Motility activation and second messenger signalling in spermatozoa from rat cauda epididymidis

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This study examined molecular mechanisms involved in the activation of motility in spermatozoa from the cauda epididymidis of rats. A 1.05-fold dilution of semen from the cauda epididymidis with 300 mmol sucrose l^{-1} did not activate motility in spermatozoa. Addition of dibutyl cAMP, pentoxifylline or Ca^{2+} to the sucrose activated motility in the short term (<30–60 min). A fivefold dilution of semen from the cauda epididymidis with a modified Tyrode's medium (BWW) activated and sustained vigorous motility that could not be attenuated with kinase inhibitors. This motility was associated with a transient increase in intracellular cAMP during the first 60 s of activation. Lower motility was activated in Ca^{2+} -deficient media but this was not associated with an increase in cAMP. A fivefold dilution with plasma from the cauda epididymidis did not activate motility. The addition of

Ca^{2+} to the sucrose induced an increase in cAMP of similar duration but lower magnitude to that associated with dilution in BWW. The results from this study indicate that the cAMP and Ca^{2+} signal transduction pathways are involved in activation of sperm motility, and that the increase in intracellular cAMP in rat spermatozoa from the cauda epididymidis undergoing motility activation is Ca^{2+} -dependent. This is the first study to report a Ca^{2+} -dependent increase in cAMP associated with motility activation in immotile mammalian spermatozoa. In light of these data, a model is proposed whereby cAMP and Ca^{2+} act as synarchic messengers, initiating a signal transduction cascade, which is independent of protein kinase A-mediated phosphorylation of flagella proteins in immotile spermatozoa from the cauda epididymidis.

Introduction

Spermatozoa are stored in extratesticular ducts in an immotile state in many animals and their motility is activated on release from the ducts, by dilution. The mechanisms involved in the activation process appear to be resolved for a number of externally fertilizing species. In sea urchin spermatozoa, sodium–proton (Na^+H^+) exchangers in the plasma membrane are activated by a voltage change that occurs at dilution. This event, which is associated with an increase in intracellular pH ($pH_{(i)}$) and activation of axonemal dynein-ATPases, can be blocked by depolarization with a high external K^+ ($K^+_{(e)}$) concentration (Christen *et al.*, 1982). Adenylyl cyclase in this species is activated by hyperpolarization (Beltran *et al.*, 1996) and the subsequent increase in adenosine 3',5'-cyclic monophosphate (cAMP) is associated with activation of motility, and phosphorylation of specific flagellar proteins (Bracho *et al.*, 1998). In trout spermatozoa, high $K^+_{(e)}$ also regulates membrane polarity and motility can be activated by a reduction in the concentration of this ion or by the addition of divalent cations (Boitano and Omoto, 1991). Activation is

accompanied by an increased amount of cAMP and phosphorylation of a 15 kDa membrane protein (for reviews, see Hamamah and Gatti, 1998; Darszon *et al.*, 1999). In cyprinid fishes such as carp, osmotic pressure is an important regulator of activation. Hypo-osmotic shock and reduction in $K^+_{(e)}$ concentration hyperpolarizes the plasma membrane. This alteration in polarity is thought to permit an influx of Ca^{2+} , essential to activate motility in these cells, in which cAMP apparently has no role (Krasznai *et al.*, 2000).

Mechanisms involved in activation of motility in mammalian spermatozoa are less well defined. The effect of dilution on the sperm membrane is poorly characterized. However, changes in Ca^{2+} , K^+ , Na^+ and H^+ status have been implicated in the activation process, as have a number of other factors (for review, see Jones and Murdoch, 1996). These factors include a reduction in viscoelasticity of the extracellular fluid surrounding quiescent spermatozoa at activation (Usselman and Cone, 1983), activation of sperm adenylyl cyclase by bicarbonate ions (Okamura *et al.*, 1985), protein kinase A (PKA)-mediated phosphorylation of flagella proteins (Tash and Bracho, 1998) and by reactive oxygen species (ROS), particularly hydrogen peroxide (H_2O_2) (Aitken, 2000).

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There is general agreement that the cyclic nucleotide adenosine 3',5'-cyclic monophosphate (cAMP) plays a role in regulating sperm function, including motility and there is also evidence to suggest that an increase in cAMP is associated with activation of motility in spermatozoa from the cauda epididymidis of hamsters (Morton *et al.*, 1974) and rats (Armstrong *et al.*, 1994). Ca^{2+} also activates motility in spermatozoa from the cauda epididymidis from both these species (Morton *et al.*, 1974; Armstrong *et al.*, 1994).

An absolute prerequisite for the study of activation of motility is a viable population of spermatozoa that have never been exposed to primary activation stimuli. The present study has followed an experimental paradigm whereby immotile spermatozoa were collected directly from the cauda epididymidis, to avoid non-specific activation. The purpose of this investigation was to use this methodology to examine the mechanisms by which motility is activated in populations of spermatozoa from the cauda epididymidis, with particular emphasis on the dynamic interactions between cAMP and Ca^{2+} in controlling this event.

Materials and Methods

Animals

Outbred sexually mature Wistar male rats aged 3–6 months were used in all experiments. The animals were housed two per cage in the Central Animal House Facility, University of Newcastle with a 12 h light:12 h dark cycle and food and water were available *ad libitum*. Animals were killed with carbon dioxide inhalation. The University of Newcastle Animal Care and Ethics Committee approved the animal experimentation.

Collection and treatment of semen from the cauda epididymidis

Semen from the cauda epididymidis was collected into a siliconized 40 μl microcapillary tube (Bacto Laboratories, Sydney) by back-flushing the distal cauda epididymidis using paraffin oil saturated with water. Semen was dispensed under paraffin oil saturated with water and maintained at 34°C in a siliconized embryo dish. The spermatozoa in this semen were immotile when examined under a stereomicroscope. Epididymal plasma was separated from spermatozoa by centrifuging the semen in a microcapillary tube for 30 min in a haematocrit centrifuge (Gelman Hawksley Ltd, Sussex). Seminal vesicle fluid was collected into a 10 μl microcapillary tube (Bacto Laboratories, Sydney) by clamping the coagulating gland and puncturing the collecting duct of the seminal vesicle. Prostatic fluid was collected from the penis of anaesthetized rats while electrically stimulating the ventral lobes of the prostate gland at 20 V and a frequency of 7 Hz (Harvard Kymograph Apparatus Ltd, Kent).

Activation of sperm motility

Two different methods of activating sperm motility were used. The first method involved diluting 1 μl samples of semen from the cauda epididymidis (containing $1.8\text{--}2.2 \times 10^9$ spermatozoa ml^{-1}) fivefold with a modified Tyrode's medium (BWW: 95 mmol NaCl l^{-1} , 4.8 mmol KCl l^{-1} , 1.7 mmol $\text{CaCl}_2 \text{ l}^{-1}$, 1.2 mmol $\text{KH}_2\text{PO}_4 \text{ l}^{-1}$, 1.2 mmol $\text{MgSO}_4 \text{ l}^{-1}$, 24.88 mmol $\text{NaHCO}_3 \text{ l}^{-1}$, 5.55 mmol glucose l^{-1} , 0.27 mmol pyruvic acid l^{-1} , 0.25 mmol lactic acid l^{-1} , 0.5 units penicillin ml^{-1} , 0.5 μg streptomycin ml^{-1} , 20 mmol Hepes buffer l^{-1} , 0.3% BSA; 300 mOsmol l^{-1}) (Biggers *et al.*, 1971). Samples (1 μl) of semen from the cauda epididymidis were also diluted fivefold with epididymal plasma or fluid from the prostatic or seminal vesicles. The second method, adapted from the procedure used by Pholpramool *et al.* (1985), Clulow *et al.* (1992), Armstrong *et al.* (1994) and Murdoch *et al.* (1999), used a volumetric pipette to deliver various chemical reagents in 50 nl of 300 mOsmol sucrose l^{-1} to 1 μl samples of semen from the cauda epididymidis. This dilution (1.05-fold) with the sucrose solution alone was examined in all studies and did not activate sperm motility. Pipettes with hermetically sealed ends were used to mix the sucrose solution with the semen sample. Motility of samples was assessed under a microscope using reflected light, before mixing, within 1 min after mixing, then every 5–15 min, depending on the experiment, at 37°C. The period over which samples were monitored was dependent on the reagent; the minimum time was 30 min and the maximum time 60 min. During monitoring, the temperature of the sample was maintained by a laboratory warming tray or stage warmer (Reichert, Vienna). At the end of the period of monitoring all samples were assessed for viability by a fivefold dilution with BWW. The ability of kinase inhibitors to prevent the attenuation of motility was examined by either including them in the BWW used to carry out fivefold dilutions or by diluting a 1 μl sample of semen from the cauda epididymidis by 1.05-fold with 300 mOsmol sucrose l^{-1} containing 20 μmol H89 l^{-1} , and then further diluting the sample fivefold with BWW containing 100 μmol H89 l^{-1} at 0, 15 and 30 min after the initial addition of the inhibitor. The concentrations of substances stated represent the final concentrations after solutions were mixed with 1 μl of semen from the cauda epididymidis. The optimal concentrations for dibutyryl cAMP (dbcAMP), pentoxifylline (PTX) and Ca^{2+} were determined from dose–response experiments.

Assessment of sperm motility

The criterion for assessing sperm motility was the 'swirling' motion of the sample, which was scored using a scale of 0–10, where 0 represents an inactive sample and 10 represents a sample of maximum activity (Pholpramool *et al.*, 1985; Clulow *et al.*, 1992;

Table 1. Reagents tested for ability to activate motility when a 1 µl sample of semen from the rat cauda epididymidis was diluted 1.05-fold with 300 mmol sucrose l⁻¹ containing the reagent

Reagent	Concentration	Motility activation
Dibutyl cAMP	2, 4, 8, 16 mmol l ⁻¹	+
Dibutyl cAMP/glucose	16, 20 mmol l ⁻¹	+
Dibutyl cAMP/acetate	16, 20 mmol l ⁻¹	+
Dibutyl cAMP/lactate	16, 10 mmol l ⁻¹	+
Pentoxifylline	2, 4, 8, 16 mmol l ⁻¹	+
Calcium (Ca ²⁺)	2, 4, 8, 16 mmol l ⁻¹	+
1,3,7-trimethylxanthine (caffeine)	2, 4, 8 mmol l ⁻¹	+
Dibutyl cGMP	5, 10, 15 mmol l ⁻¹	-
Phorbol-12-myristate-13-acetate	0.25, 2.5, 25 µmol l ⁻¹	-
Bicarbonate (HCO ₃ ⁻)	10, 20 mmol l ⁻¹	-
Sodium chloride	1, 3, 5, 10, 15, 20, 40, 60 mmol l ⁻¹	-
Glucose	5, 10, 20, 40, 60 mmol l ⁻¹	-
Acetate	10, 20 mmol l ⁻¹	-
Lactate	10 mmol l ⁻¹	-
Forskolin	25, 50, 100, 200, 400 µmol l ⁻¹	-
Cholera toxin	10 µg ml ⁻¹	-
Pertussis toxin	1, 10 µg ml ⁻¹	-
Hydrogen peroxide (H ₂ O ₂)	5 × 10 ⁻⁴ , 0.05, 5, 500 µmol l ⁻¹	-
Hydrogen peroxide (H ₂ O ₂)	2.5, 5, 20, 30, 40 mmol l ⁻¹	-
NADPH	1.25, 2.5, 5 mmol l ⁻¹	-

Values shown represent final concentrations.

+: motility activation; -: no motility activation. Motility was assessed within 1 min after dilution and then every 5–15 min for 30 or 60 min, depending on the reagent. The viability of the samples after monitoring was examined by a fivefold dilution with BWW medium. A minimum of six replicates was carried out when the test reagent activated sperm motility. A minimum of three replicates was carried out when the test reagent did not activate sperm motility.

Armstrong *et al.*, 1994; Murdoch *et al.*, 1999). A fivefold dilution with BWW, which consistently achieved motility scores of 9–10, was used as a reference, against which all motility was scored. The scoring variance between experiments, achieved by a single scorer, is estimated to be approximately 10%.

Concentration of cAMP in spermatozoa

Samples of spermatozoa ($1.4\text{--}1.8 \times 10^9$ spermatozoa ml⁻¹ for activated and $1.8\text{--}2.2 \times 10^9$ spermatozoa ml⁻¹ non-activated samples) were homogenized in trichloroacetic acid (6% w/v) with a disposable polypropylene homogenization pestle (Scientific Specialties Incorporated, Lodi, CA) at 4°C, and extracted for 10 min. After extraction, samples were centrifuged at 11 600 g (10 min at 4°C) using a Clements Orbital 100 bench centrifuge. Supernatants were extracted with 10 volumes of diethyl ether saturated with water, dried under nitrogen at 50°C and stored at -70°C until assayed. Immediately before the assay, samples were reconstituted in 150 µl acetate buffer (0.05 mol sodium acetate l⁻¹, pH 5.8, 0.02% w/v bovine serum albumin and 0.01% w/v Germall2). cAMP was then determined using the non-acetylation procedure provided by the Biotrak cAMP enzyme immunoassay system (Amersham Pharmacia Biotech, Castle Hill) in accordance with the manufacturer's

guidelines. Absorbances were read at 450 nm on a microplate reader (Bio-Rad, Hercules, CA).

Statistical analyses

For studies of activation of sperm motility, ANOVA was used to compare the effect of activating reagents on sperm motility at a selected time point (10 min). Data from cAMP analyses were transformed to the natural log where necessary and assessed using ANOVA and the Tukey Honest Significant Difference (HSD) test. Statistica 5.5 software (StatSoft Pacific Pty Ltd, Melbourne) was used for these analyses. Data are shown as mean ± SEM; the SEM is calculated from the variance among animals.

Results

Activation of sperm motility with 1.05-fold dilution

It was confirmed that diluting a 1 µl sample of semen from the cauda epididymidis with 50 nl of 300 mmol sucrose l⁻¹ (1.05-fold) did not activate sperm motility. The effects of including various reagents in the sucrose vehicle are summarized (Table 1). Only the inclusion of dbcAMP, PTX, caffeine or Ca²⁺ activated sperm motility. Dose-response experiments were carried out and the concentrations of dbcAMP, PTX and Ca²⁺ that gave

Fig. 1. Activation of sperm motility in 1 μ l of semen from the rat cauda epididymidis diluted 1.05-fold with 300 mmol sucrose l^{-1} containing (a) dibutyl cAMP (▲) or pentoxifylline (△) at final concentrations of 16 mmol l^{-1} or Ca^{2+} (■) at a final concentration of 8 mmol l^{-1} or sucrose alone (○); (b) dibutyl cAMP (▲) at a final concentration of 16 mmol l^{-1} alone or combined with glucose (×) or acetate (◆) at a final concentration of 20 mmol l^{-1} or combined with lactate (□) at a final concentration of 10 mmol l^{-1} ; (c) 16 mmol dibutyl cAMP l^{-1} , at 0 min and again at 60 min; (d) 16 mmol dibutyl cAMP l^{-1} at 0 min and then further diluted fivefold with modified Tyrode's medium (BWW) at 60 min. Values are mean $\pm$ SEM for (a) 5; (b) 6 (×, ◆), 5 (□); (c) 7 and (d) 5 animals.

the optimal effect were chosen for further analyses (Fig. 1). The inclusion of dibutyl cGMP (dbcGMP), other salts, energy substrates, reactive oxygen species, such as those generated with nicotinamide adenine dinucleotide phosphate, reduced nicotinamide adenine dinucleotide phosphate (NADPH) or hydrogen peroxide (H_2O_2), forskolin, cholera (*Vibrio cholerae*) toxin or pertussis (*Bordetella pertussis*) toxin did not activate sperm motility.

Activation of sperm motility by Ca^{2+} was rapid, but declined and ceased within 30 min (Fig. 1a). dbcAMP activated stronger sperm motility than Ca^{2+} ($P < 0.05$) and this was sustained longer but spermatozoa became virtually immotile by approximately 60 min. PTX activated lower sperm motility than dbcAMP ($P < 0.05$), which also declined by approximately 60 min (Fig. 1a). Glucose, acetate and lactate were each included with the dbcAMP, to determine whether the decline in motility within 60 min of activation with dbcAMP was due to a lack of energy substrate. The inclusion of glucose

or acetate extended the period of sperm motility to approximately 120 min. When lactate was used as the potential energy substrate, sperm motility was lower and ceased at 75 min (Fig. 1b). Glucose, acetate or lactate alone did not activate motility (data not shown). After completion of the response to an initial activation by dbcAMP, it was not possible to re-activate sperm motility even by another addition of 16 mmol dbcAMP l^{-1} at 60 min (Fig. 1c). However, the spermatozoa were viable and responded to a fivefold dilution with BWW at 60 min by showing strong sperm motility (Fig. 1d).

Activation of sperm motility with fivefold dilution

Vigorous motility was activated in spermatozoa by diluting a 1 μ l sample of semen from the cauda epididymidis fivefold with BWW (Fig. 2a) or prostatic fluid (Fig. 2b). Sperm motility activated by BWW was maintained for hours (data not shown). When Ca^{2+} was omitted from the BWW, spermatozoa were less

Fig. 2. Activation of sperm motility in 1 μl of semen from the cauda epididymidis by fivefold dilution (a) with modified Tyrode's medium (BWW) containing 1.8 mmol $\text{Ca}^{2+} \text{ l}^{-1}$ (◆), 0 mmol $\text{Ca}^{2+} \text{ l}^{-1}$ (△) or with 350 mOsmol Hepes–sucrose solution l^{-1} (●) or with plasma from the cauda epididymidis (□); (b) with prostatic fluid or seminal vesicle fluid; (c) with BWW and subsequent inhibition of motility in the same sample with a further 20-fold dilution with plasma from the cauda epididymidis (motility ceased at 60 min). (d) The effect of the protein kinase inhibitor H89 on sperm motility in 1 μl of semen from the cauda epididymidis diluted first 1.05-fold with 300 mmol sucrose l^{-1} containing 20 $\mu\text{mol H89 l}^{-1}$ and then further diluted fivefold with BWW containing 100 $\mu\text{mol H89 l}^{-1}$, at 0 (■), 15 (◆) and 30 min (▲). Motility was not inhibited and remained comparable to controls (○; fivefold dilution with BWW alone). Values are mean $\pm$ SEM for (a) 5, (b) 4, (c) 3 and (d) 3 animals.

motile ($P < 0.01$). A lower sperm motility ($P < 0.01$) was activated in samples diluted fivefold with Hepes–sucrose (350 mOsmol l^{-1}), but this motility virtually ceased within 15 min (Fig. 2a). Sperm motility was not activated by dilution with plasma from the cauda epididymidis (Fig. 2a), or seminal vesicle fluid (Fig. 2b). The effect of plasma from the cauda epididymidis was examined further by activating spermatozoa in a 1 μl sample of semen from the cauda epididymidis by fivefold dilution with BWW then further diluting the sample 20-fold with plasma 15 min later. This reduced sperm motility significantly within 15 min of application ($P < 0.01$) and spermatozoa were immotile at 60 min (Fig. 2c).

The pathway for intracellular signal transduction was examined to determine whether inhibitors of PKA activity could prevent or attenuate sperm motility activated by fivefold dilution. The cell-permeable inhibitor of PKA,

H89 (20, 100 or 500 $\mu\text{mol l}^{-1}$), or the broad-spectrum kinase inhibitor, staurosporine (5 or 25 $\mu\text{mol l}^{-1}$) was included in BWW. However, dilution with BWW containing these reagents did not prevent activation. The effect of inhibiting PKA was investigated further by allowing H89 time to access inhibitory sites within the cells by diluting a 1 μl sample of semen from the cauda epididymidis by 1.05-fold with sucrose containing 20 $\mu\text{mol H89 l}^{-1}$, and then further diluting the sample fivefold with BWW containing 100 $\mu\text{mol H89 l}^{-1}$ at 0, 15 and 30 min after the initial addition of H89. Sperm motility was not inhibited by this treatment (Fig. 2d).

cAMP content of spermatozoa

The intracellular cAMP content of spermatozoa was determined to assess the involvement of a cAMP

Fig. 3. The intracellular cAMP content in spermatozoa after 1 μ l samples of semen from the cauda epididymidis were diluted fivefold with BWW medium containing 1.8 mmol Ca²⁺ l⁻¹ (♦), BWW containing 0 mmol Ca²⁺ l⁻¹ (▲) or 1.05-fold with 300 mmol sucrose l⁻¹ containing 8 mmol Ca²⁺ l⁻¹ (■). Values are mean $\pm$ SEM for six animals.

signalling pathway in activation of sperm motility. A fivefold dilution of spermatozoa from the cauda epididymidis with BWW stimulated a sevenfold increase in cAMP in spermatozoa during the first 60 s of motility activation, compared with non-activated spermatozoa ($P < 0.01$). However, there was no increase in cAMP when Ca²⁺ was omitted from the BWW (Fig. 3) or when samples were diluted with plasma from the cauda epididymidis (data not shown). Diluting semen from the cauda epididymidis 1.05-fold with 300 mmol sucrose l⁻¹ containing 8 mmol Ca²⁺ l⁻¹ caused a significant increase in intracellular cAMP in spermatozoa after 30 s ($P < 0.01$), compared with non-activated samples. This effect could not be mimicked by Mg²⁺ at a final concentration of 8 mmol l⁻¹ or 300 mmol sucrose l⁻¹ (data not shown).

Discussion

This analysis of mechanisms of activation of sperm motility in rats used a model that allows the effects of dilution to be examined independently of the constituents of the diluent. The results shed light on potential mechanisms of activation of sperm motility and second messenger signal transduction pathways used by spermatozoa from the cauda epididymidis during the initiation of motility. dbcGMP, sodium chloride and energy substrates do not activate motility. ROS, postulated to increase pH_i and thereby act as the first messenger in the activation cascade (Aitken, 2000), too had no effect on these cells. If sperm adenylyl cyclase is involved in the initial burst of motility, it does not appear to be influenced by bicarbonate (Okamura *et al.*, 1985), because the addition of this anion failed to activate sperm

movement under the conditions used in this study. This may indicate that the soluble form of adenylyl cyclase, reported in spermatozoa to be responsive to bicarbonate (Chen *et al.*, 2000), is not the adenylyl cyclase involved in the initiation of motility in immotile spermatozoa. Similarly, forskolin, which targets the catalytic domain of most transmembrane adenylyl cyclases (Tang and Hurley, 1998) also failed to activate motility in these cells. Pertussis or cholera toxins, which catalyse the covalent incorporation of ADP-ribose into α subunits of GTP-binding proteins (Freissmuth and Gilman, 1989), also had no effect on activation of sperm motility in this population of spermatozoa (present study). However, reagents such as dbcAMP, PTX and Ca²⁺, which directly target cAMP and Ca²⁺-signalling pathways, activated sperm motility without significant dilution of epididymal semen (present study).

The immobilin hypothesis has been put forward to account for sperm quiescence in the cauda epididymidis of rats. The basic tenet of this proposal is that caudal spermatozoa are immobilized by the mechanical constraint imposed by the high viscoelasticity of plasma from the cauda epididymidis, the dilution of which permits motility (Usselman and Cone, 1983). However, the present study and earlier work (Armstrong *et al.*, 1994; Murdoch *et al.*, 1999) has clearly demonstrated that activation of sperm motility can occur in the absence of dilution. Moreover, only plasma from the cauda epididymidis of rats, hamsters and to a much lesser extent guinea pigs, contain immobilin, yet spermatozoa from other species including mice, dogs, elephants and tammar wallabies, are quiescent *in situ* in the absence of immobilin (Jones, 1973, 1978; Carr *et al.*, 1985; Clulow *et al.*, 1992; Jones and Murdoch, 1996). This finding indicates that viscosity alone does not account for quiescence during storage. However, this highly viscous fluid doubtless retards motility in samples activated in the absence of dilution, compared with those activated by fivefold dilution.

As the motility induced by directly targetting cAMP and Ca²⁺ pathways ceased within 30–60 min, additional energy substrates were provided to test the hypothesis that these factors might be rate-limiting. The inclusion of glucose or acetate with the dbcAMP extended motility for a short time only, indicating that energy substrate availability is not rate-limiting. Furthermore, a second application of dbcAMP failed to re-activate motility, indicating that the cAMP pathway is completely refractory after the initial stimulus occurs. This putative refractory state may be due to a decrease in pH_i. For example, in the absence of dilution, protons produced as a result of respiration may acidify the sperm cytoplasm, to the point where motility becomes suppressed 60 min after activation. Indeed, motility suppression in acidic media has been demonstrated in spermatozoa from bulls (Carr *et al.*, 1985), wallabies (Clulow *et al.*, 1992) and mice (Tash and Bracho, 1998). Upon dilution with a

balanced salt solution this quiescence was reversed, such that vigorous motility can be activated and maintained over time (present study). Notwithstanding, motility was activated without an apparent increase in $\text{pH}_{(i)}$, indicating that cytoplasmic alkalization is not essential to activate motility in rat spermatozoa from the cauda epididymidis.

In the present study, the finding that Ca^{2+} activated motility, and increased intracellular cAMP, indicates that this ion has a central role in activation of sperm motility in spermatozoa from the cauda epididymidis. This was further emphasized when vigorous motility was activated and sustained by dilution with BWW containing Ca^{2+} but impaired when Ca^{2+} was omitted from the BWW. The sperm motility activated by dilution with prostatic fluid may also be attributed to the availability of Ca^{2+} , present in prostasomes (Palmerini *et al.*, 1999), because the intracellular concentration of Ca^{2+} has been shown to increase in human spermatozoa after fusion with prostasomes (Kravets *et al.*, 2000).

However, it is possible that calcium is not the only factor influencing motility activation, as lower motility was induced by dilution in Ca^{2+} -free BWW or a simple solution such as Hepes–sucrose, devoid of ionic support or energy substrates (present study). Although speculative, dilution may change the gradients of a number of ions in the extracellular fluid. This would cause a voltage change across the plasma membrane, and this may be activating. In sea urchin spermatozoa, altering membrane polarity activates Na^+/H^+ exchangers (Christen *et al.*, 1982); in carp spermatozoa it facilitates activation of Ca^{2+} channels (Krasznai *et al.*, 2000); it also triggers a cascade of events associated with activation of motility in trout spermatozoa (Boitano and Omoto, 1991). The fact that dilution with plasma from the cauda epididymidis did not activate sperm motility in the present study is entirely consistent with this notion, because dilution with this fluid would not affect ionic gradients surrounding the spermatozoa. Further plasma may return the sperm membrane to its resting state because vigorous motility was inhibited by a further dilution with plasma in the present study. A plausible alternative explanation may be that plasma from the cauda epididymidis contains an inhibitor of sperm motility. Because plasma from the cauda epididymidis inhibits the motility of spermatozoa already activated with BWW, a putative inhibitor may be capable of counteracting the stimulatory effects of calcium or dilution, possibly by interacting with Ca^{2+} channels in the sperm plasma membrane.

Results from the present study show that addition of Ca^{2+} is an absolute requirement for increasing intracellular cAMP in rat spermatozoa from the cauda epididymidis. An increase in intracellular cAMP was also demonstrated in hamster spermatozoa when motility was activated by dilution with a buffer containing Ca^{2+} (Morton *et al.*, 1974). However, in that study the effects

of dilution and Ca^{2+} were confounded. In contrast, the model of activation used by the present study allows for the effects of dilution on motility to be examined independently of the composition of the diluent. As a consequence, the transient increase in intracellular cAMP in cells to which Ca^{2+} is added can be attributed directly to the Ca^{2+} . Moreover, the effect is not a general feature of divalent cations because there is no increase in cAMP when Mg^{2+} is added to spermatozoa under identical conditions. Together these data indicate that the rapid increase in cAMP in spermatozoa undergoing motility activation is Ca^{2+} -dependent. To our knowledge, this is the first definitive measure of a Ca^{2+} -dependent increase in intracellular cAMP associated with activation of motility in spermatozoa from mammalian cauda epididymidis. One possible interpretation of this data is that Ca^{2+} directly activates the sperm adenylyl cyclase involved in motility. Currently three adenylyl cyclases are known to be stimulated by Ca^{2+} (types I, III and VIII; Cooper *et al.*, 1995). Adenylyl cyclase III has been demonstrated to be present in the acrosomal membrane of rat germ cells (Defer *et al.*, 1998), but not on the sperm tail.

The mechanism(s) by which cAMP stimulates motility is still open to question. In the present study, motility could not be inhibited using a PKA-selective inhibitor, H89. One possible reason for the lack of inhibition by H89 is that it failed to cross the cell membrane and access inhibitory sites within the spermatozoa. Although this study does not provide definitive evidence that H89 can cross the cell membrane, it was purchased as a cell-permeable reagent. For this reason we have assumed that access to binding sites is not a limiting factor in this study. Another possibility is that H89 attenuated motility at amounts below those that could be detected using the current protocol for scoring sperm motility. However, the fact that moderate to low sperm motility can be activated in these cells without increasing cAMP indicates that phosphorylation of flagella proteins mediated by PKA is not essential to activate sperm motility in cauda epididymidis in rats. Additional support for this notion is derived from a study by Burton *et al.* (1999). Type II PKA, reported to be the primary isoform of PKA in mature sperm flagellum (Lieberman *et al.*, 1988; Vijayaraghavan *et al.*, 1997), was thought to bind cAMP and thus stimulate motility. However, in mouse spermatozoa, targeted disruption of the type II α regulatory subunit (RII α) gene had no deleterious effects on motility (Burton *et al.*, 1999).

An alternative possibility to cAMP mediating PKA activation is that cAMP may interact directly with a target molecule, independently of PKA activation. Synergy between cAMP and Ca^{2+} signalling pathways has been demonstrated in other types of cell (Cooper *et al.*, 1995; Mons *et al.*, 1998) and in spermatozoa by this study. It is possible that cyclic nucleotide gated channels, located on the sperm tail (Weyand *et al.*, 1994; Wiesner *et al.*,

1998) or indeed, the novel sperm ion channel, Cat Sper (Ren *et al.*, 2001), similarly located (Quill *et al.*, 2001), are potential targets for cAMP. Both serve as entry points for Ca^{2+} in activated spermatozoa. Furthermore, because exposure of spermatozoa to Ca^{2+} increased intracellular cAMP within 60 s, and application of cAMP to mouse spermatozoa increased intracellular Ca^{2+} over a similar time course (Ren *et al.*, 2001), it seems plausible that these channels form part of a regulatory feedback mechanism to alter the amounts of these two key signalling molecules in spermatozoa and control the initiation of motility.

In conclusion, the cAMP and Ca^{2+} signal transduction pathways are involved in activation of motility in immotile spermatozoa from the cauda epididymidis. Motility can be activated by dilution in Ca^{2+} -free media and it is possible that this may be due to voltage change across the plasma membrane induced by dilution. However, the presence of Ca^{2+} is essential to increase sperm motility and intracellular cAMP. Moderate to low motility can be activated in these cells without increasing cAMP, indicating that PKA-mediated phosphorylation of flagella proteins is not essential for activating motility, and leading to the suggestion that cAMP targets alternative molecules involved in the activation process in immotile spermatozoa from the cauda epididymidis from rats.

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