The Role of Calcium and Ca\textsuperscript{2+}-ATPase in Maintaining Motility in Ram Spermatozoa*

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Extracellular calcium at millimolar concentrations inhibits collective motility of ejaculated ram spermatozoa. In untreated cells, or when motility was made dependent upon glycolytic activity, there is very small inhibition, but when motility was made dependent upon mitochondrial respiration there is very high inhibition in motility by increasing extracellular Ca\textsuperscript{2+} concentration. Quercetin, which inhibits (Ca\textsuperscript{2+} + Mg\textsuperscript{2+})-ATPase activity in isolated plasma membranes, also inhibits motility mainly in cells that have been made dependent upon glycolytic activity, but there is also inhibition in untreated cells. When motility was made dependent upon mitochondrial activity, there is no inhibition but rather some stimulation in motility by quercetin. The inhibitory effect of quercetin is enhanced by increasing Ca\textsuperscript{2+} concentration in the medium. Quercetin also inhibits uptake of calcium into the cells, in a mechanism by which a calcium channel is involved. This inhibition is high only when the glycolysis is inhibited in the cells. The rate of glycolysis is decreased by quercetin or ouabain, but their effects on motility are quite different. Based on these data, it appears that the plasma membrane (Ca\textsuperscript{2+} + Mg\textsuperscript{2+})-ATPase or the Ca\textsuperscript{2+} pump have a functional role in the regulation of spermatozoa motility. This motility regulation is functioning through mechanisms which include glycolytic activity and maintenance of intracellular calcium concentrations.

The biochemical mechanisms by which external factors regulate sperm motility remain unresolved in spite of many investigations related to this topic (1–3). There is some evidence which suggests that Ca\textsuperscript{2+} has a regulatory role in the control of sperm motility. Extracellular Ca\textsuperscript{2+} strongly affects motility of intact hamster (4, 5), mouse (5), and rat (6) sperm and stimulates motility of guinea pig (7), hamster (8), sea cucumber (9), and bovine (10) spermatozoa. Ram sperm displayed little, if any, change in the presence or absence of Ca\textsuperscript{2+} at less than 5 mM (11). In bovine sperm, there appears to be an uptake of Ca\textsuperscript{2+} which parallels motility (12, 13). Although the addition of Ca\textsuperscript{2+} to intact cells can show stimulation or inhibition of motility, only inhibitory effects of Ca\textsuperscript{2+} on motility of demembranated mammalian sperm have been reported (14, 15).

A steep inward concentration gradient of Ca\textsuperscript{2+} of about 10\textsuperscript{3}–10\textsuperscript{4} times exists between the intracellular and the extracellular space of the sperm. The low intracellular Ca\textsuperscript{2+} concentration (1.1–1.0 \mu M) is maintained through operation of several possible mechanisms. One of the most known mechanisms includes the ATP-dependent calcium pump or the (Ca\textsuperscript{2+} + Mg\textsuperscript{2+})-ATPase of the plasma membrane (16, 17). This calcium pump, in ram and bull spermatozoa plasma membrane, has been partially characterized by us (18, 19). In addition, we have shown that a calcium pump is located in the head and in the tail of the spermatozoa (20). Since the motility apparatus is located in the tail, it is reasonable to assume that the calcium pump of the tail plasma membrane is involved in regulating intracellular Ca\textsuperscript{2+} concentration for motility.

We demonstrate here for the first time the effect of extracellular Ca\textsuperscript{2+} and ATPase inhibitors on collective motility of ram spermatozoa. The results suggest that both Ca\textsuperscript{2+}-ATPase and (Na\textsuperscript{+} + K\textsuperscript{+})-ATPase of the plasma membrane extra-cellular Ca\textsuperscript{2+} are involved in maintaining motility of the spermatozoa.

**EXPERIMENTAL PROCEDURES**

**Materials**

Quercetin, ouabain, antimycin A, deoxyglucose, lactate dehydrogenase, and D-800 were obtained from Sigma. 4\textsuperscript{Ca}Cl\textsubscript{2} was purchased from New England Nuclear.

**Methods**

**Semen Collection and Sperm Preparation**—Semen was collected from rams by electric induction. The fresh semen was immediately diluted with calcium-free Krebs-Ringer buffer solution (pH 7.2) containing 20 mM sodium phosphate and 25 mM fructose (21). The sperm cells were washed as described by Reichart et al. (22). The diluted semen was layered on 10% (w/v) Ficoll-400 solution prepared in Krebs-Ringer, and centrifuged at 780 X g for 10 min. The cell pellet was washed from the remaining Ficoll by centrifugation, and the final pellet was resuspended in Krebs-Ringer buffer to reach a final concentration of 4 X 10\textsuperscript{6} cells/ml.

**Motility Measurement**—Collective motility was measured in a plastic cuvette, using the multi-channel RSG system, essentially as described earlier (23, 24). The excitation light wavelength was 450 nm, and the changes in reflected light were recorded on an 8-channel polygraph. In parallel, the analogue signals were analyzed by a digital computer. Each channel was sampled at a rate of 5 Hz, and frequency analysis was conducted on line. Wave frequencies were determined for each minute, thus enabling sperm collective motility to be followed during treatment in real time (25). The collective motility measurements reflect the number of motile cells and their motility velocity. Determination of collective motility is usually done in artificial insemination centers, and it was found that the degree of this motility is directly correlated with fertility (38).

**Preparation of Sperm Plasma Membranes and Uptake of Calcium**—Plasma membrane vesicles were prepared by differential centrifugation and sucrose gradient, as we described previously (18). These membranes showed a 15-fold enrichment of the plasma membrane (Na\textsuperscript{+} + K\textsuperscript{+})-ATPase and less than 4% of the cytochrome c oxidase specific activity found in the whole cell homogenates. When examined by transmission electron microscopy, the membranes were vesicular, and mitochondria were not identified. The ATP-dependent calcium uptake, Ca\textsuperscript{2+}-ATPase, and (Na\textsuperscript{+} + K\textsuperscript{+})-ATPase activities were essentially performed as we described earlier (18).
Measurements of Lactate Production—Washed cells were suspended in Krebs-Ringer buffer (pH 7.2) containing 20 mM sodium phosphate and 28 mM fructose, and were incubated at 37 °C. The reaction was started by addition of cells. The final cell concentration was 4 × 10^6 cells/ml. At appropriate time intervals, 30-μl samples were removed and immediately frozen at −20 °C. At the end of the incubation time, the frozen samples were thawed and 60 μl of 155 mM NaCl solution was added. After centrifugation at 1650 × g for 5 min, the cell-free supernatant was analyzed for lactate (26). All data are expressed as the experimental values corrected for zero time control.

Calcium Uptake by Intact Cells—Calcium uptake by intact spermatzoa was measured in Krebs-Ringer buffer (pH 7.2) containing 20 mM sodium phosphate, 0.2 mM CaCl₂, and 10 μM of 45CaCl₂. The reaction was started by the addition of calcium. The reaction mixture was incubated at 37 °C. At appropriate time intervals, 0.1-ml samples were removed and immediately vacuum-filtered on GF/C filters that had been prewashed with buffer composed of 150 mM NaCl, 10 mM Tris (pH 7.4), and 2 mM EGTA1 (solution A). The cells trapped on the filter were washed three times with 5 ml of ice-cold solution A. The dry filters were placed in scintillation vials with 4 ml of Lumax (Lumac) solution for measurement of β-radioactivity. All data are expressed as the experimental value corrected for the zero time control.

RESULTS

Effect of Extracellular Ca²⁺ Concentration on Sperm Motility—The collective motility of washed cells was measured in the sperm motility analyzer at various Ca²⁺ concentrations. It is shown in Fig. 1 that under conditions by which the cells were exposed to antimycin A, which inhibits mitochondrial electron transport, there is a small effect of Ca²⁺ on motility. In the presence of deoxyglucose, which inhibits glycolysis, the motility of the cells is largely decreased as the Ca²⁺ concentration increased. When the two systems, the mitochondria and the glycolysis, are operated normally, there is a small decrease in motility by elevating Ca²⁺ concentration in the medium. Thus, mitochondrial driving motility is highly sensitive to elevation in extracellular Ca²⁺ concentration, while glycolytic motility does not.

Effect of Quercetin on ATP-dependent Calcium Uptake in Isolated Membranes—The flavonoid quercetin belongs to a group of substances described as inhibitors of ion-transport ATPases. Among the ATPases investigated were (Na⁺ + K⁺)-ATPase (27, 28), mitochondrial H⁺-ATPase (27, 29), sarcoplasmic reticulum (30), and red blood cells Ca²⁺-ATPase (31). Here we show that the effect of quercetin on ATP-dependent Ca²⁺ pump is isolated plasma membranes from ram spermatzoa (Fig. 2). It can be seen that quercetin inhibits Ca²⁺ uptake by the membrane and this inhibition is time- and concentration-dependent. We also found that the Ca²⁺-ATPase activity is also inhibited by quercetin (see Ref. 18).

Effect of Quercetin on Sperm Motility—Since we have shown that quercetin inhibits the Ca²⁺ pump of the sperm plasma membrane, it was interesting to know how it might affect sperm motility. The results are shown in Fig. 3 and Table I. When the mitochondria and the glycolysis are normally operated in the cells, the motility rate decreases as the quercetin concentration increases (Fig. 3A). In the presence of antimycin A (inhibits mitochondrial activity), there is rather high inhibition of motility by quercetin (Fig. 3B), whereas in the presence of deoxyglucose (which inhibits glycolysis), there is no inhibition, but rather some stimulation can be seen (Fig. 3C). The effect of Ca²⁺ on motility in the presence of quercetin is shown in Table I. It can be seen that without any addition or in the presence of antimycin A, the inhibitory effect of quercetin on motility is higher when 1 mM Ca²⁺ was added. In the presence of deoxyglucose, extracellular Ca²⁺ causes high inhibition in motility, and this inhibition is lower when quercetin was added. We can see a contradictory effect of quercetin on motility: enhancing the inhibitory effect of Ca²⁺ in normal or antimycin A-treated cells and decreasing this Ca²⁺ effect in deoxyglucose-treated cells. In order to give an explanation to these results, we followed the effect of quercetin on calcium uptake into intact cells under identical conditions.

Effect of Quercetin on Ca²⁺ Uptake into Intact Cells—We have shown in Fig. 2 that quercetin inhibits ATP-dependent calcium uptake into isolated plasma membrane vesicles. As-

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1 The abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetrasacetic acid.
Role of Calcium and ATPase in Sperm Motility

Fig. 3. Effect of quercetin on sperm collective motility. Collective motility was determined as described under "Experimental Procedures." A, regular motility; B, glycolytic dependent motility (with 5 μM antimycin A); C, mitochondria dependent motility (with 20 mM deoxyglucose). In each part, the values represent the following concentrations of quercetin: control (Δ—Δ), 50 μM (■—■), 100 μM (▲—▲), and 200 μM (□—□). Each point represents the mean of 10 measurements during 10 min, from three experiments.

Assuming that the role of this calcium pump is to excrete calcium outside the cells, it was interesting to see how quercetin might affect uptake of calcium into intact spermatozoa. It can be seen in Fig. 4 that quercetin inhibits calcium uptake into the cells, and this inhibition is much higher when deoxyglucose was present.

When the mitochondrial activity was inhibited in the cells by adding antimycin A, there was very small uptake of calcium into the cells (Fig. 4) and, of course, it was difficult to determine precisely if there was any effect of quercetin under these conditions. It seems to us that if there is any effect of quercetin in the presence of antimycin A, this increases calcium uptake rather than inhibiting it (see Fig. 4). Since antimycin A causes above 90% inhibition of calcium uptake into the cells, we suggest that about 90% of the calcium uptake has gone into the mitochondria and only about 10% into the cytosol. Similar results have been shown by Babcock et al. (10) in epididymal bovine spermatozoa.

The inhibition of calcium uptake into the cells by quercetin cannot be explained by its inhibitory effect of the plasma membrane calcium pump, if this pump excretes calcium from the cell. It is more likely that the entry of calcium through a calcium channel of the plasma membrane is inhibited by quercetin. In order to test this possibility, the uptake of calcium into the cells was determined in the presence of D-600 which is known to inhibit calcium channels. The result in Fig. 4 shows inhibition of calcium uptake into the cells by D-600, and that the inhibition is much higher in deoxyglucose-treated cells. The degree of calcium uptake for both quercetin and D-600 is very similar. There is no effect of D-600 on ATP-dependent calcium uptake in isolated plasma membrane (data not shown); thus, we can exclude any involvement of this calcium pump in the described inhibitory effect of D-600 on calcium uptake into intact cells.

Effect of Ouabain on Sperm Motility—As we mentioned before, quercetin is not a specific inhibitor of Ca2+-ATPase.
TABLE I

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>0.1 mM quercetin</th>
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<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>1 mM Ca</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 mM quercetin</td>
</tr>
<tr>
<td>5 mM antimycin A</td>
<td>100</td>
<td>80 ± 4.5</td>
</tr>
<tr>
<td>20 mM deoxyglucose</td>
<td>100</td>
<td>29 ± 3.2</td>
</tr>
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FIG. 4. Effects of quercetin and D-600 on calcium uptake in intact cell. "Ca\(^{2+}\)" uptake into intact spermatozoa was determined as described under "Experimental Procedures." The effect of quercetin and D-600 on calcium uptake was determined in the absence and presence of 20 mM deoxyglucose which inhibits glycolysis. The values indicate the following: control (O—O), deoxyglucose (A—A), 0.1 mM quercetin (Δ—a), 0.1 mM quercetin plus deoxyglucose (●—●), 1 mM D-600 (□—□), 1 mM D-600 plus deoxyglucose (■—■), 5 mM antimycin A (V—V), and 5 mM antimycin A plus 0.1 mM quercetin (▼—▼). Each point represents the mean ± S.E. of duplicates from four experiments.

In order to know if the inhibitory effect of quercetin on motility depends upon its possible inhibition of (Na\(^+\) + K\(^+\))-ATPase, we followed the effect of ouabain (specific inhibitor of (Na\(^+\) + K\(^+\))-ATPase) on sperm motility. We used 0.1 mM ouabain, which inhibits the activity of (Na\(^+\) + K\(^+\))-ATPase in isolated plasma membranes of the sperm cells (data not shown). Under these conditions, ouabain causes about 25% inhibition of glycolytic motility (in the presence of antimycin A), about 40% stimulation of mitochondrial motility (in the presence of deoxyglucose), and about 10% stimulation of normal motility (without antimycin A or deoxyglucose) (see Table II).

Effect of Quercetin and Ouabain on Glycolytic Activity—The fact that an ATPase activity is required for steady-state glycolysis was first experimentally established by Meyerhof (32). We have shown here that quercetin and ouabain which inhibit ATPase activities also inhibit glycolytic dependent motility, while no inhibition was observed on mitochondrial driven motility. Therefore, it was important to see the effect of these ATPase inhibitors on glycolytic activity. It can be seen that the glycolytic activity is inhibited by the two ATPase inhibitors, but with quercetin the inhibition is higher (Fig. 5). No further inhibition of glycolysis could be found by increasing the concentrations of ouabain or quercetin to 1 and 0.2 mM, respectively. By adding 1 mM Ca\(^{2+}\), the inhibition of glycolysis by quercetin or ouabain is relatively higher than experiments without Ca\(^{2+}\) (Fig. 5). Similar results were seen when the total lactic acid (in cells and medium) was determined (data not shown).

FIG. 5. Effects of quercetin and ouabain on glycolytic activity in the cells. Glycolytic activity was determined as described under "Experimental Procedures." The values indicate the following: control (O—O), 0.1 mM quercetin (Δ—a), 0.1 mM quercetin plus 1 mM CaCl\(_2\) (V—V), 1 mM ouabain (□—□), and 1 mM ouabain plus 1 mM CaCl\(_2\) (■—■). Each point represents the mean ± S.E. of duplicates from three experiments.

DISCUSSION

We demonstrate here for the first time the effect of extracellular calcium concentrations and ATPase inhibitors on collective motility of ram spermatozoa. The experiments presented here show high dependence on elevation in extracellular calcium concentration only when cell motility was made dependent on mitochondrial activity (Fig. 1). Under these conditions, 2 mM added Ca\(^{2+}\) causes about 90% inhibition of motility, while in cells made dependent upon glycolytic activity, motility is only 10% inhibited. We also showed that above 90% of the calcium which has been uptaken by intact spermatozoa goes into the mitochondria (see Fig. 4), and the accumulated calcium can be released from the cells by the uncoupler carbonyl cyanide m-chlorophenylhydrazone (data not shown).
not shown). These results can be explained by assuming that the entry of Ca" into the mitochondria by a process which depends upon the pH gradient in the mitochondria (33) causes reduction in ATP generation by the mitochondria (34), and the motility rate is decreased.

We suggest that high extracellular calcium is inhibitory for mitochondrial ATP synthesis, and motility under these conditions is mainly dependent upon glycolytic activity. It is important to mention that we are working with well-washed cells, in medium without added calcium, and motility of the cells continues for about 2-3 h, at a cell concentration of 4 X 10^6 cells/ml. The free Ca" concentration in these media was measured by calcium-selective electrode and found to be ~50 µM. It is assumed that under these conditions the intracellular Ca" concentration is maintained low, and motility continues for a long time.

A possible way to change intracellular calcium concentration is to affect the activity of the plasma membrane (Ca" + Mg")-ATPase which is considered to be the ATP-dependent Ca" pump. We have shown here (Fig. 2) and previously (18) that the flavonoid quercetin inhibits this Ca" pump in isolated plasma membrane vesicles from spermatozoa. It is also shown (Fig. 3, A and B) that quercetin inhibits motility at concentrations and time dependence which is similar to its inhibitory effect on the Ca" pump. When 1 mM Ca" was present, there was a higher inhibition of motility with quercetin (Table I). Calcium itself causes 20% inhibition of motility, but in the presence of quercetin, motility is 50% inhibited. When motility was made dependent upon glycolysis (in the presence of antimycin A), Ca" causes 12 and 37% inhibition of motility in absence and presence of quercetin, respectively (Table I). These results suggest that quercetin is involved in the mechanism by which Ca" affects sperm motility. When the plasma membrane Ca" pump is inhibited by quercetin, calcium may accumulate in the cells and, as a result, the motility apparatus can be blocked. It has been shown in previous works that high intracellular calcium can modify or inhibit sperm motility (35, 36). In the case of mitochondrial dependent motility (Fig. 3C) there is no decrease, but rather a slight increase in motility by quercetin. Under these conditions, extracellular calcium by itself causes high inhibition of motility, and by adding quercetin, the inhibition is decreased (see Table I). The fact that quercetin does not inhibit mitochondrial driven motility may rule that a possible direct effect of quercetin on the mitochondria or on the motility apparatus. If this is the case, we can expect to see inhibition of mitochondrial motility by quercetin, which we do not see.

The question is: why does quercetin not inhibit mitochondrial driven motility, if calcium is really accumulated in the cells, as has been suggested previously? In order to answer this question, we followed calcium uptake into the cells. It was found that quercetin and D-600 as well cause high inhibition in Ca" uptake only when the glycolytic activity was inhibited (Fig. 4).

We thus conclude that at low extracellular Ca" (~50 µM) that there is no accumulation of Ca" in deoxyglucose-treated cells. This explains why quercetin does not inhibit mitochondrial dependent motility. At high extracellular Ca" (1 mM), mitochondrial motility is inhibited probably due to an increase in intracellular Ca" concentration, even in the presence of quercetin, but quercetin causes a 30% reduction in the inhibitory effect of Ca" on motility (see Table I).

From the similar effects of quercetin and D-600 on Ca" uptake, we assume that quercetin acts as a Ca" channel blocker in addition to its effect on (Ca" + Mg")-ATPase. Since high inhibition of Ca" uptake by quercetin or D-600 can be seen only when glycolysis is inhibited, it is possible that the mentioned Ca" channel is operated at a greater rate when glycolysis is inhibited. When glycolysis is operated normally, most of the Ca" taken by the cells is transported by an alternative Ca" carrier whose activity is dependent upon glycolysis, and it is not inhibited by quercetin or D-600.

It has been suggested by Racker et al. (37) that glycolysis in Ehrlich ascites tumor cells is inhibited by ouabain or quercetin. Here we show that these two ATPase inhibitors reduce the rate of glycolysis in ram spermatozoa (Fig. 5). Inhibition of glycolysis by quercetin is higher in the presence of Ca", but this Ca" effect is seen with ouabain as well. Thus, Ca" affects glycolysis in a mechanism which is not specific to (Ca" + Mg")-ATPase or (Na" + K")-ATPase. But, we may say that the plasma membrane ATPases are involved in the regulation of sperm glycolysis and motility.

There are some similarities and differences between the effect of ouabain and quercetin on motility (Tables I and II). Both inhibit mainly glycolytic dependent motility and stimulate mitochondrial driven motility. This can be explained by the fact that glycolysis, and not mitochondrial respiration, is inhibited by these two compounds. Stimulation in motility can be a result of increasing the availability of ATP for motility, since less ATP is hydrolyzed by the ATPases which are inhibited. The results (Tables I and II), which show that quercetin inhibits motility and ouabain does not, and the 40% stimulation of mitochondrial driven motility by ouabain and very little stimulation by quercetin, suggest that quercetin affects cell motility in a way which is not identical to that of ouabain. In addition, we found that ouabain did not affect Ca" flux, and the inhibitory effect of quercetin on Ca" uptake has not changed in the presence of ouabain. Thus, we suggest that the (Ca" + Mg")-ATPase plays a role in maintaining sperm motility in a mechanism which depends upon their stimulatory effect on cell glycolysis. Racker et al. (37) also suggested that quercetin may inhibit lactic acid secretion from cells; thus, (Ca" + Mg")-ATPase and/or the (Na" + K")-ATPase participate in the mechanism of lactic acid secretion, which prevents acidification of the intracellular space. The fact that quercetin or ouabain does not inhibit mitochondrial driven motility can be explained in part by the absence of glycolytic activity. However, we cannot explain the inhibitory effect of quercetin on motility only through inhibition of glycolysis, since deoxyglucose, which completely inhibits glycolysis, causes only a slight inhibition in motility (see Fig. 3).

In summary, we suggest that the plasma membrane (Ca" + Mg")-ATPase is involved in regulating glycolysis and intracellular calcium concentration, two parameters which are part of the mechanisms regulating spermatozoa motility.

Acknowledgments—We thank Professor A. Mayevsky, Professor B. Bartov, and M. Ailenberg for their help with the sperm motility analyzer, and B. Lederbender for the careful typing of this manuscript.

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