the entire region of hamster sperm was fluorescent, prominent in head and midpiece, while rather faint in principal piece (Fig. 4C). No apparent difference was observed when extracellular NaCl concentration was changed (Fig. 4C a–d). When pH_i was determined using mTALP containing BSA, pH_i was calculated as 5.6–5.7 because of the high fluorescence at 440 nm excitation caused by BSA (Supplementary Figure 3). However, there was no difference in hamster sperm pH_i when the concentration of NaCl in mTALP varied from 75 to 150 mmol/L (Supplementary Figure 3).

Hamster sperm intracellular Ca^{2+} concentration increased as the extracellular NaCl concentration decreased

The effect of extracellular Na^+ on hamster sperm intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was investigated by fluorescent dye, fluo-4. The results are shown in

Fig. 5. The $[Ca^{2+}]_i$ value was expressed as the ratio to the fluorescence value of 75 mmol/L NaCl. Hamster sperm $[Ca^{2+}]_i$ was decreased as the extracellular Na^+ concentration increased (Fig. 5A). Significant differences were observed between 75 and 125 mmol/L NaCl, and between 75 and 150 mmol/L NaCl (Fig. 5A). Similar results were obtained when fluo-4 was loaded into sperm cells in sucrose- Ca^{2+} solution (Supplementary Figure 4).

When fluo-4-loaded spermatozoa were observed by fluorescence microscopy, a strong fluorescence was observed in acrosome in all conditions (Fig. 5B a–d). In addition, midpiece and principal piece were also fluorescent in 75 and 101 mmol/L NaCl mTALP (Fig. 5B a and b), whereas no fluorescent in flagella was observed in 125 and 150 mmol/L NaCl mTALP (Fig. 5B c and d).

The NCX inhibitors abolished the delay of hyperactivation by high concentration of Na^+

Decrease in intracellular Ca^{2+} by increase in extracellular Na^+ let us think that NCX is involved in the regulation of hamster sperm hyperactivation (Fig. 5). To examine this possibility, we investigated the effect of NCX inhibitors (SN-6, SEA0400, and KB-R7943) on hyperactivation and flagellar movement in 150 mmol/L NaCl mTALP. The results are shown in Figs 6 and 7. SN-6 and SEA0400 did not affect the percentage of motile spermatozoa up to 50 μ mol/L (Fig. 6A and D). By contrast, the percentage of motile spermatozoa was significantly decreased by 50 μ mol/L KB-R7943 at 0,

Figure 5 The effect of extracellular NaCl on hamster sperm intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). The effect of NaCl on hamster sperm $[Ca^{2+}]_i$ was examined by fluorescent dye fluo-4. (A) The determined values of $[Ca^{2+}]_i$ by extracellular Na^+ . Data are expressed as the ratio to the value of 75 mmol/L NaCl mTALP. The different superscript letters represent significant difference ($P < 0.05$). Data are expressed as mean $\pm$ S.E.M., $n=11$. (B) The fluorescent pictures of fluo-4-loaded spermatozoa in mTALP with various concentrations of NaCl (a, 75 mmol/L NaCl; b, 101 mmol/L NaCl; c, 125 mmol/L NaCl; d, 150 mmol/L NaCl). Left panels are fluorescent images and right panels are bright-field images.

Figure 6 The effect of NCX inhibitors on hyperactivation of hamster spermatozoa. The effects of NCX inhibitors (A, B and C: SN-6, D, E and F: SEA0400) on percentage of motile sperm (A and D) and time-course change of hyperactivation (B and E) and hyperactivation at 2 h (C and F) in 150 mmol/L NaCl mTALP are shown. Vehicle was ethanol and DMSO (1:1), and the final concentration of vehicle was set to 0.1%. The different superscript letters represent significant difference ($P < 0.05$). Data are expressed as mean $\pm$ S.E.M., $n = 4$.

0.5, 1.5, and 2.5 h of incubation when compared with controls (Supplementary Figure 5A).

Hyperactivation was significantly delayed in mTALP containing 150 mmol/L NaCl than in mTALP containing 101 mmol/L NaCl, as shown in Figs 1 and 3 (Fig. 6B and C). When SN-6 was added to mTALP containing 150 mmol/L NaCl, SN-6 dose dependently canceled the suppression of hyperactivation (Fig. 6B and C). At 2 h of incubation, hyperactivation in 101 mmol/L NaCl mTALP was significantly higher than that in 150 mmol/L NaCl mTALP (Fig. 6C). Addition of 50 $\mu\text{mol/L}$ SN-6 to 150 mmol/L NaCl mTALP significantly increased hamster sperm hyperactivation compared with 150 mmol/L NaCl + vehicle (Fig. 6C).

SEA0400, another inhibitor of NCX, had similar effect on hamster sperm hyperactivation (Fig. 6D, E and F). Hamster sperm hyperactivation in 150 mmol/L NaCl mTALP was accelerated by SEA0400 compared with vehicle in a dose-dependent manner, and 50 $\mu\text{mol/L}$ SEA0400 in 150 mmol/L NaCl mTALP significantly increased hyperactivation than vehicle in 150 mmol/L

NaCl mTALP at 2 h of incubation (Fig. 6E and F). KB-R7943 showed similar tendency with two other NCX inhibitors to accelerate hyperactivation in a dose-dependent manner; however, there were no significant changes in hyperactivation by KB-R7943 (Supplementary Figure 5B).

Hamster sperm $[\text{Ca}^{2+}]_i$ was significantly increased by 50 $\mu\text{mol/L}$ SN-6 and 50 $\mu\text{mol/L}$ SEA0400 compared with

Figure 7 The effect of NCX inhibitors on $[\text{Ca}^{2+}]_i$ and flagellar movement of hamster spermatozoa. The effects of 50 $\mu\text{mol/L}$ SN-6 (A, B, C, D, E and F), 50 $\mu\text{mol/L}$ SEA0400 (A), and 50 $\mu\text{mol/L}$ KB-R7943 (A) on $[\text{Ca}^{2+}]_i$ (A), beat frequency (B), bend angle (C), principal bend (D), reverse bend (E), sliding velocity (F), and waveform (G) in 150 mmol/L NaCl mTALP are shown. $[\text{Ca}^{2+}]_i$ data are represented as ratio to the value of 150 mmol/L NaCl + vehicle. Data are expressed as mean $\pm$ S.E.M., $n = 4$ (50 $\mu\text{mol/L}$ SN-6) and $n = 7$ (the others) in (A). In (B, C, D, E and F), values were determined as the mean of 15 sperm cells from three males. Asterisks indicate significant difference compared with vehicle ($P < 0.05$). (G) Flagellar waveforms in 150 mmol/L NaCl mTALP with 50 $\mu\text{mol/L}$ SN-6 at 0 h (a, activated) and at 2.5 h (b, hyperactivated) traced for three beat cycle were shown.

Figure 8 The effect of amiloride on hamster sperm hyperactivation. The effects of amiloride, an inhibitor of ENaC, on the percentage of motile spermatozoa (A) and hyperactivation (B) are shown. Vehicle was distilled water, and the concentration of the vehicle was set to 0.1%. There was no significant difference at all the concentration tested. Data are expressed as mean $\pm$ S.E.M., $n = 4$.

vehicle (Fig. 7A). KB-R7943 at 50 μ mol/L also increased $[Ca^{2+}]_i$; however, there were no significant differences between 50 μ mol/L KB-R7943 and vehicle (Fig. 7A).

Inhibition of NCX by SN-6 also affected parameters of flagellar movement (Fig. 7B, C, D, E, F and G). SN-6 (50 μ mol/L) increased beat frequency at 0 h and caused earlier decrease at 1.5 h (Fig. 7B). It did not affect bend angles (sum of principal and reverse bends) when compared with 150 mmol/L NaCl mTALP (Fig. 7C), but increased reverse bend at 2 h when we looked at principal and reverse bends separately (Fig. 7D and E). Sliding velocity increased by 50 μ mol/L SN-6 at 0 h incubation but was not affected by SN-6 at the other time of incubation (Fig. 7F). The waveform of activated spermatozoa (Fig. 7G a) and hyperactivated spermatozoa (Fig. 7G b) was apparently unaffected by SN-6 in comparison with other conditions (Fig. 2F).

Amiloride, an inhibitor of epithelial Na^+ channel (ENaC), had no effect on hyperactivation in the concentration range 0.1–20 mmol/L (Fig. 8).

Tyrosine phosphorylations on fibrous sheath proteins were not changed by extracellular Na^+

The change in capacitation-associated tyrosine phosphorylation of fibrous sheath proteins by extracellular Na^+ was investigated by western blotting (Fig. 9 and Table 3). In standard mTALP that contains 101 mmol/L NaCl, time-dependent increase in the tyrosine phosphorylation on two major bands, which was likely to correspond with the 80 and 85 kDa AKAPs, was observed (Fig. 9B arrowheads and Table 3). When extracellular Na^+ was decreased to 75 mmol/L, no significant change of the density of 80 and 85 kDa bands were observed compared with that in 101 mmol/L NaCl (Fig. 9A and Table 3). Similarly, the density of 80 and 85 kDa bands from spermatozoa in 150 mmol/L NaCl did not show significant change (Fig. 9C and Table 3). In addition, 50 μ mol/L SN-6 in 150 mmol/L NaCl mTALP (Fig. 9E) did not cause marked change in the density of 80 and 85 kDa bands when compared with those in 150 mmol/L NaCl mTALP (Fig. 9C and Table 3) and 150 mmol/L NaCl mTALP + vehicle (Fig. 9D and Table 3).

Discussion

Hyperactivation is widely assumed to be necessary for the success of fertilization *in vivo* (Yanagimachi 1994, Quill *et al.* 2003, Ho *et al.* 2009). In addition, mammalian spermatozoa have to be hyperactivated in right timing, e.g. when ovum is ovulated into the ampulla of oviduct where fertilization occurs. It was previously proposed that the balance of facilitative factors and suppressive factors for hyperactivation controls the expression timing of hyperactivation. In contrast to the abundant knowledge on several facilitative factors for hyperactivation (Fujinoki 2008, 2011, Noguchi *et al.* 2008), however, there are few known suppressive factors for hyperactivation. Estrogen $17\beta E_2$ and GABA suppress the enhancement of hyperactivation by progesterone, but do not suppress hyperactivation itself (Fujinoki 2010, Kon *et al.* 2014). The proteins secreted from seminal vesicle, semenogelin in human sperm and SVS2 in mouse sperm, are known to act as a “decapacitation factor”, but is unclear whether it affects hyperactivation or not (de Lamirande *et al.* 2001, Kawano & Yoshida 2007). In this study, we discovered the suppressive or delaying factor of the hyperactivation for the first time, i.e., Na^+ . This study could be the important piece for the full understanding of the regulatory mechanisms of fertilization *in vivo*.

In this study, we first investigated the effect of osmotic pressure, and then we unexpectedly found

Figure 9 Tyrosine phosphorylation of hamster sperm fibrous sheath proteins. The time-course changes (0, 0.5, 1, 1.5, 2, and 3 h) of tyrosine phosphorylation of hamster sperm fibrous sheath proteins in various conditions are shown. (A, B, C, D and E) Pictures of enhanced chemiluminescence (right panel) and CBB staining of the corresponding gel (left panel). (A) 75 mmol/L NaCl mTALP. (B) 101 mmol/L mTALP. (C) 150 mmol/L mTALP. (D) 150 mmol/L mTALP+vehicle. (E) 150 mmol/L mTALP+50 μ mol/L SN-6. Vehicle was ethanol and DMSO (1:1), and the final concentration was set to 0.1%. The 85 and 80 kDa AKAPs are indicated by arrowheads on the right side. The molecular sizes are shown on left side.

that extracellular Na⁺ acts as a suppressive factor on hyperactivation (Figs 1 and 3). Osmotic environments did not affect hamster sperm motility or hyperactivation in the physiological range (Figs 1, 2 and 3). The effect of extracellular Na⁺ on mouse sperm capacitation was reported previously (Fraser *et al.* 1993); however, the result was apparently contradictory. In the previous study, increase in extracellular Na⁺ caused increase in AR pattern of chlortetracycline (CTC) staining in mouse spermatozoa, indicating that capacitation and acrosome reaction were promoted by Na⁺ (Fraser *et al.* 1993). This contradiction might come from the difference of the aspect of capacitation-related event focused on hyperactivation in this study and the change of CTC staining in the previous study (Fraser *et al.*

1993). Spermatozoa are needed to be fixed before CTC staining, so the change of flagellar movement was unable to be assessed in the previous study (Fraser *et al.* 1993). Therefore, the effect of extracellular Na⁺ on hyperactivation might be overlooked in the previous study. In addition, difference of species used (hamster and mouse) may also affect the difference of results.

Extracellular Na⁺ also affected the flagellar movement of hamster spermatozoa (Fig. 2). At the onset of incubation, activated hamster spermatozoa showed higher beat frequency and lower bend angles as the concentration of NaCl in mTALP increased (Fig. 2A and B). Consequently, sliding velocity of the flagellum was significantly decreased as NaCl concentration of mTALP increased at 0, 0.5, and 1 h of incubation

Table 3 Densitometry of 85 and 80 kDa bands.

Hour	0	0.5	1	1.5	2	3
85 kDa						
75 mmol/L NaCl mTALP	0.139 $\pm$ 0.133	0.326 $\pm$ 0.141	1.043 $\pm$ 0.135	1.72 $\pm$ 0.439	1.77 $\pm$ 0.329	2.04 $\pm$ 0.513
101 mmol/L NaCl mTALP	0.269 $\pm$ 0.256	0.422 $\pm$ 0.119	3.37 $\pm$ 0.523	4.74 $\pm$ 0.916	3.71 $\pm$ 1.21	4.44 $\pm$ 2.08
150 mmol/L NaCl mTALP	0.082 $\pm$ 0.063	0.228 $\pm$ 0.033	1.09 $\pm$ 0.052	1.99 $\pm$ 0.282	2.26 $\pm$ 0.284	2.05 $\pm$ 0.469
150 mmol/L NaCl mTALP+vehicle	0.195 $\pm$ 0.083	0.541 $\pm$ 0.076	2.14 $\pm$ 0.537	3.39 $\pm$ 1.14	3.01 $\pm$ 0.944	2.53 $\pm$ 0.503
150 mmol/L NaCl mTALP+50 μ mol/L SN-6	0.073 $\pm$ 0.055	0.159 $\pm$ 0.068	2.35 $\pm$ 1.17	2.60 $\pm$ 1.16	2.28 $\pm$ 1.00	3.07 $\pm$ 1.43
80 kDa						
75 mmol/L NaCl mTALP	0.165 $\pm$ 0.138	0.114 $\pm$ 0.082	0.321 $\pm$ 0.051	0.707 $\pm$ 0.243	0.799 $\pm$ 0.276	1.20 $\pm$ 0.343
101 mmol/L NaCl mTALP	0.272 $\pm$ 0.211	0.191 $\pm$ 0.155	1.23 $\pm$ 0.152	2.41 $\pm$ 0.636	2.55 $\pm$ 0.829	2.65 $\pm$ 1.27
150 mmol/L NaCl mTALP	0.125 $\pm$ 0.105	0.044 $\pm$ 0.004	0.336 $\pm$ 0.086	0.765 $\pm$ 0.175	1.01 $\pm$ 0.029	1.30 $\pm$ 0.152
150 mmol/L NaCl mTALP+vehicle	0.210 $\pm$ 0.065	0.0569 $\pm$ 0.012	1.06 $\pm$ 0.191	2.41 $\pm$ 0.728	2.39 $\pm$ 0.672	2.20 $\pm$ 0.420
150 mmol/L NaCl mTALP+50 μ mol/L SN-6	0.025 $\pm$ 0.012	0.0431 $\pm$ 0.024	1.28 $\pm$ 0.620	1.82 $\pm$ 0.802	1.55 $\pm$ 0.649	1.80 $\pm$ 0.810

Data were expressed as the ratio to the density of the 50 kDa band of marker Magic Marks. Data were expressed as mean of three membranes $\pm$ S.E.M. Statistical analyses were done by one way ANOVA and Tukey-Kramer *post hoc* test, and there were no significant differences among them.

(Fig. 2E). This decrease in sliding velocity directly led to the suppression of VCL (Fig. 1B). Bend angle as well as sliding velocity increased as incubation time progressed after 1.5 h, and these increments seemed to be reflective of the increase in VCL (Figs 1B and 2B, E). These results suggest that not only hyperactivation but also activation, a vigorous motility at the beginning of incubation, of hamster spermatozoa was suppressed by extracellular Na^+ . In the previous study, it was reported that mouse sperm activation (swimming velocity and beat frequency) increased as the concentration of NaCl increased (Si & Okuno 1993). The regulation of hamster sperm beat frequency is likely to be regulated by NaCl in the same manner as mouse sperm. However, the results of swimming velocity were totally different. As the pattern of sperm flagellar movement (bending pattern and waveform) is quite different between mice and hamsters (Yanagimachi 1994), this contradiction might come from the difference of pattern of flagellar movement.

As incubation time progressed, beat frequency was gradually decreased, whereas bend angle and sliding velocity were gradually increased (Fig. 2A, B and E). At the same time, hyperactivation was gradually increased (Fig. 1C). Therefore, decrease of beat frequency and concomitant increase in bend angle are required for the expression of hyperactivated motility. At 2 h of incubation, the bend angle and beat frequency in 150 mmol/L NaCl mTALP was comparable to those in the other mTALPs (Fig. 2A and B). However, hyperactivation in 150 mmol/L NaCl mTALP was significantly lower at 2 h of incubation (Fig. 1C). Similarly, beat frequency, bend angle, and sliding velocity of SN-6-treated spermatozoa at 2 h showed no significant change to those in 150 mmol/L NaCl mTALP, although hyperactivation at 2 h was significantly higher than vehicle (Figs 6C and 7B, C, F). When principal and reverse bends were considered separately, the principal bend at 2 h was unchanged among all conditions, whereas the reverse bend at 2 h was significantly lower in 150 mmol/L NaCl mTALP (Figs 2C, D and 7D, E). These results indicate that an increase in reverse bend is necessary for the expression of hyperactivated motility. The parameters of hyperactivated sperm flagellar movement and waveform were unchanged by NaCl concentration and SN-6, indicating that NaCl concentration or SN-6 does not affect hyperactivation itself (Figs 2 and 7).

Seemingly, suppression of activation by Na^+ directly led to the delay in hyperactivation. However, bend angle and waveform of SN-6-treated spermatozoa did not show remarkable change as 150 mmol/L NaCl mTALP at the onset of incubation, although they showed hyperactivation significantly earlier (Figs 6 and 7). These results suggest that although Na^+ concentration affected both hyperactivation and activation, regulatory mechanisms of them are different.

The Na concentration of oviductal fluid was 158.79 ± 5.76 mmol/L (Table 2), similar to the Na^+

concentration of 125 mmol/L NaCl mTALP, which contains approximately 163 mmol/L Na^+ . At this concentration, hyperactivation was slightly delayed compared with 101 mmol/L NaCl mTALP and was significantly delayed than that in 75 mmol/L NaCl mTALP (Fig. 1C). These results suggest that oviductal environment is rather "suppressive" from the viewpoint of Na^+ concentration. It seems that the release from suppression by Na^+ plays an important role in the expression of hyperactivated motility. In fluids of male genitalia (seminal vesicle, prostate, and epididymis), Na concentration is low, which seems to facilitate hyperactivation (Table 2). In these fluids, however, concentrations of K and Ca are also quite different, so we are unable to conclude that seminal environment is facilitative or not. High concentration of K in prostatic fluid seems to be caused by destruction of cells upon collection (Table 2). However, determined concentration of K in hamster prostatic fluid was very close to that of human prostatic fluid collected by noninvasive way (Kavanagh 1985), so we assume that the way of collection of fluids affected minimum on determination of concentration.

When Cl^- decreased by replacing it with gluconate, hyperactivation was further delayed but not accelerated (Supplementary Figure 1). This suggests that delay of hyperactivation by increasing the concentration of NaCl was not caused by increase in Cl^- . However, this result also suggests that Cl^- also participates in the regulation of hyperactivation in hamster spermatozoa. However, decrease in Na^+ concentration by replacing it with choline⁺ (or NMDG⁺) caused delay of hamster sperm hyperactivation more severely than decrease in Cl^- (Supplementary Figures 1 and 2). These results suggest that the balance of Na^+ and Cl^- plays a vital role in the regulation of hyperactivation, and Na^+ is more crucial than Cl^- for hyperactivation.

In mouse spermatozoa, membrane potential is hyperpolarized during capacitation (Zeng *et al.* 1995), and such hyperpolarization is reported to be necessary for acrosome reaction (De La Vega-Beltran *et al.* 2012). In addition, capacitation-associated hyperpolarization is regulated by Na^+ conductance by ENaC (Hernandez-Gonzalez *et al.* 2006, Escoffier *et al.* 2012). In contrast to these facts, hamster sperm membrane potentials were not changed by extracellular Na^+ concentration (Fig. 4A). In addition, amiloride, an inhibitor of ENaC, did not affect hamster sperm hyperactivation (Fig. 8), suggesting that membrane potential is not involved in the regulation of hyperactivation by Na^+ . These results mean that the characteristics of spermatozoa are quite different even in hamsters and mice from the viewpoint of membrane potential.

Generally, pH_i increases during capacitation (Zeng *et al.* 1996, Nakanishi *et al.* 2001). In addition, Na^+ - HCO_3^- cotransporter (NBC) and Na^+ - H^+ exchanger (NHE) are reported to be involved in capacitation and fertilization (Demarco *et al.* 2003, Wang *et al.* 2003).

These reports led us to think that modulation of pH_i according to the concentration of extracellular Na^+ could be a cause of delay of hyperactivation. However, whole-cell pH_i value and pH_i geometry were not affected by change in extracellular Na^+ concentration (Fig. 4B, C and Supplementary Figure 3), suggesting that pH_i is not involved in the regulation of hyperactivation by Na^+ .

Finally, we found that hamster sperm $[\text{Ca}^{2+}]_i$ decreased as the extracellular Na^+ concentration increased especially at midpiece and principal piece (Fig. 5) and that the NCX inhibitors canceled the effect of extracellular Na^+ on hamster sperm hyperactivation (Figs 6 and 7A). These results indicate that the extracellular Na^+ suppresses hyperactivation by lowering $[\text{Ca}^{2+}]_i$, presumably in the flagellum via an action of NCX. This suggests that hyperactivation is suppressed by NCX before capacitation, and the inhibition of NCX activity is necessary to be hyperactivated.

In this study, we have not found the NCXs in hamster spermatozoa yet. However, the activity of Na^+ -dependent Ca^{2+} extrusion was shown in mouse spermatozoa (Wennemuth *et al.* 2003). In addition, the existence of NCX in the flagellum and its function in spermatozoa were previously reported in *Ciona* (Shiba *et al.* 2006), human (Krasznai *et al.* 2006), and herring (Vines *et al.* 2002). The existence of K^+ -dependent Na^+ - Ca^{2+} exchanger (NCKX), another transporter that has activity to exchange Na^+ and Ca^{2+} across the plasma membrane, in sea urchin spermatozoa have also been shown (Su & Vacquier 2002). These facts support the idea that NCX is present and functional in hamster spermatozoa. In those previous studies, however, full-length NCX was not (or only slightly) detected by western blotting (Krasznai *et al.* 2006, Shiba *et al.* 2006). In addition, it was discussed that the NCX in herring spermatozoa operate in reverse mode (Ca^{2+} influx), and NCX in *Ciona* spermatozoa contribute to $[\text{Ca}^{2+}]_i$ transient (Vines *et al.* 2002, Shiba *et al.* 2006). In sea (or brackish) water environment where herring or *Ciona* spawn, Na^+ exists at high concentration, which should exclusively cause efflux of intracellular Ca^{2+} . Furthermore, previous studies used KB-R7943, 3',4'-dichlorobenzamil hydrochloride (DCB), and bepridil as NCX inhibitor, which have rather lower specificity to NCX than SEA0400 and SN-6 (Matsuda *et al.* 2001, Iwamoto *et al.* 2004). Actually, 50 $\mu\text{mol/L}$ KB-R7943 significantly decreased the percentage of motile spermatozoa (Supplementary Figure 5A), indicating harmful side effect. Taken together, the precise physiological role of NCX in spermatozoa is still controversial, and thus, further studies are needed to elucidate which type of NCX (or NCKX) is involved in the regulation of hamster sperm capacitation.

The concentration of SN-6 and SEA0400 used in this study (50 $\mu\text{mol/L}$) was rather high because forward mode of NCX (Ca^{2+} extrusion) is less sensitive to these

inhibitors than reverse mode (Ca^{2+} influx) (Iwamoto *et al.* 2004, Lee *et al.* 2004). However, we assume that concentrations of these inhibitors does not matter as the percentage of motile spermatozoa was not affected by these concentrations (Fig. 6A and D), in contrast to KB-R7943 (Supplementary Figure 5A).

It is widely accepted that the tyrosine phosphorylation is strongly associated with capacitation (Visconti *et al.* 1995, 1998, Visconti & Kopf 1998). Especially, tyrosine phosphorylation on AKAPs of fibrous sheath during capacitation was widely observed in human and hamster spermatozoa (Carrera *et al.* 1996, Jha & Shivaji 2002). In this study, tyrosine phosphorylation on two major bands, which was likely to correspond with the 80 and 85 kDa AKAPs respectively, was observed among all samples and no significant change was seen among them (Fig. 9A, B, C, D, E and Table 3). These results suggest that a well-known tyrosine phosphorylation-dependent signaling pathway of capacitation progressed normally regardless of extracellular Na^+ concentration. However, hyperactivation was delayed as extracellular Na^+ concentration increased (Figs 1 and 3), suggesting that hyperactivation was controlled by the other pathway, which is independent of the conventional tyrosine phosphorylation-dependent pathway in our experimental condition. Recent study showed that mouse sperm hyperactivation could be induced by transient $[\text{Ca}^{2+}]_i$ increase by ionophore without activation of cAMP-dependent tyrosine phosphorylation pathways (Tateno *et al.* 2013). In this study, we showed that hamster sperm $[\text{Ca}^{2+}]_i$ increased when extracellular Na^+ decreased, presumably because of decline of NCX activity by decrease in extracellular Na^+ (Figs 6 and 7A). Indeed, it was previously shown that the inward NCX current (i.e., Ca^{2+} efflux) was changed depending on the external Na^+ concentration in the range close to our experimental conditions (0–150 mmol/L Na^+) in rat odontoblasts (Tsumura *et al.* 2010). Collectively, it is suggested that hamster sperm hyperactivation could be regulated by Ca^{2+} -dependent and tyrosine phosphorylation-independent pathway simultaneously with tyrosine phosphorylation-dependent pathway such as mouse sperm, and such Ca^{2+} -dependent and tyrosine phosphorylation-independent pathway may lead to hyperactivation when extracellular Na^+ concentration decreased. Otherwise, calmodulin-dependent kinase (CaM kinase)-dependent phosphorylation, which was shown in mouse spermatozoa (Schlingmann *et al.* 2007), may act as modulatory pathway for hyperactivation in hamster spermatozoa.

In conclusion, this study showed that hamster sperm hyperactivation is altered by extracellular Na^+ concentration. Extracellular Na^+ suppresses sperm activation and hyperactivation, and the removal of suppression by extracellular Na^+ leads to the expression of hyperactivated motility. This suppression of hyperactivation is suggested to be regulated via an action of NCX.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-15-0367>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this work.

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