Channels for water efflux and influx involved in volume regulation of murine spermatozoa

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Abstract

The nature of the membrane channels mediating water transport in murine spermatozoa adjusting to anisotonic conditions was investigated. The volume of spermatozoa subjected to physiologically relevant hypotonic conditions either simultaneously, or after isotonic pre-incubation, with putative water transport inhibitors was monitored. Experiments in which quinine prevented osmolyte efflux, and thus regulatory volume decrease (RVD), revealed whether water influx or efflux was being inhibited. There was no evidence that sodium-dependent solute transporters or facilitative glucose transporters were involved in water transport during RVD of murine spermatozoa since phloretin, cytochalasin B and phloridzin had no effect on volume regulation. However, there was evidence that Hg^{2+} - and Ag^{+} -sensitive channels were involved in water transport and the possibility that they include aquaporin 8 is discussed. Toxic effects of these heavy metals were ruled out by evidence that mitochondrial poisons had no such effect on volume regulation.

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Introduction

The difference in osmolality between luminal fluids in the epididymis, seminal vesicles and female tract imposes an osmotic challenge on spermatozoa upon ejaculation (Cooper & Barfield 2006). For the mouse, spermatozoa have to regulate their volume before fertilisation occurs (Yeung *et al.* 2006) or infertility ensues (Yeung *et al.* 2000). The channels through which osmolytes are shed from murine spermatozoa to drive water efflux in response to cell swelling have been identified as K^{+} channels (Barfield *et al.* 2005), Cl^{-} channels (Yeung *et al.* 2005) and $\text{K}^{+}\text{--Cl}^{-}$ co-transporters (Klein *et al.* 2006), but the route of water is as yet unknown. Knowledge of this may be useful to provide new diagnostic tests of sperm function, suggest new therapies for infertility, improve cryopreservation protocols or design male contraceptives mimicking natural infertility stemming from inadequate volume regulation (Cooper & Barfield 2006).

Water cannot pass through occupied K^{+} channels, as the water of hydration is stripped off the ions before they enter the selectivity filter (Armstrong 2003, Saparov & Pohl 2004) and as the water can only diffuse slowly across the lipid bilayers, its transport across plasma membranes occurs mainly through aquaporins (AQPs) at 10- to 100-fold higher rates (see Agre *et al.* 2002). Among mammalian cells, 13 members of the AQP family have so

far been identified; each being dominantly present in different tissues with most individual cell types having more than one isoform (see Castle 2005, Verkman 2005). Isoforms of AQPs used by spermatozoa for water transport have not been identified, although the presence of some proteins has been reported (Table 1).

In studies of osmolyte channels, inhibitors specific for certain channels have been proved useful in their pharmacological identification (Cooper & Yeung 2007); however, there are no specific inhibitors for AQP and HgCl_2 is the most established classical inhibitor used (see Yang *et al.* 2006). The mechanism of action and inhibitory site are known to be the binding of Hg^{2+} to the cysteine residue (cys-187 in AQP1, as in most other AQPs) near to one of the two Asn-Pro-Ala (NPA) motifs that form the water pore of different AQP isoforms (Preston *et al.* 1993, see also Agre 2006).

Besides AQPs, there are other membrane proteins that participate in osmotic water transport; although they are present at lower densities than AQPs, their biological importance relative to AQPs has not been studied in most tissues (Verkman & Mitra 2000, see Table 1). Inhibitors of facilitative glucose transport, cytochalasin B and phloretin, decrease reversibly the osmotic water transport across the plasma membranes of several cell types (Table 1), but they are not entirely specific in their action. Nevertheless, the observation that phloretin inhibits water transport in ovine and human spermatozoa (Curry *et al.* 1995) and

Table 1 Putative channels involved in water transport and their presence in spermatozoa.

Channels and transporters involved in water transport		
AQP	Aquaporins	Meinild <i>et al.</i> (1998), Verkman & Mitra (2000) and Verkman (2005)
SLC5A1	Sodium–glucose co-transporter	Loo <i>et al.</i> (2002)
GLUT (SLC2As)	Glucose transporters	Fischbarg <i>et al.</i> (1989, 1990)
SLC14A1	Urea transporter	Yang & Verkman (2002)
	Anion channels	Pohl (2004)
CFTR	Cystic fibrosis transmembrane	
	Conductance regulator	Hasegawa <i>et al.</i> (1992)
VRAC	Volume-regulated anion channels	Nilius (2004)
Inhibitors of water channels		
Hg	AQP1	Preston <i>et al.</i> (1993) and Niemietz & Tyerman (2002)
	AQP2, AQP5, AQP6	Agre & Kozono (2003)
	AQP3	Kuwahara <i>et al.</i> (1997)
	AQP8	Nielsen <i>et al.</i> (2007)
	AQP9	Castle (2005)
	Not MIP, AQP4, AQP7	Ishibashi <i>et al.</i> (1997) and Agre <i>et al.</i> (2002)
	AQP11?*	Gorelick <i>et al.</i> (2006), Ishibashi (2006) and Yakata <i>et al.</i> (2007)
Ag	AQP1	Niemietz & Tyerman (2002)
	AQP8	Bienert <i>et al.</i> (2007)
TEA	AQP1	Brooks <i>et al.</i> (2000), Agre & Kozono (2003) and Detmers <i>et al.</i> (2006)
Phloretin	AQP9	Tsukaguchi <i>et al.</i> (1998, 1999)
Cytochalasin B, phloretin	Glucose transporters	Fischbarg <i>et al.</i> (1989, 1990)
Cytochalasin B, phloretin	SLC2A1, SLC2A2, SLC2A3, SLC2A4	Haber <i>et al.</i> (1993), Maher <i>et al.</i> (1996), Kasahara & Kasahara (1997), Angulo <i>et al.</i> (1998), Martin <i>et al.</i> (2003) and Walker <i>et al.</i> (2005)
	Not SLC2A5	Burant <i>et al.</i> (1992), Burant & Davidson (1994) and Helliwell <i>et al.</i> (2000)
Cytochalasin B	SLC2A8, SLC2A9	Doege <i>et al.</i> (2000), Augustin <i>et al.</i> (2004) and Gomez <i>et al.</i> (2006)
Phloridzin	SLC5A1	Loo <i>et al.</i> (1996, 1999, 2002), Yang & Verkman (1998, 2002) and Zeuthen <i>et al.</i> (2001)
Presence of putative water channel proteins in spermatozoa		
SLC5A10 not SLC5A1	Dog	Rigau <i>et al.</i> (2002)
AQP7	Rat, man	Calamita <i>et al.</i> (2001) and Saito <i>et al.</i> (2004)
AQP8	Rat	Calamita <i>et al.</i> (2001)
Not AQP1	Ram, human	Curry <i>et al.</i> (1995) and Liu <i>et al.</i> (1995)
SLC2A3, SLC2A8, SLC2A9	Mouse	Urner & Sakkas (1999), Schurmann <i>et al.</i> (2002), Gomez <i>et al.</i> (2006) and Kim & Moley (2007)
SLC2A3, SLC2A5	Pig, man	Burant <i>et al.</i> (1992) and Sancho <i>et al.</i> (2007)

*, suspected but not proven.

phloridzin, an inhibitor of sodium-coupled solute transporters, may block water influx into murine spermatozoa (Barfield *et al.* 2005) suggests that the approach of using transport inhibitors may throw light on the water channels employed by spermatozoa during regulatory volume decrease (RVD).

In the present work, the types of water channels involved in physiological sperm volume regulation were probed using various inhibitors of water transport. The volume of viable spermatozoa, ascertained from their forward scatter of a laser beam, was measured by a flow cytometer, since this permits the simultaneous determination of cell vitality. Transfer to a hypotonic medium within the physiological range should trigger osmotic water influx and a related increase in cell volume which will induce RVD. Quinine, an effective inhibitor of sperm RVD, causes unopposed influx of water into the cell by blocking the K⁺ efflux responsible for driving water outwards for RVD. This swelling will be hampered if water transport is prohibited by co- or pre-incubation with putative water transport inhibitors. Therefore, failure of quinine to swell the treated sperm will provide clear evidence of inhibition of water influx.

Results

Long time-course protocol

Control and quinine-treated spermatozoa

When murine spermatozoa were released into hypotonic medium, and first measured at 5 min, the cell volume gradually increased to reach a steady state by 30 min. In the presence of quinine, the cells were far larger than controls at the first time point studied, but volume slightly decreased between 15 and 30 min (Fig. 1). The large difference in the extent of response of the controls among mice (compare Figs 1 and 2) was unanticipated and may reflect the osmotic load of spermatozoa from different mice. As a consequence, all results from each experiment were expressed as a ratio of the control values.

Phloridzin-treated cells

None of the concentrations of phloridzin tested influenced the vitality of the spermatozoa, as indicated by the exclusion of the DNA stain propidium iodide (PI; Table 2). Two-way ANOVA revealed that there were no

Figure 1 The effect of phloridzin on volume regulation under long time-course conditions. The relative volume of murine epididymal spermatozoa (mean $\pm$ S.E.M.; ordinate) with time after osmotic challenge (abscissa) when transferred to hypotonic (330 mmol/kg) medium alone (filled black circles, control), hypotonic medium containing 800 μ mol/l quinine alone (open black circles), phloridzin alone at 100 μ mol/l (filled red triangles), 500 μ mol/l (filled blue diamonds) or 1000 μ mol/l (filled green squares) or with 800 μ mol/l quinine together with phloridzin at 100 μ mol/l (open red triangles), 500 μ mol/l (open blue diamonds) or 1000 μ mol/l (open green squares). At least 5000 spermatozoa were registered at each time point from each of seven epididymides. Spermatozoa from the same mice were used for all treatments.

statistically significant differences in cell volume between control cells and those treated with phloridzin at any concentration, or between cells treated with quinine alone or with quinine together with phloridzin

Figure 2 The effect of mercury on volume regulation under long time-course conditions. The relative volume of murine epididymal spermatozoa (mean $\pm$ S.E.M.; ordinate) with time after osmotic challenge (abscissa) when transferred to hypotonic (330 mmol/kg) medium alone (filled black circles, control), hypotonic medium containing 800 μ mol/l quinine alone (open black circles), Hg^{2+} alone at 10 μ mol/l (filled cyan squares), 30 μ mol/l (filled red up triangles), 100 μ mol/l (filled blue diamonds) or 300 μ mol/l (filled green down triangles) or with 800 μ mol/l quinine together with 100 μ mol/l Hg^{2+} (open blue diamonds). Statistical differences: *, between test and control at the same time point; §, between test and quinine treated at the same time point; $P < 0.05$. At least 5000 spermatozoa were registered at each time point from each of eight epididymides. Spermatozoa from the same mice were used for all treatments.

(Fig. 1). As phloridzin had no effect in the long time-course experiments, it was not studied in the short time-course protocol.

Mercury-treated cells

None of the concentrations of Hg^{2+} tested reduced the viability of spermatozoa (Table 2). As a trend in volume was observed, with Hg -treated cells shrinking as control cells increased in volume, one-way ANOVA was applied at the 5- and 45-min time points. At 5 min, cells incubated with 30 and 100 μ mol/l Hg^{2+} and 100 μ mol/l Hg with quinine were significantly larger than the controls. By 45 min all concentrations of Hg^{2+} , except 10 μ mol/l, reduced cell volume to below that of the control cells. Cells pre-incubated in Hg^{2+} and quinine were significantly smaller than cells in quinine alone (Fig. 2).

Initial time-course protocol

Control and quinine-treated spermatozoa

When murine epididymal spermatozoa were first pre-incubated for 10 min in isotonic medium (430 mmol/kg) and then transferred to hypotonic medium, the cells maintained their volume, first measured at 20 s, for the entire 5 min with a slight increase in size during the last 3 min. In the presence of quinine, cells were far larger than controls at the first time point and maintained this volume over the 5 min studied (Figs 3–5).

Mercury-treated cells

At the concentration tested (100 μ mol/l), Hg^{2+} had no effect on cell viability (Table 2). Spermatozoa pre-incubated with Hg^{2+} for 10 min before hypotonic challenge retained their small size during the time when control cells were slightly increasing in size. At 20 s, there were no differences between the volumes of Hg^{2+} -treated spermatozoa and the controls, but Hg^{2+} prevented the increase in size normally produced by quinine. By 305 s, the mercury-treated cells were significantly smaller than the controls (which had increased in size). Thus, the slight cell swelling under control conditions, reflecting the inefficiency of RVD, was abolished by Hg^{2+} (Fig. 3).

Silver-treated cells

At the concentration tested (30 μ mol/l), Ag^+ had no effect on cell viability (Table 2). At 20 s, cells treated with silver and quinine were significantly smaller than those incubated in quinine alone and this was maintained during incubation until 305 s (Fig. 4). Spermatozoa incubated with Ag^+ before hypotonic challenge swelled to volumes between those of the control and quinine-treated spermatozoa; but the variability was such that they were not significantly different from either group (Fig. 4).

Table 2 Viability of murine spermatozoa during incubation with channel inhibitors.

Treatment	% viable cells (mean $\pm$ S.E.M.)			
	Min			
Long time-course	5	15	30	45
Control	77.1 $\pm$ 3.3	70.2 $\pm$ 2.7	67.5 $\pm$ 3.8	64.6 $\pm$ 2.9
Quinine (800 μ mol/l)	77.4 $\pm$ 2.6	73.2 $\pm$ 2.6	62.1 $\pm$ 3.7	69.0 $\pm$ 6.2
Phloridzin (100 μ mol/l)	72.7 $\pm$ 3.3	65.9 $\pm$ 3.3	60.0 $\pm$ 4.6	60.0 $\pm$ 2.2
Phloridzin (500 μ mol/l)	74.9 $\pm$ 3.7	67.5 $\pm$ 2.8	63.3 $\pm$ 2.9	62.0 $\pm$ 2.5
Phloridzin (1000 μ mol/l)	78.1 $\pm$ 3.0	68.6 $\pm$ 2.1	63.6 $\pm$ 2.0	37.7 $\pm$ 2.3
Phloridzin (100 μ mol/l) + quinine (800 μ mol/l)	77.6 $\pm$ 3.9	69.5 $\pm$ 3.7	67.1 $\pm$ 3.1	68.8 $\pm$ 5.3
Phloridzin (500 μ mol/l) + quinine (800 μ mol/l)	76.5 $\pm$ 2.0	71.2 $\pm$ 2.4	65.5 $\pm$ 5.0	60.1 $\pm$ 4.3
Phloridzin (1000 μ mol/l) + quinine (800 μ mol/l)	89.1 $\pm$ 1.1	76.1 $\pm$ 2.4	65.2 $\pm$ 4.3	71.8 $\pm$ 4.3
Control (800 μ mol/l)	78.1 $\pm$ 3.0	76.5 $\pm$ 1.1	73.5 $\pm$ 3.1	69.5 $\pm$ 3.8
Quinine (800 μ mol/l)	80.1 $\pm$ 4.6	75.4 $\pm$ 5.1	70.0 $\pm$ 5.8	74.6 $\pm$ 1.4
HgCl ₂ (10 μ mol/l)	75.8 $\pm$ 4.0	67.1 $\pm$ 5.0	65.9 $\pm$ 5.8	68.5 $\pm$ 8.1
HgCl ₂ (30 μ mol/l)	88.4 $\pm$ 2.5 ^a	77.0 $\pm$ 3.3	77.8 $\pm$ 2.6	76.7 $\pm$ 3.5
HgCl ₂ (100 μ mol/l)	84.6 $\pm$ 1.2	78.5 $\pm$ 1.6	74.4 $\pm$ 2.1	73.5 $\pm$ 0.9
HgCl ₂ (300 μ mol/l)	86.2 $\pm$ 2.2	82.0 $\pm$ 1.7	78.5 $\pm$ 2.6	76.4 $\pm$ 2.6
HgCl ₂ (100 μ mol/l) + quinine (800 μ mol/l)	79.0 $\pm$ 2.1	76.0 $\pm$ 2.4	74.3 $\pm$ 3.1	72.0 $\pm$ 1.7
Initial time-course	Min (0–5)			
Control	62.3 $\pm$ 3.2			
Quinine (800 μ mol/l)	68.9 $\pm$ 2.3			
HgCl ₂ (100 μ mol/l)	67.7 $\pm$ 3.1			
HgCl ₂ (100 μ mol/l) + quinine (800 μ mol/l)	76.4 $\pm$ 0.9			
AgNO ₃ (30 μ mol/l)	66.1 $\pm$ 2.1			
AgNO ₃ (30 μ mol/l) + quinine (800 μ mol/l)	74.1 $\pm$ 1.9			
Phloretin (800 μ mol/l)	79.3 $\pm$ 3.6			
Phloretin (800 μ mol/l) + quinine (800 μ mol/l)	85.6 $\pm$ 0.9 ^a			
Control	68.2 $\pm$ 3.8			
Quinine	74.4 $\pm$ 2.4			
Phloretin (800 μ mol/l) + furosemide (1000 μ mol/l)	89.0 $\pm$ 0.9 ^a			
Phloretin (800 μ mol/l) + TEA (5 mM)	88.9 $\pm$ 0.6 ^a			
Phloretin (800 μ mol/l) + quinine (800 μ mol/l) + furosemide (1000 μ mol/l)	87.9 $\pm$ 1.3 ^a			
Phloretin (800 μ mol/l) + quinine (800 μ mol/l) + TEA (5 mM)	87.1 $\pm$ 1.4 ^a			
Control	72.3 $\pm$ 1.5			
Quinine	71.3 $\pm$ 1.5			
Cytochalasin B (5 μ mol/l)	74.2 $\pm$ 3.4			
Cytochalasin B (20 μ mol/l)	77.0 $\pm$ 1.7			
Cytochalasin B (40 μ mol/l)	75.6 $\pm$ 2.4			
Cytochalasin B (5 μ mol/l) + quinine (800 μ mol/l)	77.9 $\pm$ 0.4			
Cytochalasin B (20 μ mol/l) + quinine (800 μ mol/l)	73.5 $\pm$ 1.3			

^aSignificantly different from the controls.

Phloretin-treated cells

The viability of phloretin- and quinine-treated spermatozoa was not depressed below that of the controls (Table 2). At the first (20 s) time point, cells treated with phloretin and transferred to quinine were as large as

Figure 3 The effect of mercury on volume regulation under initial time-course conditions. The relative volume of murine epididymal spermatozoa (mean $\pm$ S.E.M.; ordinate) with time after osmotic challenge (abscissa) with pre-incubation in isotonic (430 mmol/kg) medium alone (circles) for 10 min and then transfer to hypotonic (330 mmol/kg) medium alone (filled black circles, control) or hypotonic medium containing 800 μ mol/l quinine alone (open black circles), or pre-incubation in isotonic medium containing 100 μ mol/l Hg²⁺ (squares) and transfer to hypotonic medium containing 100 μ mol/l Hg²⁺ (filled red squares) or 800 μ mol/l quinine together with 100 μ mol/l Hg²⁺ (open red squares). Statistical differences: *, between test and control at the same time point; §, between test and only quinine treated at the same time point; $P < 0.05$. Between 20 000 and 60 000 spermatozoa were registered over the entire period from each of seven epididymides. Spermatozoa from the same mice were used for all treatments.

untreated cells in quinine alone, but their size diminished during incubation so that by 305 s they were significantly smaller than both those incubated in quinine alone and the controls. Cells incubated in phloretin alone were smaller than the controls (Fig. 5).

Cytochalasin B-treated cells

Another glucose transporter inhibitor, cytochalasin B, at 5, 20, 40 μ mol/l, with or without 800 μ mol/l quinine, had no effect on sperm viability (Table 2). Unlike the other facilitative glucose transporter inhibitor phloretin, none of the concentrations tested had an influence on sperm volume or their response to quinine (data not shown).

Phloretin + tetraethylammonium (TEA)- or phloretin + furosemide-treated cells

In attempts to determine the cause of the volume decline in the presence of phloretin and quinine, additional inhibitors were provided with phloretin in the pre- and post-incubation media; the voltage-gated K-channel inhibitor TEA and the potassium-chloride co-transporter (KCC, SLC12A) inhibitor furosemide. Murine spermatozoa incubated with TEA or furosemide, either alone or each with quinine, were significantly more viable than the control cells (Table 2). In the absence of phloretin, neither compound alone altered swelling in the presence of quinine (data not shown).

Figure 4 The effect of silver on volume regulation under initial time-course conditions. The relative volume of murine epididymal spermatozoa (mean $\pm$ S.E.M.; ordinate) with time after osmotic challenge (abscissa) with pre-incubation in isotonic (430 mmol/kg) medium alone (circles) for 10 min and then transfer to hypotonic (330 mmol/kg) medium alone (filled black circles, control) or hypotonic medium containing 800 μ mol/l quinine alone (open black circles), or pre-incubation in isotonic medium containing 30 μ mol/l Ag^+ (squares) and transfer to hypotonic medium containing 30 μ mol/l Ag^+ (filled red squares) or 800 μ mol/l quinine together with 30 μ mol/l Ag^+ (open red squares). Statistical differences: §, between test and only quinine treated at the same time point; $P < 0.05$. Between 20 000 and 60 000 spermatozoa were registered over the entire period from each of eight epididymides. Spermatozoa from the same mice were used for all treatments.

In half of the animals used, and in both epididymides of the same mice, both TEA and furosemide separately were partially able to delay the decline in volume occurring in the presence of quinine after pre-incubation in phloretin (responders; Fig. 6), whereas in another four mice (non-responders), the decline in volume in the phloretin- and quinine-treated spermatozoa still occurred in the presence of furosemide or TEA (Fig. 6). At the first (20 s) time point, there were no differences between volumes of cells incubated with any treatment; none was different from the phloretin plus quinine control. By 305 s, significant differences were observed between the responders and non-responders to either furosemide or TEA in the cells given phloretin with quinine.

Possible toxic effects of heavy metal inhibitors

Two mitochondrial poisons, sodium azide and rotenone, at concentrations known to block oxidative phosphorylation and motility of spermatozoa (Van Dop *et al.* 1977, Aitken *et al.* 1997), were incubated with spermatozoa before hypo-osmotic challenge. Comparison of sperm volumes at 20 and 305 s demonstrated that there were no significant differences in cell volume between the controls and those in azide or rotenone or between cells incubated in azide or rotenone with quinine and those incubated in quinine alone (Fig. 7).

Figure 5 The effect of phloretin on volume regulation under initial time-course conditions. The relative volume of murine epididymal spermatozoa (mean $\pm$ S.E.M.; ordinate) with time after osmotic challenge (abscissa) with pre-incubation in isotonic (430 mmol/kg) medium alone (circles) for 10 min and then transfer to hypotonic (330 mmol/kg) medium alone (filled black circles, control) or hypotonic medium containing 800 μ mol/l quinine alone (open black circles), or incubation in isotonic medium containing 800 μ mol/l phloretin (squares) and transfer to hypotonic medium containing 800 μ mol/l phloretin alone (filled red squares) or 800 μ mol/l quinine together with 800 μ mol/l phloretin (open red squares). Statistical differences: *, between test and control at the same time point; §, between test and quinine treated at the same time point; $P < 0.05$. Between 20 and 60 000 spermatozoa were registered over the entire period from each of seven epididymides. Spermatozoa from the same mice were used for all treatments.

Discussion

Under the conditions in the long time-course protocol, osmotic water entrance into spermatozoa should be opposed by osmolyte efflux by RVD mechanisms, which in turn should be inhibited by the K-channel blocker quinine. This is demonstrated by the quinine-induced swelling as water enters the cells unopposed. Simultaneous addition of phloridzin, an inhibitor of water/solute transport, did not affect cell volume while acting alone; nor did it affect the quinine-induced swelling, suggesting that sodium-dependent glucose transporters are not involved in water transport during RVD.

Under similar conditions, however, the effects of Hg^{2+} were difficult to interpret. Cells were initially larger than controls, but this was followed by a decrease in volume. These results are compatible with the concept that when RVD becomes activated by the hypo-osmotic challenge, which should be immediate as in the controls, Hg^{2+} first inhibits water efflux more than influx, perhaps related to the time it takes to penetrate the cell and to bind to and inhibit the sulphhydryl groups of the channels. Cell swelling caused by the initial inhibitory activity may trigger other, Hg-insensitive, water transport mechanisms to allow water efflux driven by osmolyte efflux in RVD. These may include Cl^- /anion channels that also allow water passage (Hasegawa *et al.* 1992, Pohl 2004). With this protocol, in the transfer from

Figure 6 The effect of TEA, furosemide and phloretin on volume regulation under initial time-course conditions. The relative volume of murine epididymal spermatozoa (mean $\pm$ S.E.M.; ordinate) with time after osmotic challenge (abscissa) after incubation in isotonic (430 mmol/kg) medium containing 800 μ mol/l phloretin for 10 min and then transfer to hypotonic (330 mmol/kg) medium containing 800 μ mol/l phloretin and 800 μ mol/l quinine (open red squares), 800 μ mol/l phloretin and 800 μ mol/l quinine with 5 mM TEA (green squares), or 800 μ mol/l phloretin and 800 μ mol/l quinine with 1 mM furosemide (blue diamonds). Spermatozoa from four of the eight animals responded to the additional inhibitors, TEA or furosemide in delaying volume decline (closed symbols), whereas four did not (open symbols). Statistical differences: *, between additional TEA or furosemide and no TEA or furosemide control at the same time point; $P < 0.05$. Between 20 000 and 60 000 spermatozoa were registered over the entire period from each of 8 (TEA or furosemide) or 16 (no TEA; furosemide control) epididymides. Spermatozoa from the same mice were used for all treatments.

epididymal fluid (high osmolality, high K^+) to the test medium, spermatozoa are subjected to both osmotic and ionic changes. Changes in membrane potential would activate ion pumps, as it occurs during copulation.

To eliminate the problematical interpretation related to an anticipated delay in the action of Hg, the metal ion was allowed to interact with the functional groups on the transport proteins before hypo-osmotic challenge. In this experimental design (termed the initial time-course protocol), spermatozoa were pre-incubated in a medium with the mean osmolality of epididymal fluid, i.e. close to isotonic conditions (Cooper *et al.* 2008), so that osmotic challenges would be minimised during pre-incubation with the putative inhibitors without quinine. Thereafter, they were subjected to a physiological osmotic challenge in the drug in the absence or presence of quinine. In this protocol, sperm membrane repolarisation would occur under isotonic conditions, so that the subsequent transfer to the test medium would largely reflect an osmotic response.

If the pre-incubation were long enough for the water channel inhibitor to become effective, any immediate effects on water entry into the cell would become apparent; therefore, the cell volume was monitored at the earliest time-point (20 s) and continuously for 5 min. Furthermore, as quinine blocks osmolyte efflux during RVD, resulting in cell swelling by unopposed entry of

Figure 7 The effect of metabolic poisons on volume regulation under initial time-course conditions. The relative volume of murine epididymal spermatozoa (mean $\pm$ S.E.M.; ordinate) with time after osmotic challenge (abscissa) with pre-incubation in isotonic (430 mmol/kg) medium alone (circles) for 10 min and then transfer to hypotonic (330 mmol/kg) medium alone (filled black circles, control) or hypotonic medium containing 800 μ mol/l quinine alone (open black circles), pre-incubation in isotonic medium containing 20 μ mol/l rotenone (squares) and transfer to hypotonic medium containing 20 μ mol/l rotenone alone (filled red squares) or 800 μ mol/l quinine together with 20 μ mol/l rotenone (open red squares), or incubation in isotonic medium containing 20 μ mol/l sodium azide (diamonds) and transfer to hypotonic medium containing 20 μ mol/l sodium azide alone (filled blue diamonds) or 800 μ mol/l quinine together with 20 μ mol/l sodium azide (open blue diamonds). Between 20 000 and 60 000 spermatozoa were registered over the entire period from each of eight epididymides. Spermatozoa from the same mice were used for all treatments.

water, any blockade of water entry would be demonstrated by cell volumes similar to those of the controls even in the presence of quinine.

Indeed, after pre-incubation, Hg^{2+} was able to block the volume increase that normally occurs under hypotonic conditions when RVD is inhibited by quinine, which suggests that Hg^{2+} blocks water entry. Because both AQP8 and AQP7 proteins are present in rat spermatozoa (Calamita *et al.* 2001) and Hg^{2+} inhibits AQP8 (Nielsen *et al.* 2007), but not AQP7 (Ishibashi *et al.* 1997), AQP8 may be mediating the water influx into murine spermatozoa. In the presence of quinine, blockade of water influx was also blocked by Ag^+ since the volume of the treated cells was no different from that of control cells. Since AQP8 is Ag^+ sensitive (Biernert *et al.* 2007), this is further evidence that AQP8 may be involved in water influx in spermatozoa. However, in the absence of quinine, Ag^+ alone allowed water influx to some extent, since cell volumes were intermediate between those of the controls and those with quinine. The water channel involved here is not known.

The ability of murine spermatozoa to transport water during volume regulation in the presence of sodium azide and rotenone, at concentrations known to prevent sperm motility and mitochondrial activity (Aitken *et al.* 1997), suggests that the effects of Hg^{2+} and Ag^+ on water transport were not due to any general toxic actions but

more specific effects on water transport, most likely the binding of Hg^{2+} to the cysteine residue (cys-187) near to one of the two Asn-Pro-Ala (NPA) motifs that form the water pore of different AQP isoforms (Preston *et al.* 1993, see also Agre 2006).

Evidence for the involvement of glucose transporters in water transport includes the ability of specific inhibitors of glucose transport, cytochalasin B and phloretin to decrease reversibly the osmotic water transport across the plasma membranes of several cell types (Fischbarg *et al.* 1989, 1990). Previous studies have suggested that they may be involved in spermatozoa since water transport in ovine and human spermatozoa are sensitive to phloretin (Curry *et al.* 1995). Although glucose transporters have been detected in spermatozoa (Table 1), in this study neither phloretin nor cytochalasin B prevented water influx in the presence of quinine, making the glucose transporter an unlikely candidate as a water channel in murine spermatozoa.

In the presence of quinine and phloretin, cell volume was the same as that of cells incubated in quinine alone, indicating that water influx had not been inhibited by phloretin. Furthermore, neither was water efflux inhibited, since with time the spermatozoa shrank to volumes smaller than the controls. This suggests that phloretin promotes osmolyte efflux through channels insensitive to quinine. In the absence of quinine, phloretin reduced sperm volume below the level of the control cells. This implies that under the control experimental conditions, RVD was not efficient enough to prevent hypo-osmotic swelling completely and phloretin either inhibited water entry or was promoting water efflux. It is unlikely that these effects are due to abnormal metabolism through phloretin's inhibition of glucose transport via the glucose transporter, since the BWB medium contains the respiratory substrates lactate and pyruvate. Although phloretin can inhibit AQP9 (Castle 2005), where antibodies have been used on epididymal sections, there are no reports of this AQP in luminal spermatozoa (Ruz *et al.* 2006, Da Silva *et al.* 2007).

Experiments designed to determine the possible alternative RVD mechanism in the presence of quinine and phloretin, leading to the observed water efflux, showed that both TEA and furosemide were able, in the same animals, to delay the onset of RVD. Unlike quinine, TEA itself has no effect on RVD of murine spermatozoa (Barfield *et al.* 2005), indicating that quinine-sensitive and TEA-insensitive voltage-sensitive K^+ channels are preferentially utilised for K^+ efflux in initial RVD. However, in the presence of quinine and phloretin in swollen cells, TEA-sensitive channels were clearly activated after a short delay. TEA blocks voltage-sensitive K^+ channels, small conductance, Ca^{2+} -sensitive K^+ channels (SK_{Ca} ; Coetzee *et al.* 1999) and large conductance K^+ channels (BK_{Ca} ; Rosenfeld *et al.* 2001), so it may be blocking K^+ channels operating in swollen, but not normal, cells. Furosemide alone is capable of blocking RVD of murine spermatozoa

with a delayed time course under long time-course conditions (Klein *et al.* 2006) and the $\text{K}-\text{Cl}$ co-transporter it blocks in the present study is presumably operating earlier in swollen cells in the presence of quinine and phloretin in the responder animals.

That spermatozoa from only four of eight mice responded in this way reflects the between-male variability in osmotic responses. These responses may depend, among other things, on the ability of the spermatozoa to take up osmolytes, the ability of the epididymis to provide them, the osmolalities experienced within that epididymis, the length of time stored within the epididymis, which depends on sexual activity and nocturnal seminal emissions, and the expression of the osmolyte and water channels.

In summary, there is no evidence that sodium-dependent glucose transporters, glucose transporters or AQP9 are involved in water transport during RVD of murine spermatozoa; but the Hg^{2+} - and Ag^+ -sensitive AQP8 is a likely candidate as water channel.

Materials and Methods

Animal experiments

All experiments were done on adult male mice of the C57BL/6N strain (Charles River, Sulzfeld, Germany) and were conducted according to the German Federal Law on the Care and Use of Laboratory Animals (licence G67/2001). Spermatozoa were obtained from the cauda epididymidis as described previously (Barfield *et al.* 2005) and were subjected to two experimental protocols. In the first (long time-course) procedure, spermatozoa were subjected to a hypotonic insult of physiological proportions at the same time as the inhibitors, as described previously (Barfield *et al.* 2005). Briefly, segments of the caudal tubule were excised and their contents transferred to BWB medium containing 4 mg BSA/ml (Biggers *et al.* 1971) with osmolality raised with NaCl to 330 mmol/kg similar to that of female tract fluids (Yeung *et al.* 2000; BWB₃₃₀) and incubated for up to 45 min at 37 °C. Water channel inhibitors with or without quinine (800 $\mu\text{mol/l}$) were present from the outset. At 10–15 min intervals, aliquots were diluted with the same incubation medium containing 6 $\mu\text{g/ml}$ PI, gently mixed and drawn into the flow cytometer. The transfer from epididymal fluid to the lower osmolality, lower K^+ experimental medium will also repolarise the membranes of the spermatozoa that are essentially in K^+ -rich epididymal fluid. This will activate the ion pumps maintaining membrane potential in the same way as it occurs naturally upon ejaculation. However, ion loss during RVD is passive.

In the second protocol (initial time-course), the tubule contents were transferred to an albumin (4 mg/ml)-containing BWB medium with osmolality raised with NaCl to 430 mmol/kg (BWB₄₃₀; similar to that of epididymal fluid (Yeung *et al.* 1999) and close to isotonic with cauda epididymal spermatozoa (Cooper *et al.* 2008)) for pre-incubation at 37 °C in the presence of water transport inhibitors but not quinine. After 10 min, the sample was transferred to BWB₃₃₀ containing 6 $\mu\text{g/ml}$ PI as well as the inhibitor with or

without 800 µmol/l quinine and read immediately and continuously for 5 min in the flow cytometer. In this procedure, changes in membrane potential would occur in the 10-min pre-incubation period under isotonic conditions, but the subsequent challenge would be purely osmotic.

For each protocol, spermatozoa from the same mice were used for all conditions in a given replicate of an experiment.

Flow cytometry

The flow cytometer was a FACS 500 (Beckmann–Coulter Epics, Krefeld, Germany) using 488 nm laser as excitation source, a 19 °C for forward scatter detection and red PI fluorescence detection for gating of live cells. The machine alignment was checked on the day of use with fluorescent spheres (Fluorospheres, Beckmann–Coulter) in order to ensure that voltages and signals were reproducible between studies. For the long time-course protocol, samples were read from 5 min and then every 10 or 15 min for 45 min. For the initial time-course protocol, samples were read as soon as possible after sperm dispersion and 20 s after injection into the equipment (the time taken for the sample to load automatically and fill the dead space of sample tubing) in continuous reading mode for 5 min. Data were collected and displayed in sequential 15 s bins. Data presented here are solely from viable cells that excluded the vital dye PI. The volume of spermatozoa was calculated from a standard curve obtained from the forward scatter of fluorescent spheres of known diameter (3, 4 and 5 µm: Duke Scientific Corporation Palto Alto, CA, USA). A linear relationship between volume (fl: y) and forward scatter (mean channel number: x) was obtained ($y=0.211x-41.336$) with coefficients of correlation (r) of 0.980 and determination (r^2) of 0.961. From this relationship, the mean ($\pm$ S.E.M.) volume of 160 control spermatozoa in hypotonic medium was 73 ± 1 fl and 82 ± 1 fl in the absence and presence of quinine respectively. In order to determine changes with time, absolute cell volumes were expressed as ratios of the first control samples (5 min in the long time-course protocol and 20 s in the initial time-course protocol).

Inhibitors

All reagents were the purest grade from Sigma. Quinine–HCl, HgCl₂ and AgNO₃ were made up in water, whereas phoretin, cytochalasin B, phloridzin and furosemide were made up in DMSO. The final percentage of DMSO (0.2% v/v) had no effect on cell size (data not shown). Stock solutions of sodium azide (100 mM in water) and rotenone (20 mM in DMSO) were also prepared. In order to rule out the possibility that the metal ion effects were due to general toxicity, sperm volume and the response to quinine under hypotonic conditions were also measured when spermatozoa were pre-incubated (under initial time-course conditions) with the mitochondrial poisons at a final concentration of 20 µM.

Statistical analysis

Only the data from the long time-course experiments with phloridzin conformed to a normal distribution with equal variance and could be analysed by two-way ANOVA. No

matter how transformed, the remaining data could not be made to meet the required assumptions; so the optimal statistical analysis (two-way ANOVA) could not be applied. One-way ANOVA on normally distributed data, otherwise Kruskal–Wallis one-way ANOVA on ranks, was applied at relevant time points to illustrate the trends observed. When significant changes were found ($P<0.05$), this was followed by all pairwise comparisons or against the relevant controls as appropriate (with Dunn's test for non-Gaussian distributions and the Holm–Sidak method for Gaussian) with the statistical program SigmaStat (Systat Software, Version 3.5, Erkrath, Germany).

Declaration of interest

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

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