Biochemical Identification of Dynein-ATPase Activity in Human Sperm

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Dynein-ATPase is the intracellular motor for sperm motility. In the present work we assayed the dynein-ATPase activity in an axoneme-containing fraction of human sperm, free of plasma membranes, in normozoospermic and asthenozoospermic donors. Axoneme-containing fractions were isolated from semen samples obtained from healthy donors with either normozoospermia or asthenozoospermia, as indicated by a sperm motility lower than 50% (WHO grade a + b). The dynein-ATPase activity was assayed and partially characterized. The dynein-ATPase activity in the axoneme-containing fractions was identified as Mg^{2+}-dependent ATPase activity inhibited by 10 μM vanadate. This inhibition was not seen when the assay was done in the presence of 1 mM norepinephrine. The dynein-ATPase activity is Mg^{2+}-dependent, Li^{+}-sensitive, and insensitive to 2 mM ouabain, 1 μM oligomycin, and 1 μM thapsigargin. The dynein-ATPase activity was significantly lower ($p < 0.001$) for asthenozoospermic donors as compared to normozoospermic donors. This is a straightforward dynein-ATPase assay that can be used to obtain data of functional interest in clinical or experimental settings.

Key words: Human Sperm, Dynein-ATPase, Axoneme

Introduction

The axoneme is a structure located within the sperm tail, composed of more than 250 proteins, and responsible for the generation of sperm motility (Inaba, 2003). Electron microscopy images show a typical arrangement of 9 pairs or doublets of peripheral microtubules surrounding 2 central microtubules (9 + 2), an arrangement characteristic of the axoneme of most mammals (Afzelius, 1959). Dyneins work as intracellular motors that project from the peripheral doublets of the axoneme, in an arm-like fashion. Each doublet has two arms: an outer dynein arm and an inner dynein arm, which have a crucial role in the tubular sliding that generates sperm motility. Sperm motility is proportional to the number of force-generating dynein molecules present in the axoneme (Lindemann, 2003). Absence of the dynein arms, as seen in some syndromes, leads to impairment of sperm motility and male infertility (Neesen *et al.*, 2001).

Gibbons (1965, 1974) has shown that dyneins exhibit an Mg^{2+}-dependent ATPase activity, which has been proposed to function as follows (Oiwa and Sakakibara, 2005). As soon as ATP binds to dynein, this protein is released from the microtubules and there is a conformational change. ATP is then hydrolyzed and there is a rebinding of dynein to the microtubules. The products of ATP hydrolysis, *i.e.* phosphate and ADP, are released from dynein slowly, and the release of ADP is the rate-limiting step of the whole process. The rebinding of dynein to the microtubules accelerates the ADP release (Omoto and Johnson, 1986). The dynein-ADP intermediate is thought to be involved in the interaction of dynein with the microtubules and the resultant force generation.

Only a few reports have tried to correlate the dynein-ATPase activity in the human sperm with

the effect of different drugs on the sperm motility (D'Cruz *et al.*, 2000; Romac *et al.*, 1994). One of the main problems with these studies arises from the fact that the isolation and ATPase assay of the dyneins require lengthy and laborious steps of purification. Furthermore, the biochemical identification of the dynein-ATPase activity can be masked by the presence of several Mg^{2+}-dependent phosphatase activities, *e.g.*, the plasma membrane Na^+/K^+- and Ca^{2+}-ATPases, the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), the secretory pathway Ca^{2+}-ATPase (SPCA1), the calmodulin kinase IV (CaMKIV), the H^+-ATPase from the mitochondrial membrane, the alkaline phosphatase from the plasma membrane, and other unspecific phosphatases. In the present work, in light of the fact that the dynein-ATPase activity is inhibited by micromolar amounts of vanadate (Gibbons and Gibbons, 1987; Shpetner *et al.*, 1988) and that this inhibition is reversed by norepinephrine (Omoto and Moody, 1988), we developed an easy way to biochemically identify the dynein-ATPase activity in axoneme-containing fractions of human sperm.

Material and Methods

Semen samples

Semen samples were obtained by masturbation into a sterile plastic container from healthy donors with either normal semen parameters or with asthenozoospermia, in agreement with the guidelines of the World Health Organization (WHO, 1999) and in accordance with the ethical standards established by the Declaration of Helsinki, as revised in Tokyo in 2004. The study protocol was approved by the Bioethics Committee of IVIC, Caracas, Venezuela, and all donors gave informed signed consent. The subjects were requested to have 3–4 d of abstinence before the collection. The median abstinence time was 3.0 d. The semen samples were analyzed with respect to semen volume, sperm motility and sperm concentration as soon as the ejaculates had liquefied (within 45–60 min), and then used to isolate the axoneme-containing fractions. The sperm concentration and motility were determined by CASA (Hamilton Thorne, Beverly, MA, USA). All the chemicals and reagents used in the present work were of analytical grade and were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Isolation of axoneme-containing fractions

Axoneme-containing fractions were obtained by a modification of a method previously described (Olson *et al.*, 1987). After liquefaction, the semen samples were resuspended with 9 ml of buffer A [50 mM 2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris), pH 7.4, 4 °C; 100 mM NaCl]. The suspension was then centrifuged at 59,764 × *g* for 10 min at 4 ºC with a SW41Ti rotor in a Beckman Ultracentrifuge. The supernatant was discarded and the pellet was resuspended in buffer B (10 mM Tris, pH 7.4, 4 °C; 0.5 mM EDTA; and a protease inhibitor cocktail composed of 0.5 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml pepstatin A). The suspension was then homogenized twice using a high-performance disperser Ultraturrax T-25 instrument (IKA) at 13,500 rpm for 5 s with an S25N-18G dispersing element. 3 ml of the suspension were placed on top of a discontinuous gradient of sucrose (3 ml 50% and 3 ml 20%). The gradient was centrifuged at 111,132 × *g* for 60 min with a rotor SW41Ti in a Beckman ultracentrifuge. The resulting pellet was washed, resuspended in buffer B, and utilized to assay the dynein-ATPase activity.

SDS pretreatment of the sperm fraction

In order to avoid the presence of membrane vesicles, the sperm fractions were pretreated with a mixture of 2% sodium dodecyl sulfate (SDS), 1% bovine serum albumin (BSA) and 25 mM imidazole, pH 7.2, 37 °C, as previously described (Marín *et al.*, 1986). A 240-μl aliquot of the fraction (approx. 0.4 mg protein/ml) was pretreated with SDS/BSA/imidazole to give a final content of 0.050% SDS, 0.025% BSA, 0.625 mM imidazole, pH 7.2, at 37 °C. The fractions were incubated for 20 min at 37 °C, and then assayed for ATPase activity. Protein was determined by the Bio-Rad micromethod based on Bradford (1976).

Incubation media for ATPase assays

The Na^+/K^+-ATPase activity was assayed with the following incubation medium (final concentrations): 50 mM Tris, pH 7.0 at 37 ºC; 2 mM $MgCl_2$; 100 mM NaCl; 20 mM KCl; 2 mM Tris-ATP; with and without 2 mM ouabain. The Na^+/K^+-ATPase activity was calculated as the difference between the amounts of phosphate liberated in the test

tubes in the presence and absence of ouabain, an inhibitor of this enzyme.

The Ca^{2+}-ATPase activity was assayed with the following incubation medium (final concentrations): 50 mM Tris, pH 7.4, 37 ºC; 3 mM $MgCl_2$; 0.1 mM ouabain; 80 mM NaCl; 15 mM KCl; 0.1 mM EGTA; 2 mM ATP; and 0.1 μM calmodulin; with and without 55 μM $CaCl_2$ (0.1 μM free Ca^{2+}). The Ca^{2+}-ATPase activity was calculated as the difference between the amounts of phosphate liberated in the test tubes in the presence and absence of 0.1 μM free Ca^{2+}.

The dynein-ATPase activity was assayed with the following incubation medium (final concentrations): 150 mM Tris, pH 8.0, 37 ºC; 2 mM $MgCl_2$; 2 mM Tris-ATP; 2 mM ouabain; 1 μM oligomycin; 1 μM thapsigargin (Sigma-Aldrich, St. Louis, MO, USA). The dynein-ATPase activity was calculated as the difference between the amounts of phosphate liberated in the presence and absence of 10 μM vanadate (as sodium orthovanadate). The inhibitory effect of vanadate on the ATPase activity was completely blocked by the presence of 1 mM norepinephrine in the incubation medium.

ATPase assays

The ATPase assays were determined as follows. An 180 μl-aliquot of the specific incubation medium was prewarmed at 37 °C for 2 min. The reaction was started by addition of 20 μl of the sperm fraction (0.1–0.2 mg protein ml^{-1}) previously treated with SDS. After 30 min of incubation, 300 μl of a stop solution containing (final contents) 0.72% SDS, 0.48% molybdic acid, 2.8% ascorbic acid, and 2.18% hydrochloric acid were added to the test tubes, which were placed on ice for 10 min, and then mixed with 500 μl of a solution containing (final contents) 2% sodium arsenite, 2% sodium citrate, and 2% acetic acid. The tubes were then rewarmed at 37 °C for 10 min, and the developed colour was measured in a Sunrise (Tecan) spectrophotometer at 705 nm. All samples were run in quadruplicate. Activity was expressed as nmol of Pi liberated per mg of protein per min, after subtraction of a blank run in parallel without the sperm suspension, which was added after the reaction was stopped. For the used incubation time (30 min), the ATPase activity was time-linear for all the samples assayed (Marín *et al.*, 1986; Proverbio *et al.*, 1986).

Statistics

The results are expressed as means ± standard error for the number of indicated assays with different preparations in each case. The standard errors of the differences were calculated for paired data. Data analysis was performed with the Student's *t* test, "*n*" being the number of repeated assays with different preparations. Only probabilities < 0.05 were accepted.

Results and Discussion

Axoneme-containing fractions from human sperm were tested for possible cross-contamination with plasma membranes. This was assessed by assaying the activity of well-known enzyme markers of the sperm plasma membrane such as the Na^+/K^+- and the Ca^{2+}-ATPase (Koçak-Toker *et al.*, 2002; Schuh *et al.*, 2004). The results of these assays are shown in Tables I and II. The axoneme-containing fractions show a Mg^{2+}-ATPase activity and neither Na^+/K^+-ATPase (Table I) nor Ca^{2+}-ATPase (Table II) activities. This is a clear

Table I. Na^+/K^+-ATPase activity in axoneme-containing fractions of human sperm.

Incubation medium	ATPase activity [nmol Pi/mg prot · min]	Na^+/K^+-ATPase activity [nmol Pi/mg prot · min]
a) Mg^{2+} + ouabain	117 ± 5	
b) Mg^{2+} + Na^+ + K^+	123 ± 6	6 ± 3 (b–a)
c) Mg^{2+} + Na^+ + K^+ + ouabain	122 ± 5	1 ± 3 (b–c)

The ATPase assay was carried out as follows (final concentrations): (a) 150 mM Tris-HCl, pH 7.0, 37 ºC; 2 mM $MgCl_2$; 2 mM Tris-ATP; 2 mM ouabain; (b) 50 mM Tris-HCl, pH 7.0, 37 ºC; 2 mM $MgCl_2$; 100 mM NaCl; 20 mM KCl; 2 mM Tris-ATP; (c) 50 mM Tris-HCl, pH 7.0, 37 ºC; 2 mM $MgCl_2$; 100 mM NaCl; 20 mM KCl; 2 mM Tris-ATP; 2 mM ouabain. Na^+/K^+-ATPase activity was calculated by subtracting either the activity value in row (b) minus the one in row (a) or the value in row (b) minus the one in row (c). Values are expressed as means ± standard error, $n = 6$. The standard error for the Na^+/K^+-ATPase activity was calculated for paired data. There are no significant differences among the activity values of groups a, b, and c.

Table II. Ca^{2+}-ATPase activity in axoneme-containing fractions of human sperm.

Incubation medium	ATPase activity [nmol Pi/mg prot · min]	Ca^{2+}-ATPase activity [nmol Pi/mg prot · min]
a) Mg^{2+}	127 ± 4	
b) Mg^{2+} + Ca^{2+}	128 ± 5	1 ± 2 (b–a)
c) Mg^{2+} + Ca^{2+} + calmodulin	128 ± 4	1 ± 3 (c–a)

The ATPase assay was carried-out as follows (final concentrations): (a) 150 mM Tris-HCl, pH 7.4, 37 ºC; 3 mM $MgCl_2$; 0.1 mM ouabain; 0.1 mM EGTA; 2 mM ATP; (b) 50 mM Tris-HCl, pH 7.4, 37 ºC; 3 mM $MgCl_2$; 0.1 mM ouabain; 80 mM NaCl; 15 mM KCl; 0.1 mM EGTA; 2 mM ATP; 55 μM $CaCl_2$ (0.1 μM free Ca^{2+}); (c) 50 mM Tris-HCl, pH 7.4, 37 ºC; 3 mM $MgCl_2$; 0.1 mM ouabain; 80 mM NaCl; 15 mM KCl; 0.1 mM EGTA; 2 mM ATP; 55 μM $CaCl_2$ (0.1 μM free Ca^{2+}); 0.1 μM calmodulin. Ca^{2+}-ATPase activity was calculated by subtracting either the activity value in row (b) minus the one in row (a) or the value in row (b) minus the one in row (c). Values are expressed as means ± standard error, $n = 6$. The standard error for the Ca^{2+}-ATPase activity was calculated for paired data. There are no significant differences among the activity values of groups a, b, and c.

Table III. Vanadate-sensitive ATPase activity in axoneme fractions of human sperm in the presence and absence of $MgCl_2$.

Addition	ATPase activity [nmol Pi/mg prot · min]		Vanadate-sensitive ATPase activity [nmol Pi/mg prot · min]
	– vanadate	+ vanadate	
a) None	104 ± 3	78 ± 3	26 ± 2*
b) EDTA	5 ± 2	2 ± 2	3 ± 2

The ATPase assay was carried out as follows (final concentrations): (a) 150 mM Tris-HCl, pH 8.0, 37 ºC; 2 mM $MgCl_2$; 2 mM Tris-ATP; 2 mM ouabain; (b) 50 mM Tris-HCl, pH 8.0, 37 ºC; 2 mM Tris-ATP; 2 mM ouabain; 1 mM EDTA. The ATPase assay was carried out in the presence and absence of 10 μM vanadate. The vanadate-sensitive ATPase activity was calculated by subtracting the activity value in the presence and absence of vanadate. Values are expressed as means ± standard error, $n = 6$. The standard error for the vanadate-sensitive ATPase activity was calculated for paired data.

* $p < 0.001$ vs. vanadate-sensitive ATPase activity in the absence of Mg^{2+} (row b).

indication that the axoneme-containing fractions from human sperm are practically free of plasma membranes.

The Mg^{2+}-ATPase activity of the axoneme-containing fractions was partially inhibited by vanadate, reaching maximal inhibition at a concentration of 5 μM (data not shown). Vanadate has been shown to be a potent inhibitor of P-type ATPases (Carafoli, 1992), myosin-ATPase (Goodno, 1979), SERCA (O'Neal *et al.*, 1979), and dynein-ATPase (Gibbons *et al.*, 1978; Kobayashi *et al.*, 1978; Shimizu and Johnson, 1983). Since vanadate inhibits the dynein-ATPase and also the sperm flagellar motility (Gibbons *et al.*, 1978; Kobayashi *et al.*, 1978), the vanadate-sensitive Mg^{2+}-ATPase activity of the axoneme-containing fractions shown in our results might represent the dynein-ATPase activity of the human sperm. The remaining vanadate-insensitive Mg^{2+}-ATPase activity of the preparations is probably the result of the activity of different ATPases and phosphatases present in the human sperm (Harper *et al.*, 2005; Tomes *et al.*, 2004).

The Mg^{2+}-dependence of the vanadate-sensitive ATPase activity of the axoneme-containing fractions was studied, and the results, shown in Table III indicate clearly that the vanadate-sensitive ATPase activity is Mg^{2+}-dependent. The optimum ATP concentration for the dynein-ATPase activity was found to be between 1 and 2 mM, with a K_m value around 150 μM (data not shown).

Fig. 1 shows the effect of the pH value of the incubation medium on the Mg^{2+}-ATPase activity of the axoneme-containing fractions, in the presence and absence of 10 μM vanadate. The vanadate-sensitive Mg^{2+}-ATPase activity shows an optimal pH value at around 8.0, which is in agreement with the optimal alkaline pH value found for dynein-ATPases of bull, sea urchin, and trout sperm (Belles-Isles *et al.*, 1986; Gatti *et al.*, 1989; Gibbons and Fronk, 1979).

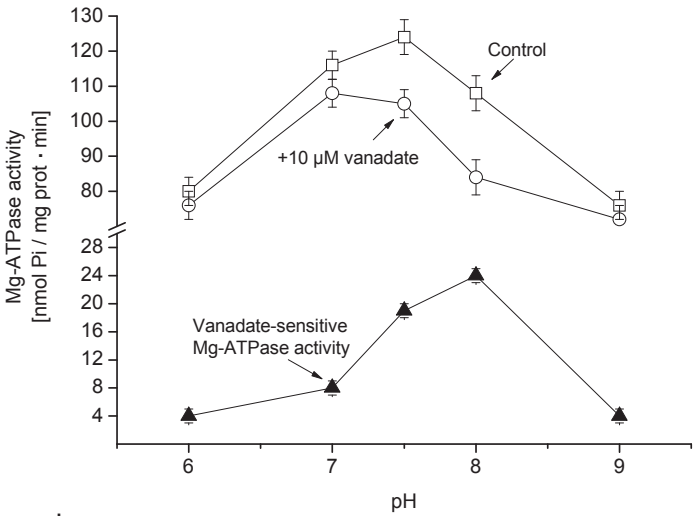

Fig. 1. Effect of the pH value of the incubation medium on the Mg^{2+}-ATPase activity of axoneme-containing fractions of human sperm. The Mg^{2+}-ATPase activity was assayed in the following medium: 150 mM Tris-HCl, pH 8.0, 37 ºC; 2 mM $MgCl_2$; 2 mM Tris-ATP; with and without 10 μM vanadate. Values are expressed as means ± standard error for n = 17. The vanadate-sensitive Mg^{2+}-ATPase activity was calculated as the difference of Mg^{2+}-ATPase activity in the presence and absence of 10 μM vanadate. The standard errors of the differences were calculated for paired data. Data analysis was performed with Student's t test. * $p < 0.001$ vs. vanadate-sensitive ATPase activity at the indicated pH.

The vanadate-sensitive Mg^{2+}-ATPase activity of the axoneme-containing fractions from human sperm was tested for several ATPase inhibitors: 2 mM ouabain [Na^+/K^+-ATPase inhibitor (Koçak-Toker *et al.*, 2002)], 1 μM oligomycin [mitochondrial Mg^{2+}-ATPase inhibitor (Erkkila *et al.*, 2006)], 1 μM thapsigargin [SERCA inhibitor (Lawson *et al.*, 2007)] and LiCl [dynein-ATPase inhibitor (Gibbons and Gibbons, 1984)]. The results, shown in Table IV, indicate that only LiCl, an inhibitor of dynein-ATPase, produced an important inhibition of the vanadate-sensitive Mg^{2+}-ATPase activity of the axoneme-containing fraction. All the other tested inhibitors had no effect on the ATPase activity. This result is very important, since it is in agreement with the finding that low concentrations of Li^+ reversibly inhibit the microtubule-based movement of reactivated sea urchin sperm flagella through inhibition of the dynein-ATPase activity (Gibbons and Gibbons, 1984).

It has been shown that norepinephrine effectively impedes and reverses the vanadate inhibition of the dynein-ATPase by reducing vanadate (+V) to oxovanadium (+IV) (Omoto and Moody, 1988). Consequently, the effect of 1 mM norepinephrine on the Mg^{2+}-ATPase activity of our preparations was assayed in the presence and absence of 10 μM vanadate. The results are shown in Fig. 2. It can be seen that the inhibition of the Mg^{2+}-ATPase activity by vanadate is completely abolished by the presence of norepinephrine in the assay medium.

Table IV. Effect of various inhibitors on the vanadate-sensitive Mg^{2+}-ATPase activity in axoneme-containing fractions of human sperm.

Inhibitor	Vanadate-sensitive Mg^{2+}-ATPase activity [nmol Pi/mg prot · min]
None	25 ± 4
Ouabain (2 mM)	26 ± 2
Oligomycin (1 μM)	24 ± 3
Thapsigargin (1 μM)	25 ± 3
LiCl (6 mM)	7 ± 2*

The Mg^{2+}-ATPase, was assayed in the following medium (final concentrations): 150 mM Tris-HCl, pH 8.0, 37 ºC; 2 mM $MgCl_2$; 2 mM Tris-ATP. The vanadate-sensitive Mg^{2+}-ATPase activity was calculated as the difference of Mg^{2+}-ATPase activity in the presence and absence of 10 μM vanadate (see Fig. 1). Activity values are expressed as means ± standard error, n = 6.
* $p < 0.001$ vs. no addition.

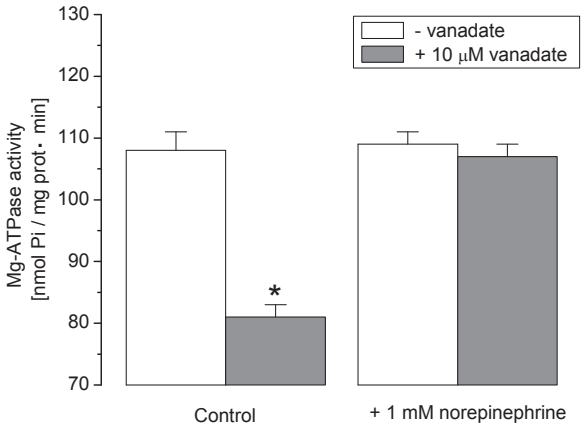

Fig. 2. Effect of 1 mM norepinephrine (final concentration) on the Mg^{2+}-ATPase activity of axoneme-containing fractions of human sperm. The Mg^{2+}-ATPase activity was assayed in the following medium: 150 mM Tris-HCl, pH 8.0, 37 °C; 2 mM $MgCl_2$; 2 mM Tris-ATP; 1 mM EDTA; in the presence and absence of 10 μM vanadate. Norepinephrine was added simultaneously with vanadate to the assay medium, without any preincubation. Activity values are expressed as means ± standard error, $n = 6$. * $p < 0.001$ vs. assay in the absence of vanadate and vs. assay in the presence of vanadate and norepinephrine.

To the best of our knowledge, there are two other works reporting dynein-ATPase activity determination in human sperm preparations (D'Cruz *et al.*, 2000; Romac *et al.*, 1994). However, in the first one D'Cruz *et al.* (2000) did not assay the effect of the different inhibitors of the ATPase activities. In the other one Romac *et al.* (1994) did not explore the effect of norepinephrine on the dynein-ATPase activity inhibited by vanadate, the effect of thapsigargin or the sensitivity of the enzyme to the pH value of the incubation medium. In the present work we considered only the vanadate-sensitive, ouabain-insensitive, oligomycin-insensitive, thapsigargin-insensitive, and Li^{+}-sensitive Mg^{2+}-ATPase activity of axoneme-containing fractions as the dynein-ATPase activity, while Romac *et al.* (1994) considered the ouabain-insensitive and oligomycin-insensitive Mg^{2+}-ATPase activity of demembranated spermatozoa as the dynein-ATPase activity.

The developed dynein-ATPase assay is a straightforward test and it can be used to identify pathological forms of motility that are associated with changes in the dynein-ATPase activity. In fact, we used the test to assay the activity of this enzyme in samples from asthenozoospermic donors, and we found it to be lower than that from normozoospermic donors [(11 ± 2) nmol Pi/mg protein · min versus (25 ± 3) nmol Pi/mg protein · min, respectively]. This dynein-ATPase assay can be used to test the effect of new drugs that could inhibit this ATPase activity and therefore the sperm motility.

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Afzelius B. (1959), Electron microscopy of the sperm tail. Results obtained with a new fixative. J. Biophys. Biochem. Cytol. **5**, 269.

Belles-Isles M., Chapeau C., White D., and Gagnon C. (1986), Isolation and characterization of dynein ATPase from bull spermatozoa. Biochem. J. **240**, 863–869.

Bradford M. M. (1976), A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248–254.

Carafoli E. (1992), P-type ATPases. Introduction. J. Bioenerg. Biomembr. **24**, 245–247.

D'Cruz O. J., Vassilev A., and Uckun F. M. (2000), Studies in humans on the mechanism of potent spermicidal and apoptosis-inducing activities of vanadocene complexes. Biol. Rep. **62**, 939–949.

Erkkila K., Kyttanen S., Wikstrom M., Taari K., Hikim A. P., Swerdloff R. S., and Dunkel L. (2006), Regulation of human male germ cell death by modulators of ATP production. Am. J. Physiol. Endocrinol. Metab. **290**, E1145–1154.

Gatti J. L., King S. M., Moss A. G., and Witman G. B. (1989), Outer arm dynein from trout spermatozoa. Purification, polypeptide composition, and enzymatic properties. J. Biol. Chem. **264**, 11450–11457.

Gibbons I. R. (1965), Chemical dissection of cilia. Arch. Biol. **76**, 317–352.

Gibbons I. R. (1974), Mechanisms of flagellar motility. In: The Functional Anatomy of the Spermatozoon

(Afzelius B. A., ed.). Pergamon Press, Oxford, pp. 127–140.
Gibbons I. R. and Fronk E. (1979), A latent adenosine triphosphatase form of dynein 1 from sea urchin sperm flagella. J. Biol. Chem. **254**, 187–196.
Gibbons B. H. and Gibbons I. R. (1984), Lithium reversibly inhibits microtubule-based motility in sperm flagella. Nature **309**, 560–562.
Gibbons B. H. and Gibbons I. R. (1987), Vanadate-sensitized cleavage of dynein heavy chains by 365-nm irradiation of demembranated sperm flagella and its effect on the flagellar motility. J. Biol. Chem. **15**, 8354–8359.
Gibbons I. R., Cosson M. K., Evans J. A., Gibbons B. H., Houck B., Martinson K. H., Sale W. S., and Tang W.-J. Y. (1978), Potent inhibition of dynein adenosine triphosphatase and of the motility of cilia and sperm flagella by vanadate. Proc. Natl. Acad. Sci. USA **75**, 2220–2224.
Goodno C. C. (1979), Inhibition of myosin ATPase by vanadate ion. Proc. Natl. Acad. Sci. USA **76**, 2620–2624.
Harper C. V., Wootton L., Michelangeli F., Lefièvre L., Barrat C. L. R., and Publicover S. J. (2005), Secretory pathway Ca^{2+}-ATPase (SPCA1) Ca^{2+} pumps, not SERCAs, regulate complex $[Ca^{2+}]_i$ signals in human spermatozoa. J. Cell Sci. **118**, 1673–1685.
Inaba K. (2003), Molecular architecture of the sperm flagella: Molecules for motility and signaling. Zool. Sci. **20**, 1043–1056.
Kobayashi T., Martensen J., Nath J., and Flavin M. (1978), Inhibiton of dynein ATPase by vanadate and its possible use as a probe for the role of dynein in cytoplasmatic motility. Biochim. Biophys. Acta **16**, 146–154.
Koçak-Toker N., Aktan G., and Aykaç-Toker G. (2002), The role of Na,K-ATPase in human sperm motility. Int. J. Androl. **25**, 180–185.
Lawson C., Dorval V., Goupil S., and Leclerc P. (2007), Identification and localisation of SERCA 2 isoforms in mammalian sperm. Mol. Hum. Reprod. **13**, 307–316.
Lindemann C. (2003), Structural-functional relationships of the dynein, spokes, and central-pair projections predicted from an analysis of the forces acting within a flagellum. Biophys. J. **84**, 4115–4126.
Marín R., Proverbio T., and Proverbio F. (1986), Inside-out basolateral plasma membrane vesicles from rat kidney proximal tubular cells. Biochim. Biophys. Acta **858**, 195–201.
Neesen J., Kirschner R., Ochs M., Schmiedl A., Habermann B., Mueller C., Holstein A. F., Nuesslein T., Adham I., and Engel W. (2001), Disruption of an inner arm dynein heavy chain gene results in asthenozoospermia and reduced ciliary beat frequency. Human. Mol. Gen. **10**, 1117–1128.
Oiwa K. and Sakakibara H. (2005), Recent progress in dynein structure and mechanism. Curr. Op. Cell Biol. **17**, 98–103.
Olson G., Winfrey V., Winer M., and Davenport G. (1987), Outer acrosomal membrane of guinea pig spermatozoa: isolation and structural characterization. Gamete Res. **17**, 77–94.
Omoto C. K. and Johnson K. A. (1986), Activation of the dynein adenosinetriphosphatase by microtubules. Biochemistry **25**, 419–427.
Omoto C. and Moody M. (1988), Kinetics of vanadate dissociation: estimation of the rate by inhibitor inactivation. Anal. Biochem. **168**, 377–344.
O'Neal S. G., Rhoads D. B., and Racker E. (1979), Vanadate inhibition of sarcoplasmatic reticulum Ca^{2+}-ATPase and other ATPases. Biochem. Biophys. Res. Commun. **89**, 845–850.
Proverbio F., Proverbio T., and Marín R. (1986), Na^+-ATPase is a different entity from the $(Na^+ + K^+)$-ATPase in rat kidney basolateral plasma membranes. Biochim. Biophys. Acta **858**, 202–205.
Romac P., Žanić-Grubišić T., Čulić O., Cvitković P., and Flogel M. (1994), Sperm motility and kinetics of dynein ATPase in astheno- and normozoospermic samples after stimulation with adenosine and its analogues. Hum. Rep. **9**, 1474–1478.
Schuh K., Cartwright E. J., Jankevics E., Bundschu K., Liebermann J., Williams J. C., Armesilla A., Emerson M., Oceandy D., Knobeloch K. P., and Neyses L. (2004), Plasma membrane Ca^{2+} ATPase 4 is required for sperm motility and male fertility. J. Biol. Chem. **279**, 28220–28226.
Shimizu T. and Johnson K. A. (1983), Presteady state kinetic analysis of vanadate-induced inhibition of the dynein ATPase. J. Biol. Chem. **258**, 13833–13840.
Shpetner H. S., Paschal B. M., and Vallee R. B. (1988), Characterization of the microtubule-activated ATPase of brain cytoplasmic dynein (MAP1C). J. Cell Biol. **107**, 1001–1009.
Tomes C. N., Roggero C. M., De Blas G., Saling P. M., and Mayorga L. S. (2004), Requirement of protein tyrosine kinase and phosphatase activities for human sperm exocytosis. Dev. Biol. **265**, 399–415.
WHO (World Health Organization) (1999), WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction, 4th ed. Cambridge University Press, Cambridge.