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Transport mechanism for calcium and phosphate in ram spermatozoa

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Calcium uptake into ejaculated ram spermatozoa is highly enhanced by the addition of extracellular phosphate. Under identical conditions, extracellular calcium stimulates the uptake of phosphate by the cells. Both calcium and phosphate uptake are comparably inhibited by the sulfhydryl reagent mersalyl. The I_{50} was found to be 6.36 and 10.14 nmol mersalyl per mg protein for phosphate and calcium uptake, respectively. Calcium uptake is inhibited by mersalyl whether phosphate is present or not. Extracellular fructose causes a 5-fold increase in calcium uptake. When fructose and phosphate are present in the cell's medium, there is an additive effect, which indicates that two independent systems are involved in calcium transport into the cell. Ruthenium red, which blocks Ca^{2+} transport into the mitochondria, causes 70% and 95% inhibition of calcium uptake in the absence or in the presence of fructose, respectively. Ruthenium red does not affect phosphate uptake unless calcium was present in the incubation medium. The stimulatory effect of fructose upon calcium uptake can be mimicked by L-lactate and can be inhibited by the glycolytic inhibitor 2-deoxyglucose. Fructose and L-lactate stimulate mitochondrial respiration in a comparable way. Oligomycin, which inhibits mitochondrial ATP synthesis, does not inhibit Ca^{2+} uptake. This indicates that ATP is not involved in the mechanism by which mitochondrial respiration stimulates Ca^{2+} uptake. The calcium channel blocker, verapamil, inhibits Ca^{2+} uptake in the presence or absence of extracellular phosphate. The phosphate-dependent calcium transport mechanism is more sensitive to verapamil than is the phosphate-independent transporter. In summary, the data indicate that the plasma membrane of mammalian spermatozoa contains a calcium/phosphate symporter, a phosphate-independent calcium carrier and a calcium-independent phosphate carrier.

Introduction

Motility and capacitation are two processes which occur in spermatozoa which are regulated by intracellular calcium concentrations ($[Ca]_i$) [1–6]. The selection of calcium as an intracellular messenger, requires precise regulation of its concentration. In mammalian spermatozoa, the systems that regulate $[Ca^{2+}]_i$ involve the Na^+/Ca^{2+} antiporter [7,8] and the ATP-dependent calcium pump [9–11] of the plasma membrane and the mitochondria [12]. Very little information has been accumulated about the transport mechanism(s) involved in calcium movement into the cell. It has been suggested that a Na^+/Ca^{2+} antiporter of the plasma membrane is involved in Ca^{2+} transport into bovine sperm [8]. This Na^+/Ca^{2+} carrier can

Abbreviations: FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetracetic acid; Mops, morpholinepropanesulfonate.

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be blocked by caltrin, a protein purified from bovine seminal plasma [5,8,13,14]. In a recent paper, we have suggested that a calcium-phosphate carrier is located in the plasma membrane of bovine spermatozoa [15]. In this current paper, we show for the first time comparable data for calcium and phosphate uptake into ram spermatozoa. We present direct proof of the existence of a calcium-phosphate transporter in sperm plasma membrane.

Materials and Methods

Semen was collected from rams by electric induction. The fresh semen was immediately diluted (1 : 1) with buffer 1 containing 110 mM NaCl, 5 mM KCl, 10 mM Mops (pH 7.4). The sperm cells were washed twice by centrifugation at $600 \times g$ for 10 min at room temperature. The final cell pellet was resuspended in buffer 1.

Uptake of ^{45}Ca by sperm suspensions was determined by the filtration technique. Cells ($3 \cdot 10^8$/ml) were incubated in buffer 1 containing 0.2 mM $CaCl_2$ and 2 μCi $^{45}CaCl_2$. The concentration of phosphate or fructose, when used, was 0.5 mM and 10 mM, respectively. After the appropriate incubation time at 37°C, 0.1 ml was removed and immediately vacuum-filtered on GF/C filters. The cells trapped on the filter were washed three times with 5 ml of solution composed of buffer 1 containing 2 mM $CaCl_2$. The dry filters were counted in scintillation vials with 4 ml of Lumax (Lumac Corp.).

Uptake of $[^{32}P]P_i$ by sperm suspensions was determined by the filtration technique, as described above for ^{45}Ca uptake. Cells ($3 \cdot 10^8$/ml) were incubated in buffer 1 containing 0.5 mM P_i and 5 μCi $[^{32}P]P_i$. The concentration of calcium or fructose, when used, was 0.2 mM and 10 mM, respectively. Incubation, filtration and counting conditions were described above for ^{45}Ca uptake. The cells trapped on the filter were washed three times with 5 ml of solution composed of buffer 1 containing 5 mM P_i. All data for ^{45}Ca and $[^{32}P]P_i$ uptake, are expressed as the experimental value corrected for the zero-time control.

Mitochondrial respiration of the cells was monitored at 37°C using a Clark oxygen electrode. The total protein content was 2.2 mg/10^8 cells. The experiments shown were each performed upon a single preparation of sperm pooled from one or two ejaculates, and are representative of three or more identical experiments performed with other such pools.

Results

Effect of phosphate on calcium uptake

Calcium uptake into ejaculated ram sperm is dependent on the concentration of added phosphate. As shown in Fig. 1, a 2-fold stimulation in calcium uptake results from the addition of 0.5 mM phosphate. The calcium uptake in absence of phosphate is 70% inhibited by Ruthenium red (or FCCP, data not shown), a result which indicates that 70% of the calcium taken up is accumulated in the sperm mitochondria. The dependency of calcium uptake upon extracellular calcium or phosphate concentrations is shown in Fig. 2. In the absence of added phosphate, the cell suspensions contain no detectable extracellular phosphate (data not shown). This finding indicates that phosphate is not released from the cells.

Effect of calcium on phosphate uptake

In Fig. 3 it is shown that phosphate uptake into ejaculated ram sperm is dependent upon extracellular phosphate concentrations. The uptake of

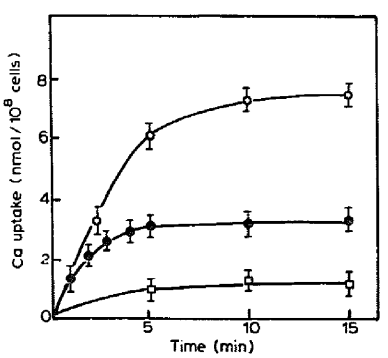

Fig. 1. Effect of phosphate on calcium uptake. Sperm cells were incubated in buffer 1 which contained 0.2 mM $CaCl_2$, and the uptake of Ca^{2+} into cells was determined. The symbols represent: control (●), 0.5 mM P_i (○) and 1.5 μM Ruthenium red (□).

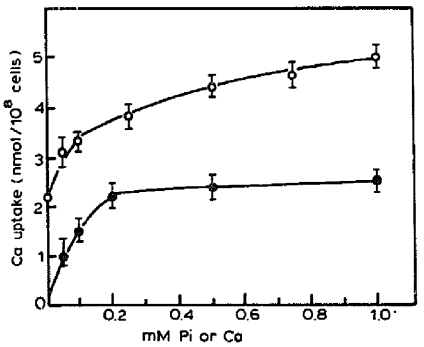

Fig. 2. Effect of calcium or phosphate concentrations on calcium uptake. Cells were incubated in buffer 1 for 3 min at 37 °C and the uptake of ^{45}Ca into the cells was measured. In the experiments using P_i concentrations, buffer 1 contained 0.2 mM $CaCl_2$. The symbols represent: calcium concentrations. (●) and P_i concentrations (○).

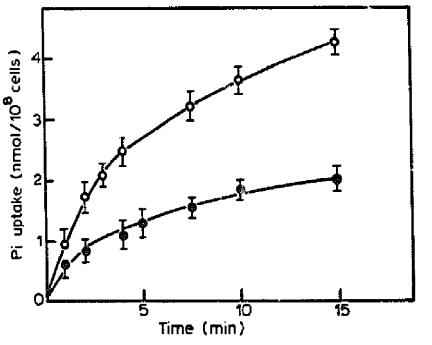

Fig. 4. Effect of calcium on P_i uptake. Sperm cells were incubated in buffer 1 containing 0.5 mM P_i and the uptake of $[^{32}P]P_i$ into the cells was determined. The values are given for results with 0.2 mM $CaCl_2$ (○) without Ca^{2+} and with 0.1 mM EGTA (●).

phosphate is stimulated by about 2-fold including 0.2 mM calcium in the cell suspension (Fig. 4).

Effect of mersalyl

Mersalyl is a sulfhydryl reagent which does not penetrate via the plasma membrane of sperm cells [15]. It is well documented that mersalyl inhibits phosphate transport into the mitochondria [16]; therefore, it is important to mention that mersalyl does not penetrate via the plasma membrane.

The effect of mersalyl concentrations on phosphate uptake into ram spermatozoa is shown in Fig. 5. There is no inhibition in phosphate uptake at mersalyl concentrations up to 20 μM, and above this concentration inhibition is increased by enhancing the concentrations of mersalyl. At 14 nmol mersalyl per 10^8 cells there is 50% inhibition of phosphate uptake. The effect of mersalyl on calcium uptake in the presence and absence of phosphate is shown in Fig. 6. Since calcium uptake in the absence of phosphate is very low, we

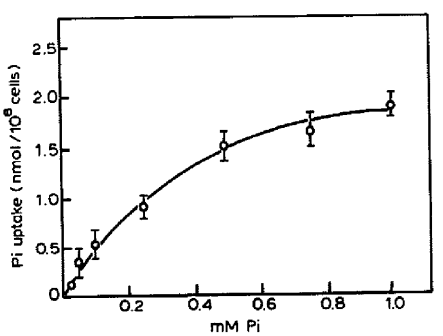

Fig. 3. Effect of phosphate concentrations on P_i uptake. Sperm cells were incubated in buffer 1 containing 0.1 mM EGTA and various concentrations of phosphate and without added calcium. The incubation was performed for 3 min at 37 °C.

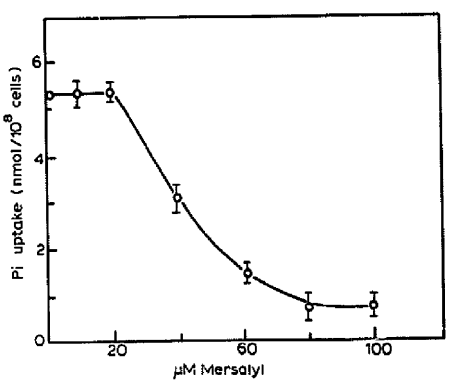

Fig. 5. Effect of mersalyl on phosphate uptake. Cells were preincubated in buffer 1 containing 0.5 mM P_i, 0.2 mM $CaCl_2$, 10 mM fructose for 5 min at 37 °C with increased concentrations of mersalyl. The reaction was started by adding $[^{32}P]P_i$ and the phosphate taken up was determined after 3 min of incubation. 10 μM mersalyl is equivalent to 3.33 nmol mersalyl per 10^8 cells.

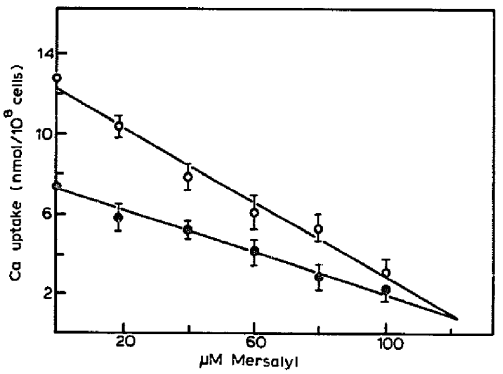

Fig. 6. Effect of mersalyl on calcium uptake. Sperm cells were preincubated in buffer 1 containing 0.5 mM P_i (●) or 10 mM fructose (○) for 5 min at 37°C with increased concentrations of mersalyl. The reaction was started by adding 0.2 mM $CaCl_2$ and the uptake of calcium was determined after 3 min of incubation. 10 μM mersalyl is equivalent to 3.33 nmol mersalyl per 10^8 cells.

added fructose in order to increase the phosphate-independent calcium uptake. It can be seen that increasing the mersalyl concentration causes linear inhibition in calcium uptake, with 50% inhibition at 22.3 nmol mersalyl per 10^8 cells. There are no differences in the effect of mersalyl whether phosphate is present or not.

Effect of substrates on calcium and phosphate uptake

It is well known that phosphate transport into the mitochondria is coupled to calcium transport, although they have two different carriers. Therefore, it was interesting to determine whether inhibition of calcium transport into the mitochondria will affect phosphate transport into the cell. First, we wanted to discover how Ruthenium red, which inhibits calcium transport into the mitochondria, would affect calcium uptake into the cell in the presence of phosphate and fructose. The data in Table I indicate that extracellular fructose or phosphate causes 5- or 3-fold increase in calcium uptake, respectively. In the presence of phosphate plus fructose there is an additive effect. Ruthenium red causes 65% or 95% inhibition of Ca^{2+} uptake in the absence or in presence of fructose, respectively. A 3-fold stimulation in calcium uptake

TABLE I

EFFECT OF RUTHENIUM RED ON CALCIUM UPTAKE

Cells incubated in buffer 1 containing 0.2 mM $CaCl_2$, and the ^{45}Ca taken up was determined after 3 min at 37°C. The concentration of Ruthenium red was 1.5 μM.

Additions	Ca uptake (nmol/10^8 cells)	
	control	Ruthenium red
None	2.3±0.5	0.78±0.13
0.5 mM P_i	7.2±0.7	2.54±0.18
10 mM fructose	11.6±0.8	0.59±0.09
10 mM fructose+0.5 mM P_i	14.7±1.0	1.43±0.14

results by adding phosphate and there is no stimulation, but rather 30% inhibition in the presence of fructose. The stimulatory effect of phosphate on calcium uptake can be seen in the presence of fructose as well. Thus, the data indicate that the stimulatory effect of phosphate on calcium transport can be observed under conditions where mitochondrial calcium transport is inhibited. The effect of Ruthenium red on phosphate uptake in the presence of fructose and calcium is shown in Table II. A 2- or 6-fold stimulation in phosphate uptake results from the addition of calcium or calcium plus fructose, respectively. There is no effect of fructose in the absence of calcium. In the presence of Ruthenium red there is still a 1.3- or 2.5-fold stimulation in phosphate uptake when calcium or calcium plus fructose, respectively, are present. Ruthenium red inhibits phosphate transport when calcium only is present in the medium. The fact that phosphate uptake in the presence of Ruthenium red, is 2.5-fold stimulated by adding

TABLE II

EFFECT OF RUTHENIUM RED ON PHOSPHATE UPTAKE

Cells were incubated in buffer 1 containing 0.5 mM P_i, and the uptake of $^{32}P_i$ into the cells was measured after 3 min at 37°C.

Additions	P_i uptake (nmol/10^8 cells)	
	control	Ruthenium red
None	1.05±0.16	1.34±0.17
0.2 mM $CaCl_2$	2.15±0.22	1.81±0.27
10 mM fructose+0.1 mM EGTA	1.21±0.21	1.64±0.15
10 mM fructose+0.2 mM $CaCl_2$	6.54±0.33	3.32±0.28

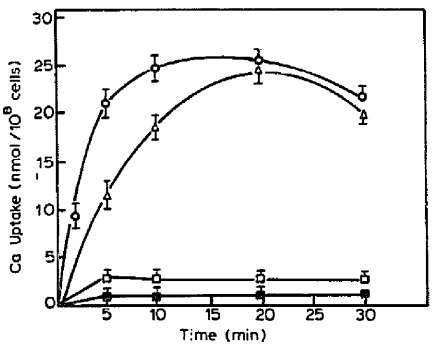

Fig. 7. Effect of fructose and L-lactate on Ca^{2+} uptake. Sperm cells were incubated in buffer 1 containing 0.2 mM $CaCl_2$ and substrate and the uptake of ^{45}Ca was determined at 37 °C. The symbols represent: no substrate (□), 10 mM fructose (△), 10 mM L-lactate (○) and 10 mM fructose or L-lactate with 1.5 μM Ruthenium red (■).

calcium plus fructose, conditions under which calcium uptake is more than 90% inhibited, indicate that calcium stimulates phosphate transport via the plasma membrane.

The fact that calcium transport in the presence of fructose is 95% inhibited by Ruthenium red, indicates that the mechanism by which fructose stimulates calcium transport into the cell is via stimulation of mitochondrial activity. In order to test this possibility, we examined the effect of various mitochondrial substrates and fructose, in the presence of glycolytic inhibitor, on calcium uptake and respiration. The effect of the sperm mitochondrial substrate L-lactate on calcium uptake is shown in Fig. 7. It can be seen that the effect of L-lactate is similar to the effect of fructose, both of which are above 90% inhibited by Ruthenium red. From Table III we can see that the stimulatory effect of fructose on calcium uptake is 85% inhibited after preincubation of cells with the glycolytic inhibitor 2-deoxyglucose. The fact that 2-deoxyglucose does not affect the calcium uptake which is dependent upon L-lactate or phosphate (Table III) indicates a specific effect of 2-deoxyglucose on glycolytic activity and not on the calcium transport mechanism. The effect of various glycolytic and mitochondrial substrates on calcium uptake can be seen in Table IV. The calcium uptake activity is related to the various

TABLE III

THE EFFECT OF 2-DEOXYGLUCOSE ON CALCIUM UPTAKE

Cells were preincubated in buffer 1 containing 40 mM 2-deoxyglucose for 1 h at 25 °C, then diluted four times in buffer 1 containing 0.2 mM $CaCl_2$ and various components. The uptake of ^{45}Ca was determined after incubation for 5 min at 37 °C.

Additions	Ca uptake (nmol/10^8 cells)	
	control	2-deoxyglucose
None	2.28±0.57	2.22±0.40
0.5 mM P_i	3.70±0.70	3.77±0.39
10 mM fructose	14.75±1.37	4.25±0.50
10 mM L-lactate	15.01±1.05	15.78±0.81

substrates according to the sequence: fructose = glucose = lactate > β-hydroxybutyrate > glycerol. In order to ensure that glycerol does not cause any damage to the plasma membrane, its effect was determined in the presence of phosphate or fructose. It can be seen from Table IV, that there is no inhibitory effect of glycerol on calcium uptake under these conditions.

Effect of substrates on respiration

The effect of fructose and L-lactate on sperm respiration is shown in Fig. 8. As can be seen, a 2.1- or 2.5-fold stimulation in respiration results from the addition of fructose or L-lactate, respectively. There is only a very small effect when

TABLE IV

THE EFFECT OF VARIOUS SUBSTRATES ON CALCIUM UPTAKE

Cells were incubated in buffer 1 containing 10 mM of substrate and ^{45}Ca uptake was determined after 5 min incubation at 37 °C.

Additions	Ca uptake (nmol/10^8 cells)
None	1.80±0.51
0.5 mM P_i	3.66±0.68
Fructose	15.81±1.55
Glucose	15.00±1.57
L-Lactate	15.87±1.10
β-Hydroxybutyrate	9.45±1.48
Glycerol	5.05±1.08
Glycerol + 0.5 mM P_i	7.28±1.05
Glycerol + fructose	18.60±1.49

L-lactate is added in the presence of fructose. These data indicate that fructose and L-lactate affect calcium uptake into the cell via the same mechanism.

Effect of oligomycin

The data presented so far indicate that calcium transport into sperm cells is controlled by mitochondrial activity. The question remains as to how mitochondrial respiration can affect calcium transport via the plasma membrane. One of the possibilities suggests the involvement of ATP in the process. In order to test this possibility, ATP levels in the cells were affected using the mitochondrial ATPase inhibitor, oligomycin. A close evaluation of the changes in ATP levels was made by following sperm motility, which was sufficient to ensure that oligomycin was active. It can be seen from Table V that oligomycin inhibits completely the motility which was dependent upon mitochondrial activity (without external substrate

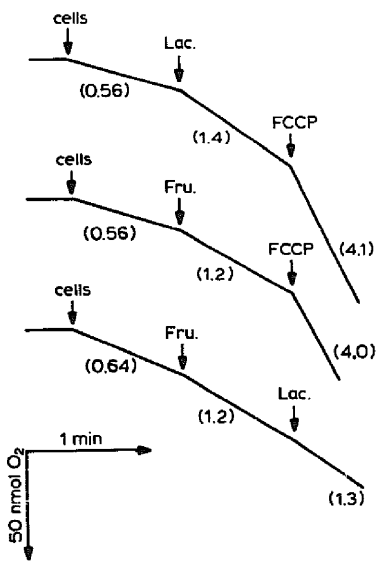

Fig. 8. Effect of fructose and L-lactate on mitochondrial respiration. The respiration of $2 \cdot 10^8$ cells/3 ml was determined in buffer 1 containing 0.2 mM $CaCl_2$ at 37 °C. Fructose or L-lactate were added to final concentration of 10 mM. The concentration of the uncoupler FCCP was 2 μM. The numbers in parenthesis represent the respiration rate in μmol $O_2/10^8$ cells per h.

TABLE V

EFFECT OF OLIGOMYCIN ON CALCIUM UPTAKE AND CELL MOTILITY

Cells were preincubated in buffer 1 with 20 nmol oligomycin/10^8 cells for 30 min at 25 °C. The uptake of ^{45}Ca was determined in buffer 1 containing 0.2 mM $CaCl_2$ and phosphate or substrate as indicated. Cell motility was determined with a light microscope; (+) indicates good motility and (−) indicates no motility.

Additions	Ca uptake (nmol/10^8 cells)		Motility	
	control	oligomycin	control	oligomycin
None	0.87±0.03	1.54±0.03	+	−
0.5 mM P_i	1.76±0.03	2.09±0.02	+	−
10 mM fructose	6.82±0.05	9.90±0.07	+	+
10 mM fructose + 0.5 mM P_i	9.13±0.07	10.71±0.08	+	+
10 mM L-lactate	6.64±0.07	10.23±0.09	+	−
10 mM L-lactate + 0.5 mM P_i	9.24±0.07	12.43±0.10	+	−

or with lactate as the only substrate). This result indicates a large decrease in ATP levels under these conditions. In the presence of fructose, oligomycin had no effect on cell motility, which indicates that the ATP level is high. No motile cells were seen when oligomycin was added in the absence of added substrate, a result which indicates that the motility, under these conditions, is dependent upon mitochondrial respiration. It is shown in Table V, that oligomycin does not cause any inhibition in calcium uptake, as one would expect if ATP were involved in the mechanism of calcium transport via the plasma membrane. In fact, there is always some stimulation of calcium uptake by oligomycin. It is possible that the drop in ATP level caused by oligomycin causes a reduction in Ca^{2+}/Mg^{2+}-ATPase activity. Since this enzyme pumps calcium out of the cell, a reduction in its activity will result in elevation of the calcium content in the cell.

Effect of verapamil

It is shown in Fig. 9, that verapamil, which is considered to be a voltage-dependent calcium blocker, causes inhibition of calcium uptake in the presence of extracellular phosphate or fructose. A 50% inhibition was found at 0.6 and 1.36 mM verapamil for phosphate or fructose-stimulated

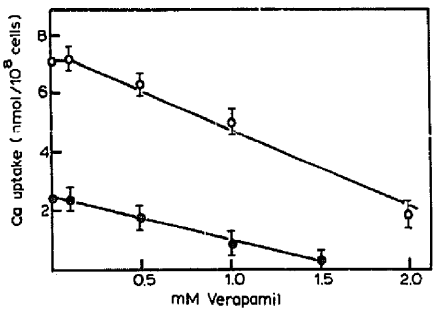

Fig. 9. Effect of verapamil on calcium uptake. Cells were incubated in buffer 1 containing 0.2 mM $CaCl_2$ and increased concentrations of verapamil. Calcium uptake was determined after incubation for 3 min at 37°C. The values represent incubations in the presence of 0.5 mM P_i (●) and 10 mM fructose (○).

calcium uptake, respectively. Thus, it seems that the phosphate-dependent calcium transport mechanism is more sensitive to verapamil in comparison to the phosphate-independent mechanism.

Discussion

The data presented here support possible mechanisms for the enhanced effect of phosphate and fructose on calcium uptake into ejaculated ram spermatozoa. We also present data which deal with the effect of calcium and fructose on phosphate uptake. In a recent paper [15], we suggested that the plasma membrane of bovine spermatozoa, contains a calcium-phosphate carrier. In this current paper, we present data which further support this proposal. The data also indicate that a phosphate-independent calcium carrier and a calcium independent phosphate carrier are involved in calcium and phosphate uptake. A stimulation of about 2-fold in calcium uptake or in phosphate uptake was found when phosphate or calcium, respectively, were added. Mersalyl inhibits calcium uptake as well as phosphate uptake into the cells in a comparable way (Figs. 5 and 6). Similar results were obtained for bovine spermatozoa [15,17]. This indicates that a common mechanism for calcium and phosphate transport exist in sperm cells. Mersalyl inhibits calcium uptake whether phosphate is present or not (Fig. 6). This indicates that the calcium carrier itself is inhibited by mersalyl. Thus, the inhibition of calcium uptake in the presence of extracellular phosphate results from the inhibition of a calcium carrier and not necessarily from the inhibition of a phosphate carrier, as was found in liver mitochondria [16]. Since mersalyl does not penetrate via the plasma membrane [15], we conclude that phosphate stimulates calcium transport by affecting a transport mechanism which is located in the sperm plasma membrane. To exclude the possibility that extracellular phosphate enhances calcium uptake due to its effect on sperm mitochondria, we determined calcium and phosphate uptake in Ruthenium red treated cells. Ruthenium red, inhibits the mitochondrial calcium uniport without effecting the calcium carrier of the plasma membrane [18]. Although calcium uptake is 95% inhibited by Ruthenium red, a 3-fold stimulation of calcium uptake can still be seen when phosphate is added (Tables I and II). This indicates that phosphate affects calcium transport via the plasma membrane. The fact that calcium stimulates phosphate uptake into the cells and this effect of calcium is only 50% inhibited by Ruthenium red (Table II) indicates that calcium affects phosphate transport via the plasma membrane. Ruthenium red inhibits phosphate uptake in the presence of calcium only, a result which indicates that a phosphate carrier is not inhibited directly, under these conditions. The fact that in the presence of fructose, Ruthenium red causes 95% inhibition of calcium uptake and only 50% inhibition of phosphate uptake, can be explained by considering the existence of a calcium pump in the plasma membrane [10] and the finding that 40% of the phosphate taken up is incorporated into non-permeable nucleotides [17].

We have shown that fructose strongly stimulates calcium uptake without added phosphate, but there is no stimulation of phosphate uptake unless calcium is added (Tables I and II). These data indicate that fructose affects phosphate transport via its effect on calcium transport. Since this effect of fructose cannot be found when the glycolytic activity is inhibited (Table III), we conclude that glycolytic metabolites stimulate calcium and phosphate uptake. The additive effect of fructose and phosphate on Ca^{2+} uptake indicates that two separate systems for calcium transport exist in the cells.

The enhanced effect of fructose on calcium uptake is due to the supply of L-lactate, via the glycolysis, to the mitochondria. L-Lactate alone (Table III) and other mitochondrial substrates, also support calcium uptake into the cells. The similar stimulation of respiration and calcium uptake by fructose and L-lactate (Fig. 8) indicates that calcium uptake is increased by stimulating mitochondrial respiration. In order to explore the mechanism by which mitochondrial activity may affect calcium transport via the plasma membrane, we speculated that ATP might be involved. The results with oligomycin (Table V) are in agreement with our data from bovine sperm, from which calcium uptake and direct determination of cell ATP levels were measured (Breitbart, H., Welbie, R. and Lardy, H.A., work in preparation).

Thus, we conclude that ATP is not involved in the mechanism by which the mitochondria affects calcium transport.

The data with verapamil (Fig. 9) indicate that phosphate-dependent calcium uptake is relatively more sensitive to verapamil than that which is phosphate-independent. This result supports the rest of the data in this paper, which indicate the existence of two carriers for calcium transport located in the plasma membrane of ram spermatozoa. One system is a phosphate-independent carrier and the second is a phosphate-dependent transporter. In addition, the plasma membrane contains a calcium-independent phosphate transport mechanism.

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