Role of volume-stimulated osmolyte and anion channels in volume regulation by mammalian sperm

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The ability to maintain cellular volume is an important general physiological function. Swelling induced by hypotonic stress results in the opening of channels, through which ions exit with accompanying water loss (regulatory volume decrease, RVD). RVD has been shown to occur in mammalian sperm, primarily through the opening of quinine-sensitive potassium channels. However, as yet, direct evidence for the participation of anion channels in sperm RVD has been lacking. The chloride channel type CIC-3 is believed to be involved in RVD in other cell types. Using electronic cell sizing for cell volume measurement, the following results were obtained. (i) The anion channel blockers 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), tamoxifen and 4,4'-diisothiocyanostilbene-2,2'-disulphonic acid (DIDS) increased hypotonic swelling in concentration-dependent fashion, whereas verapamil (P-glycoprotein inhibitor) had little effect. The most potent, NPPB and DIDS, blocked RVD without affecting cell membrane integrity at effective concentrations. (ii) When gramicidin was included to dissipate Na⁺/K⁺ gradients, major secondary swelling was observed under hypotonic conditions. This secondary swelling could be reduced by NPPB, and suppressed completely by replacing chloride in the medium with sulphate, an ion which does not pass through chloride channels. It was deduced that the initial hypotonic swelling activated an anion channel through which chloride ions could then enter freely down a concentration gradient, owing to the lack of a counter-gradient of potassium. (iii) Taurine, an osmolyte often involved in RVD, does not appear to play a role in sperm RVD because lengthy preincubation with taurine did not alter sperm RVD response. Our observations provide direct evidence that a chloride channel (possibly CIC-3) is involved in the process of volume regulation in mammalian sperm.

Key words: cell volume/chloride transport/CIC-3/RVD/sperm

Introduction

When cells encounter hypo- or hypertonic environments, they tend to swell or shrink owing to the influx or efflux of water during re-establishment of osmotic equilibrium. The resultant dilution or concentration of the intracellular components, as well as local distortion of structural elements (cytoskeleton, plasma membrane etc.), can have profound effects on cell function (for review, see Lang et al., 1998). Studies have revealed that many animal cell types are able to maintain their volume after osmotic challenge, thereby avoiding the consequences of excessive volume changes (reviewed by Hoffmann and Dunham, 1995). During its life, the spermatozoon experiences considerable changes in its environment, most notably during maturation within the epididymis and at ejaculation (Cooper, 1986; Cooper and Yeung, 2003). In the distal cauda the environment is significantly hypertonic, thus on ejaculation the spermatozoon experiences hypertonic stress as it encounters an environment of osmolality similar to that of blood plasma. Hence it is not surprising that the spermatozoon has been found to exhibit volume regulatory abilities, particularly regulatory volume decrease (RVD) in the face of hypertonic challenge.

These abilities may be crucial to natural fertility. Human sperm with compromised volume regulatory ability were found to be unable to migrate through mucus (Yeung and Cooper, 2001). In a comparison of caput, corpus and cauda sperm from fertile and infertile transgenic mice, it was found that sperm from the infertile strain showed abnormal motility characteristics commensurate with poor volume regulation (Yeung et al., 1999). The ability to regulate volume in the face of osmotic challenge appears to develop during maturation: when sperm from wild-type mouse were exposed to hypotonic conditions, corpus and cauda cells returned to a smaller volume after initial slight swelling whereas cells from the caput swelled but did not subsequently shrink (Yeung et al., 2002). One may deduce that any disruption of this aspect of epididymal maturation would likely hinder the transport of sperm in the female tract with serious consequences. The physiological relevance of sperm volume regulation is therefore clear, and a detailed understanding of the mechanisms involved and their potential lesions will provide insight into subtle aspects of male infertility.

Cell volume is essentially determined by the intracellular content of osmotically active solutes relative to the osmolarity of the extracellular fluid. Thus RVD essentially involves a mechanism for transferring low Molecular weight osmolytes (K⁺, Cl⁻, organic anions, and small organic solutes) out of the cell, so as to re-establish osmolyte equilibrium across the cell membrane; water loss accompanies the loss of these osmolytes, whence hypotonic swelling is halted and reversed (see reviews by Grinstein et al., 1984; Sarkadi and Parker, 1991; Al-Habori, 1994; O’Neill, 1999). The net efflux of the osmolytes results from a swelling-induced activation of specific transport pathways. In the case of most animal cell types, K⁺ and Cl⁻ leave the cell by parallel activation of separate volume-sensitive transporters.
K⁺ and anion channels; organic osmolyte efflux can also take place through a volume-sensitive anion channel. RVD mechanisms in sperm are as yet poorly defined. The involvement of quinine-inhibitable potassium channels has been demonstrated for bull, boar, human and dog sperm (Kulkarni et al., 1997; Petrounkina et al., 2001a, 2004; Yeung et al., 2001). While studying mouse sperm exposed to hypotonic conditions, Yeung et al. (1999) were able to block RVD by treatment with inhibitors of anion channels as well as K⁺-channels. However, thus far, this has been the only report of involvement of anion channels in sperm RVD.

Anion channels are often called chloride channels, as Cl⁻ is the most abundant anion in plant and animal tissues (Jentsch and Günther, 1997). There can be several types of Cl⁻ channels. Most abundant anion in plant and animal tissues (Jentsch and et al., 1994), whereas human and boar sperm volumes are both more similar to human sperm in their shape and cell volume: the mouse, boar and human acrosome reaction (Melendrez and Meizel, 1995; Espinosa et al., 1996) and boar (Melendrez and Meizel, 1996); there is evidence that similar channels participate in the mouse, boar and human acrosome reaction (Melendrez and Meizel, 1995; Espinosa et al., 1998; Bray et al., 2002).

In the present study, we have investigated the role of anion channels in mammalian sperm RVD using boar sperm as models. Although mouse sperm have become established models for studies relevant to human sperm physiology, boar sperm appear to be very suitable models for studies on cell volume regulation. Boar sperm are more similar to human sperm in their shape and cell volume: mouse sperm volume has been estimated to be ~80 μm³ (Du et al., 1994), whereas human and boar sperm volumes are both ~25 μm³ (Gilmore et al., 1995, 1996; Curry et al., 1996; Devireddy et al., 2004) and their resulting osmotically active cell volumes are comparable. Both boar and human sperm behave as linear osmometers over a significantly narrower range than do mouse sperm (185–900 and 145–900 mosmol/kg, as against 75–1200 mosmol/kg: Gilmore et al., 1995, 1996; Willoughby et al., 1996). Moreover, the extent of prolonged volume swelling that sperm can withstand before irreversible loss of functional cell integrity occurs is similar for boar and human (~1.1 times their isotonic volume: Gao et al., 1995; Gilmore et al., 1996), whereas the osmotic tolerance of mouse sperm is significantly higher (1.24 times their isotonic volume: Willoughby et al., 1996). Although the way in which these parameters may affect volume regulation is not yet defined, in studying the process it would seem preferable to use as models for human sperm a species as closely physically and functionally similar as possible.

Materials and methods

In essence, our experimental approach to studying sperm volume control consisted of diluting washed sperm samples into isotonic or hypotonic medium at 39°C, and taking sub-samples at timed intervals thereafter for analysis of the population volume distributions. Effects were judged largely by comparing the modal volumes of such distributions. Much of the methodology used was based on earlier studies of sperm volumetric behaviour (Petrounkina et al., 2000, Petrounkina et al., 2001a). These earlier publications may be consulted for further details and explanation of the approach.

Chemicals

Unless otherwise stated, chemicals were obtained from Merck AG (Germany), Alexis GmbH (Germany) and Sigma AG (Germany).

Semen sources

Semen was mostly obtained from boars of hybrid BHZP race held at the GFS Aschenberg AI station. It was diluted in commercial BTS extender (Beltsville Thawing Solution: see Johnson et al., 1988) and delivered to the Institute in an insulated container, after which it was stored at 17°C for 24–72 h.

For some experiments, semen was obtained from three fertile crossbred animals from the Institute of Reproductive Medicine’s boar colony. These samples were collected (generally twice a week) by the ‘gloved hand’ method via sterile gauze (to remove gel). Immediately after collection the semen was transferred to the laboratory, diluted in BTS extender to a concentration of 0.8–1.0 × 10⁸ cells/ml and stored as above.

Semen processing

Aliquots of diluted semen (3–5 ml) were washed through a two-step gradient of 35% and 70% isotonic Percoll–saline (Vincent and Nadeau, 1984). After removal of the supernatant layers, the loose sperm pellet was resuspended in residual 70% Percoll to a final concentration of ~2 × 10⁸ cells/ml. Prior to incubation, semen samples were maintained throughout at a minimum of 25°C. Processed samples were used for the experimental studies within 1 h of washing.

Media

Two variants of a HEPES-buffered saline medium were used as the vehicles for volumetric measurements. The isotonic variant (isoHBSM; 300 mosmol/kg) consisted of 137 mmol/l NaCl, 10 mmol/l glucose, 2.5 mmol/l KOH, and 20 mmol/l HEPES buffered with NaOH to pH 7.4 at 39°C (Harrison et al., 1993). The hypotonic variant (hypoHBSM; 180 mosmol/kg) was prepared by adjusting the NaCl content to ~76 mmol/l. In experiments requiring low external chloride content, a Na₂SO₄-based analogue was used. Isotonic sodium sulphate medium (isoSHM; 300 mosmol/kg) consisted of 112 mmol/l sodium sulphate, 10 mmol/l glucose, 2.5 mmol/l KOH, and 20 mmol/l HEPES buffered with NaOH to pH 7.4 at 39°C. Hypotonic sodium sulphate medium (hypoSHM; 180 mosmol/kg) was prepared by adjusting the sodium sulphate content to ~62 mmol/l. To minimize detection of particulate ‘noise’ during cell volume measurements, all media were passed through a 0.2 μm filter before use.

Sperm treatment protocol for volumetric measurements

Specific details for each experimental series are given in the relevant Experiments and results section. The general protocol was as follows. Aliquots of washed sperm suspensions were diluted into isoHBSM at 39°C containing predetermined concentrations of effectors (final sperm cell concentration 0.5–1 × 10⁸/ml). After 5 min preincubation, samples (40–80 μl) were transferred to 5 ml of either hypoHBSM or isoHBSM containing the same concentration of effector as the parent medium, after which they were incubated further at 39°C (final sperm concentration ~1 × 10⁸ cells/ml). The isotonic and hypotonic sperm suspensions were sampled for cell volume measurement after predetermined periods (2–20 min). IsoHBSM and hypoHBSM without added effector were used as control media. Where an effector was added from a stock solution prepared in alcohol or dimethyl sulphoxide, the final concentration of solvent in the testing solution was in all cases <0.5% (v/v).

Cell volume measurement

At each sampling time-point, a single sample from each incubated sperm suspension was passed through a CASY 1 cell counter (Schaerfe Systems GmbH, Germany), which produced cell volume information on the basis of cell frequency distribution within 1024 electronic ‘volume’ channels. The capillary measuring chamber was 60 μm in diameter, the sample volume setting was 200 μl and the size scale 10 μm; each sampling obtained data from >10,000 cells.

Because the electrical conductivity of the hypotonic media was lower than that of the isotonic media, a correction factor (1.10) was applied to data recorded from sperm suspensions in hypotonic media (obtained by comparison of volume measurements of standard 3.4 μm latex beads: see Petrounkina et al., 2000). Care was taken to flush the measuring chamber.
with 400 µl of the appropriate medium between measurements in different media; on each such occasion, prior to analysis of the sperm samples, test counts were made on media without cells.

**Analysis of volumetric data**

Unless otherwise stated, the analyses used the modal values of the volume distribution (corrected for the different osmotic conditions); these values were obtained directly from the cell volume measurements using the CASY software. (Note that the modal volume has been shown to be a more sensitive parameter of volume change than the mean volume—see Petrunkina and Töpfer-Petersen, 2000.)

The relative volume shift $V_r$ was used as a measure of the volume regulation in response to hypotonic conditions. It was defined as $V_r = V_{\text{hyp}}/V_{\text{iso}}$, where $V_{\text{hyp}}$ was the modal value of the hypotonic volume distribution and $V_{\text{iso}}$ was the modal value of the isotonic volume distribution. In the situation where several sperm subpopulations contributed to a distribution, the values pertaining to the most abundant osmotically active subpopulation were used, since that was considered most representative of general sperm behaviour. A cell subpopulation was considered as osmotically active when $V_r > 1$.

The observed effects of chloride channel inhibitors were verified by analysis of variance, $t$-test and non-parametric analysis (SAS Software: General Linear Model).

Unless otherwise stated, values presented are means ± SEM. $P < 0.05$ was considered statistically significant.

**Sperm quality estimations**

Sperm morphology was examined in samples taken after delivery of diluted semen from the AI station, or from the native ejaculates shortly after semen collection (for methodology, see Petrunkina et al., 2001a, and references therein). In general, only samples with a low percentage of morphological abnormalities (from highly fertile boars) were used. A few samples with relatively poor morphology (from lower fertility boars) were included in studies of gramicidin and taurine responses.

Membrane integrity was checked using propidium iodide staining (2.5 µg/ml) in combination with flow cytometry (Dako Galaxy; DakoCytomation GmbH, Germany). In the two-peak fluorescence intensity histogram recorded in the FL3 channel (red fluorescence), the lower intensity peak represented plasma membrane-intact (live) cells while the higher intensity peak represented dead cells with defective plasma membranes. The percentage of dead cells was calculated using FloMax Software (Versus. 2.0, 1999; Partec GmbH, Germany). The dose-dependency of membrane damage by effector drugs was checked in washed and incubated samples, comparing three higher concentrations without added effector. In time-course experiments, membrane integrity was determined at 5 and 20 min incubation in both iso- and hypotonic media.

**Experiments and results**

**Effect of chloride channel blockers on RVD: dose dependence**

The following blockers were investigated: 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), 4,4'-diisothiocyanostilbene-2,2'-disulphonic acid (DIDS), verapamil, tamoxifen, and 1,9-dideoxyforskolin (DDFSK). Sperm were preincubated in isoHBSM with a range of concentrations of each blocker for 5 min (10 min for tamoxifen and DDFSK), and then diluted into isoHBSM and hypoHBSM containing the same concentration of blocker; volume analyses were made after a further 5 min (NPPB, DIDS, verapamil) or 20 min (tamoxifen, DDFSK). Each drug concentration was tested on three or four independent sperm samples. The results are shown in Figure 1.

NPPB, a well-known chloride channel inhibitor, caused a dose-dependent increase in $V_r$ at concentrations between 2 and 20 µmol/l (Figure 1a). At 20 µmol/l the modal hypotonic volume was ~43.0 fl ($V_r 3.80$) as compared to 13.2 fl ($V_r 1.25$) in the absence of the drug. At higher concentrations, the effect of NPPB effect decreased.

**Figure 1.** Concentration dependence of the effect of chloride channel inhibitors on volume response to hypotonic stress. Sperm were incubated at 39°C with the given concentration of the test drug for 5 min under isonic conditions. They were then diluted into either isonic or hypotonic medium at 39°C containing the same drug concentration. Volumetric measurements were made after a further 5 min (NPPB, DIDS, verapamil) or 20 min (tamoxifen, DDFSK). All concentrations were tested on three or four independent sperm samples. Results are presented in terms of $V_r$ (i.e. volume in hypotonic medium/volume in isonic medium; see Materials and methods).
Although at 200 μmol/l there was an increase in the percentage of dead cells (under isotonic conditions, 20.4 versus 8.7% in control; under hypotonic conditions, 23.7 versus 15.5% in control), the majority of cells remained intact. A concentration of 20 μmol/l was chosen for subsequent RVD time-course experiments, a level which did not negatively affect sperm viability.

DIDS, a VSOAC and ClC-3 blocker, caused large increases in $V_i$ even at 1 μmol/l, the lowest concentration tested (Figure 1b). At a level of 10 μmol/l, the modal hypotonic volume was $\sim 33.5 \text{ fl} (V_r 2.49)$ as compared to 13.2 fl ($V_r 1.25$) in the absence of the drug. This concentration, which did not affect sperm viability, was chosen for time-course experiments.

Verapamil, an inhibitor of P-glycoprotein as well as of Ca$^{2+}$ channels, failed to show significant effects on sperm cell volume (Figure 1c). (P-glycoprotein has been linked with volume-activated chloride efflux; no longer considered to be the actual VSOAC, it is now thought to play a regulatory role: see Valverde, 1999; Idriss et al., 2000.) Only slight increases in $V_i$ at higher concentrations were observed (between 15 and 25%). Cell viability was not affected. The concentration at which the greatest increase in $V_i$, due to increasing levels of cell death (even at 5 μmol/l tamoxifen, $\sim 40\%$ of sperm were dead, under isotonic as well as hypotonic conditions). A concentration of 1 μmol/l was chosen for time-course experiments.

DDFSK, a forskolin analogue inactive towards adenylyl cyclase but known to be a chloride channel inhibitor, also had little effect on cell volumes after 5 min exposure to hypotonic conditions. But after 20 min, 1 μmol/l (the lowest dose tested) caused a marked increase in $V_i$ (Figure 1e), though there was no loss in sperm viability. Under these conditions the hypotonic cell volume was 35.2 fl ($V_r 2.95$) as compared with 23.4 fl ($V_r 1.90$) in the absence of the drug. A concentration of 1 μmol/l was chosen for time-course experiments.

**Effect of chloride channel blockers on RVD: time course**

Sperm were preincubated in the presence of 20 μmol/l NPPB, 10 μmol/l DIDS, 30 μmol/l verapamil, 1 μmol/l tamoxifen, or 1 μmol/l DDFSK; control samples were preincubated in the absence of any effector. After 5 min preincubation (10 min for tamoxifen and DDFSK), samples were diluted into iso- or hypo-HBSM containing the same additive as the parent preincubation medium and sampled for volume analysis after 2, 5, 10 and 20 min (NPPB, DIDS and verapamil), or after 5 and 20 min (tamoxifen and DDFSK). The results are shown in Figures 2 and 3 respectively. Each effector was tested on three independent sperm samples.

NPPB did not cause any statistically significant effects after 2 and 5 min dilution (Figure 2a). However, after 10 min, the hypotonic volume had increased markedly (29.9 versus 17.8 fl in the control; $P < 0.05$); this swelling was maintained during the full 20 min of incubation (Figure 2a). In the control, swelling that was already clearly advanced after 2 min decreased thereafter. No significant effect of NPPB was observed on isotonic sperm volume.

DIDS caused the hypotonic volume to increase markedly already after 2 min dilution (Figure 2b). Thereafter, cell volume increased further, reaching a maximum after 20 min exposure to hypotonic conditions (30.6 versus 15.9 fl in the control; $P < 0.05$). In these experiments, swelling in the control, seen after 2 min exposure, remained almost constant throughout. No significant effect of DIDS was observed on isotonic sperm volume.

No statistically significant effects were observed after addition of 30 μmol/l verapamil to preincubation medium and sampling medium (Figure 2c). Both isotonic and hypotonic volumes remained

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**Figure 2.** Time-course of the effect of NPPB, DIDS and verapamil on volume response to hypotonic stress. Sperm were preincubated at 39°C for 5 min with either 20 μmol/l NPPB or 10 μmol/l DIDS or 30 μmol/l verapamil; they were then diluted into the same concentration of blocker in either hypoHBSM or isoHBSM at 39°C. As controls, parallel samples were incubated and diluted similarly in the absence of any channel blocker. Cell volume estimations were made 2, 5, 10 and 20 min after dilution. Each blocker was tested on three independent sperm samples. • = blocker-containing hypoHBSM; ○ = control hypoHBSM; ▽ = blocker-containing isoHBSM; ▼ = control isoHBSM.
almost unchanged; only slight swelling as compared to control values was observed in verapamil-incubated suspensions.

Tamoxifen affected sperm swelling more slowly than the other effectors (Figure 3a). Also, sperm suspensions incubated in the presence of tamoxifen showed some increase in isotonic volume (after 20 min incubation, 18.3 versus 12.2 fl in the control, \( P < 0.05 \)). However, hypotonic volume was affected to a greater extent (after 20 min, 37.9 versus 18.2 fl in the control, \( P < 0.05 \)). As a result, \( V_t \) was increased significantly (2.07 versus 1.49 in control) and RVD was abolished.

DDFSK also abolished RVD (Figure 3b). Although hypotonic volume was not affected by the drug after 5 min of exposure to hypotonic conditions (28.8 versus 28.5 fl in control), modal cell volume after 20 min had increased to 35.2 fl whereas in the control it had decreased to 23.4 fl. At 20 min, therefore, \( V_t \) was 2.96 versus 1.49 in the control.

In these time-course experiments, at the levels used, none of the drugs had any effect on sperm viability (treated samples showed 7–18% dead, control samples 9–19% dead).

**Gramicidin effect on cell volume response to hypotonic conditions**

Gramicidin forms channels in cell membranes which allow free passage of monovalent cations. We tested its effect on sperm swelling under iso- and hypotonic conditions in order to observe the effect of collapsing the electrochemical gradients of \( K^+ \) and \( Na^+ \), and hence examine more specifically the involvement of chloride (anion) channels in cell volume control. In preliminary experiments we noted that gramicidin caused considerable secondary swelling under hypotonic conditions although it had very little effect on cell volume under isotonic conditions. Since in a NaCl-based environment intracellular chloride levels are markedly below extracellular levels, we hypothesized that this secondary swelling was due to the influx of water that accompanied uncontrolled influx of chloride through a swelling-operated anion channel (in company with influx of monovalent cations through the gramicidin channels).

Therefore, because sulphate only passes poorly through swelling-activated chloride channels (Grinstein et al., 1984 and references therein), we compared the effect of gramicidin on hypotonic swelling of sperm in a Na$_2$SO$_4$-based medium with that in the standard NaCl-based HB medium. After preincubation in HB medium without additive, sperm were diluted into either iso- or hypo-HBS medium with that in the standard NaCl-based HB medium. After 5 min incubation, the hypotonic volume was ~29.4 fl as compared with ~15–20 fl in the absence of gramicidin (see Figure 2). However, when chloride was replaced with sulphate (hypoSHM), there was very little hypotonic swelling throughout the incubation period. The difference between the two media was significant at 10 and 20 min of incubation (\( P < 0.05 \)).

These results supported strongly our hypothesis that the enhanced gramicidin-induced swelling in hypo-HBSM was due to entry of chloride down the concentration gradient via a chloride channel. To confirm the concept, we tested the effect of NPPB on the rate of swelling caused by gramicidin in HB medium. Sperm from six different ejaculate samples were preincubated in the absence of any

**Figure 3.** Time-dependency of the effect of (a) tamoxifen and (b) DDFSK on sperm volume response to hypotonic stress. Sperm from six different ejaculate samples were preincubated for 10 min with 1 \( \mu M \) tamoxifen or DDFSK; they were then diluted into the same concentration of blocker in either hypo-HBS or iso-HBS. As controls, parallel samples were incubated and diluted similarly in the absence of any channel blocker. Cell volume estimations were made 5 and 20 min after dilution. Each blocker was tested on 3 independent sperm samples. I: blocker-containing hypo-HBS; III: control hypo-HBS; III: blocker-containing iso-HBS; O: control iso-HBS.

**Figure 4.** Effect of replacement of chloride with sulphate on gramicidin-induced hypotonic swelling. Sperm were preincubated in HB medium in the presence of 500 nmol/l gramicidin. The results are shown in Figure 4. During incubation under isotonic conditions, regardless of medium, sperm volume remained close to that measured in the absence of gramicidin (~13–14 fl; cf. Figure 2). Under hypotonic conditions, the sperm swelling considerably in gramicidin-containing chloride-based medium (hypo-HBS); after 5 min incubation, the hypotonic volume was ~29.4 fl compared with ~15–20 fl in the absence of gramicidin (see Figure 2). However, when chloride was replaced with sulphate (hypoSHM), there was very little hypotonic swelling throughout the incubation period. The difference between the two media was significant at 10 and 20 min of incubation (\( P < 0.05 \)).

Materials and Methods for composition of these media.) Cell volume estimations were made 5, 10 and 20 min after dilution. Replicate experiments were performed on three independent sperm samples. I: hypotonic sulphate-based medium; O: hypotonic chloride-based medium; ▼: isotonic sulphate-based medium; ▽: isotonic chloride-based medium.
cells to build up their intracellular stores. Then aliquots (40–60 μl) were washed through a Percoll gradient as et al. (1972) suggested that the osmolyte might be involved in the sperm fluid is high (Johnson et al., 1972; Cooper, 1986), and Johnson (1997). Together with its metabolite hypotaurine, taurine’s concentration in both sperm and epididymal fluid is high (Johnson et al., 1972; Cooper, 1986), and Johnson et al. (1972) suggested that the osmolyte might be involved in osmoregulation in sperm. In a preliminary test of this hypothesis, we washed samples of extended semen through a Percoll gradient as described above but in the presence of 5 mmol/l taurine. After washing, the residual sperm pellets (in Percoll–saline–taurine) were kept for 45 min at ambient temperature in order to allow taurine-deficient cells to build up their intracellular stores. Then aliquots (40–60 μl of the ‘loaded’ suspensions) were transferred to isoHBSM containing 5 mmol/l taurine and preincubated at 38°C for a further 5 min. Finally, subsamples of the preincubated suspensions were diluted into taurine-free iso- and hypo-HBSM and incubated further. Cell volumes were measured after 2, 5, 10 and 20 min. Parallel sperm samples processed in the absence of taurine act as controls. Over- all, there was no positive effect of taurine on RVD (Figure 6); if anything, taurine-treated samples showed poorer RVD than controls, but, due to wide sample variation, the difference was not significant (P = 0.36).

### Taurine effect on RVD in boar sperm

During the experiments described above, it was noted that there was much variation between ejaculates (see Figures 1–5). One possible explanation is that the ejaculates varied in taurine content whereas the sperm’s RVD response between different ejaculates might be due to variations in semen taurine content. Taurine is an important osmolyte in many cell types and has been shown to play a role in regulatory volume decrease (Kirk, 1997). Together with its metabolite hypotaurine, taurine’s concentration in both sperm and epididymal fluid is high (Johnson et al., 1972; Cooper, 1986), and Johnson et al. (1972) suggested that the osmolyte might be involved in osmoregulation in sperm. In a preliminary test of this hypothesis, we washed samples of extended semen through a Percoll gradient as described above but in the presence of 5 mmol/l taurine. After washing, the residual sperm pellets (in Percoll–saline–taurine) were kept for 45 min at ambient temperature in order to allow taurine-deficient cells to build up their intracellular stores. Then aliquots (40–60 μl of the ‘loaded’ suspensions) were transferred to isoHBSM containing 5 mmol/l taurine and preincubated at 38°C for a further 5 min. Finally, subsamples of the preincubated suspensions were diluted into taurine-free iso- and hypo-HBSM and incubated further. Cell volumes were measured after 2, 5, 10 and 20 min. Parallel sperm samples processed in the absence of taurine act as controls. Over- all, there was no positive effect of taurine on RVD (Figure 6); if anything, taurine-treated samples showed poorer RVD than controls, but, due to wide sample variation, the difference was not significant (P = 0.36).

### Discussion

While the main driving force for RVD is the efflux of potassium ions down a concentration gradient through a volume-activated potassium channel, chloride ions must be enabled to exit in tandem with the potassium ions in order to maintain electrochemical neutrality. It is the water efflux which accompanies the ionic efflux that brings about a reduction in swelling. Our studies provide clear evidence of the existence in boar sperm of a volume-activated chloride channel that plays an important role in regulatory volume decrease (RVD) following swelling induced by hypotonic stress.

The evidence for participation of chloride channels in RVD in boar sperm was provided by the inhibitory effect on RVD of NPPB, DIDS, tamoxifen and DDFSK. All have been reported to block the outwardly rectifying volume-sensitive anion channels (VERSU- SOAC) (see reviews by Strange et al., 1996; Kirk, 1997; Jentsch et al., 2002). Tamoxifen can also inhibit both PKC (see Gundimeda et al., 1996) and P-glycoprotein (see Tominaga et al., 1995; Idri ss et al., 2000); there is evidence that PKC might be involved in the channel activation mechanism that leads to RVD, possibly via the multidrug resistance P-glycoprotein, which itself has been shown not to be a chloride channel but which appears to act as a regulator of VSOAC (see Bond et al., 1998; Idri ss et al., 2000; Zhong et al., 2002). However, in our study, tamoxifen was inhibitory at 1 μmol/l, a concentration clearly lower than that reported to be needed for PKC inhibition (IC50 = 10 μmol/l; see Calbiochem Catalog and Technical Resource, 2003/2004). Moreover, verapamil, which inhibits P-glycoprotein but does not inhibit VSOAC (Tominaga et al., 1995; Idris s et al., 2000), failed to show any effect on the time-course of RVD in boar sperm, confirming that the inhibition of RVD by tamoxifen is mainly due to its effects on a chloride channel.

Further important evidence for the existence of a swelling-activated chloride channel in boar sperm was provided by our experiments using gramicidin. This compound forms pores in the plasma membrane that enable free exchange of monovalent cations. Since cell volume is thought to depend upon parallel activation of both potassium and chloride channels, we sought to examine the involvement of chloride channels in RVD more directly by using gramicidin to over-ride the potassium channel and collapse the potassium...
gradient. Any change in volume would then stem from chloride ion movement; cations would follow chloride in order to maintain electroneutrality (electrochemical considerations demand that anion flux must be accompanied by cation flux and vice versa). We found that, although gramicidin had no effect on sperm volume under isotonic conditions, the ionophore not only abolished RVD after hypotonic stress but caused enhanced (secondary) swelling. We inferred that this swelling resulted from an influx of chloride ions through a volume-activated chloride channel (with accompanying influx of water). If a suitable channel were open, such an influx would take place because the intracellular chloride concentration is low (largely due to the fact that within cells negatively charged proteins as well as other intracellular anions replace chloride in maintaining ionic balance). We were able to demonstrate the validity of our inference in two ways. When chloride was replaced by sulphate in the external medium (sulphate passes only very poorly through swelling-activated chloride channels: Grinstein et al., 1984 and references therein), gramicidin no longer caused secondary swelling. Secondly, the gramicidin-induced swelling in chloride medium was significantly reduced by the chloride channel blocker NPPB. That the chloride channel was only open in swollen sperm could be deduced by the lack of effect of gramicidin on isotonic sperm volume: although a chloride gradient was undoubtedly present across the sperm membrane, the anion was unable to enter under these conditions. Similar observations and deductions were made by Grinstein et al. (1982), who reported a gramicidin-induced swelling of peripheral blood lymphocytes in chloride-based media.

Assuming that the swelling-activated chloride channel in boar sperm is the channel termed VSOAC, the latter’s molecular identity has not been elucidated and indeed is still controversial. While as many as eight different candidates have been proposed in the past, and most have since been invalidated (see Clapham, 1998). Of those remaining, two front runners are still favoured: CIC-3 and CIC-2 (see Jentsch et al., 2002). During preliminary western blotting experiments (see Figure 7 for a typical result), we found that an anti-CIC-3 antibody from Sigma regularly revealed several bands in all five boar sperm extracts examined whereas an anti-CIC-2 antibody from Calbiochem revealed bands (differing from those revealed by the anti-CIC-3 antibody) in only two of the extracts. On this basis, it would seem more likely that CIC-3 is the VSOAC in boar sperm, in accord with the most recent findings in other cell types (Jin et al., 2003; Wang et al., 2003). However, although a similar CIC-3 antibody blocks VSOAC activity in muscle cells, Xenopus oocytes and gastric epithelial cells (Duan et al., 2001; Jin et al., 2003), its specificity has been called into question because of its interaction with several other proteins as well as CIC-3 itself (Weylandt et al., 2001; see also Jin et al., 2003; Wang et al., 2003). Furthermore, the antibody did not reveal in the sperm extracts a band of molecular weight 90–92 kDa (the size ascribed to CIC-3 in other cell types: Jin et al., 2003; Wang et al., 2003). Given these uncertainties, work is proceeding in our laboratory to elucidate the molecular identity of the sperm VSOAC and explain the size anomaly.

An aspect of our RVD observations that interests us particularly is the great variation between ejaculates in the sperm population’s ability to regulate its volume after hypotonic shock. As pointed out in the Introduction, this ability may have an important association with fertilizing ability. It may also be a crucial factor in the sperm’s ability to survive freezing and thawing during cryopreservation, when the cell encounters large osmotic gradients. During our study, we noted that sperm samples could be crudely classified into ‘good’ and ‘bad’ responders, in terms of the degree of RVD that they exhibited. ‘Good’ responders showed initial swelling with reduced or constant volume after 20 min exposure to hypotonic conditions; ‘bad’ responders showed no reduction in volume after initial swelling and even a slow increase in volume during the 20 min exposure. There are various possible explanations for these differences. In an earlier report (Petrunkina et al., 2001a), we speculated that they might be due to differences either in number or in functionality of volume-sensitive chloride channels. The ‘bad’ responders would be those with fewer functional channels, as they would be unable to lose chloride effectively. Such ‘bad’ responders would also show limited hypotonic swelling in the presence of gramicidin: an insufficient functionality of functional chloride channels would limit the amount and rate of chloride influx. An alternative explanation is that samples vary in respect of sperm intracellular chloride levels, whence the cells’ ability to release chloride in tandem with potassium ions would vary. ‘Bad’ responders would be those sperm with low chloride levels, in which a large gradient against external levels would greatly inhibit chloride release. In this case, however, ‘poor’ responders would show enhanced gramicidin-induced swelling because the influx of chloride down the large concentration gradient would be greater. Unfortunately, due to the large general variation between ejaculates, the limited number (six) of ejaculates in which gramicidin swelling behaviour was compared with RVD response has not allowed us to distinguish between these two alternative explanations thus far. We also considered the possibility that ‘good’ responders are able to regulate their volume by making use of other osmolytes in addition to potassium and chloride. Taurine is involved in RVD in many cell types (see Kirk, 1997). The level of (hypotaurine is very high in boar epididymal plasma, and an osmoregulatory role of taurine in sperm physiology has been suggested (Johnson et al., 2003).
1972). We therefore isolated and incubated sperm samples in the presence or absence of taurine before testing their RVD response, the idea being to compare samples assumedly with high taurine content with those of lesser or depleted content. However, no significant effect of taurine on volume regulation was observed. Yeung et al. (2003) have also reported that taurine does not appear to act as a potential osmolyte in human sperm.

Although no molecular explanation for RVD differences between sperm samples is yet available, this does not preclude a link between RVD response and fertilizing potential. During the studies described above, we were able to obtain fertility data for eight boars. Of these, the sperm from four showed good volume regulation \( V_r \) at 20 min < \( V_r \) at 5 min: classical RVD time course—see Figure 2a, whereas the others showed only moderate or poor regulation. The results of the analysis of the data for these eight boars are shown in Table I. High RVD appeared to be related to better fertility. Of course, the number of animals involved is far too low to be able to draw firm conclusions as yet, but the hypothesis that the ability to regulate volume is linked to fertility warrants further investigation; preliminary data from our laboratory have already indicated a link between volume control ability and fertility in bulls (Petrunkina et al., 2001b). Thus testing of RVD could become a useful and important sperm fitness parameter.

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### References


Clapham DE (1998) The list of potential volume-sensitive chloride currents (Table I). Values are means ± SEM. The values with different superscripts differ significantly between the rows.

Fertility data were provided by the GFS Aschenberg AI station for the time-period October 2002–July 2003 (during which the RVD experiments were performed). The fertility index represents the deviation from the mean percentage farrowing rate for the AI station for this period. It was kindly calculated for us by Dr H.Brandt (Institute for Animal Breeding and Domestic Animal Genetics, Giessen), using his own computer program. For the boars in question, the indices were based on >30 inseminations from each animal from which >20 piglet litters were born; the average farrowing rate was calculated from data for some 150 boars used for insemination on ~100 farms.


### Table I. Relationship between regulatory volume decrease (RVD) and fertility

<table>
<thead>
<tr>
<th>Boar sperm RVD class</th>
<th>No. of animals</th>
<th>( V_r ) at 5 min</th>
<th>( V_r ) at 20 min</th>
<th>RVD</th>
<th>Fertility index*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very good</td>
<td>4</td>
<td>2.04 ± 0.33</td>
<td>1.50 ± 0.22</td>
<td>0.54 ± 0.26^</td>
<td>+2.60 ± 0.83</td>
</tr>
<tr>
<td>Moderate–poor</td>
<td>4</td>
<td>1.66 ± 0.34</td>
<td>2.33 ± 0.57</td>
<td>−0.67 ± 0.24B</td>
<td>−0.32 ± 3.19</td>
</tr>
</tbody>
</table>

*Fertility data were provided by the GFS Aschenberg AI station for the time-period October 2002–July 2003 (during which the RVD experiments were performed). The fertility index represents the deviation from the mean percentage farrowing rate for the AI station for this period.


Yeung CH, Sonnenberg-Riethmacher E and Cooper TG (1999) Infertile spermatozoa of c-ros tyrosine kinase receptor knockout mice show flagellar angulation and maturational defects in cell volume regulatory mechanisms. Mol Hum Reprod 6,1062–1069.


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