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Regulation of calcium transport in bovine spermatozoa

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Calcium uptake into bovine epididymal spermatozoa is enhanced by introducing phosphate in the suspending medium (Babcock et al. (1975) J. Biol. Chem. 250, 6488–6495). This effect of phosphate is found even at a low extracellular Ca^{2+} concentrations (i.e., 5 μM) suggesting that phosphate is involved in calcium transport via the plasma membrane. Bicarbonate (2 mM) cannot substitute for phosphate, and a relatively high bicarbonate concentration (20 mM) causes partial inhibition of calcium uptake in absence of P_i. In the presence of 1–2 mM phosphate, 20 mM bicarbonate enhances Ca^{2+} uptake. The data indicate that the plasma membrane of bovine spermatozoa contains two carriers for Ca^{2+} transport: a phosphate-independent Ca^{2+} carrier that is stimulated by bicarbonate and a phosphate-dependent Ca^{2+} carrier that is inhibited by bicarbonate. Higher phosphate concentrations (i.e., 10 mM) inhibit Ca^{2+} uptake into intact cells (compared to 1.0 mM phosphate) and this inhibition can be relieved partially by 20 mM bicarbonate. This effect of bicarbonate is inhibited by mersalyl. Calcium uptake into the cells is enhanced by adding exogenous substrates to the medium. There is no correlation between ATP levels in the cells and Ca^{2+} transport into the cell. ATP levels are high even without added exogenous substrate and this ATP level is almost completely reduced by oligomycin, suggesting that ATP can be synthesized in the mitochondria in the absence of exogenous substrate. Calcium transport into the sperm mitochondria (washed filipin-treated cells) is absolutely dependent upon the presence of phosphate and mitochondrial substrate. Bicarbonate cannot support Ca^{2+} transport into sperm mitochondria. There is good correlation between Ca^{2+} uptake into intact epididymal sperm and into sperm mitochondria with the various substrates used. This indicates that the rate of calcium transport into the cells is determined by the rate of mitochondrial Ca^{2+} uptake and respiration with the various substrates.

Introduction

A natural consequence of the selection of calcium ion as an intracellular messenger is the necessity for tight regulation of its concentration. In mammalian spermatozoa, intracellular calcium plays a pivotal role in the control of sperm motility and capacitation [1–6]. Proposed regulatory sites of spermatozoa intracellular calcium include both the plasma membrane [7–12] and the mitochondria [13]. The fact that sperm cytosolic, ionized calcium is maintained at a low level (0.1 μM) [14] in a medium of millimolar calcium supports the concept of the plasma membrane as the primary regulatory site. However, though several groups have described systems involved in sperm calcium secretion, ATP-dependent calcium pump [7,9–11] Na^+/Ca^{2+} antiporter [12], little is known about the mechanism(s) involved in calcium transport into the cell.

It has been suggested that the plasma membrane Na^+/Ca^{2+} antiporter is involved in Ca^{2+} transport into bovine spermatozoa [12]. Moreover, this carrier has been proposed as the site of calcium uptake inhibition by caltrin; a protein purified from bovine seminal plasma [5,12,15]. Recently, we presented data on a bovine plasma membrane calcium transporter which is inhibited by mersalyl, but insensitive to low concentrations of verapamil [16].

In the present paper we demonstrate the effect of phosphate, bicarbonate and various substrates on calcium uptake into bovine epididymal spermatozoa. These data indicate that the plasma membrane contains a calcium-phosphate transporter which is stimulated by bicarbonate, and phosphate-independent calcium transporter which is inhibited by bicarbonate. In addition,

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we present data which indicate that under conditions of limiting mitochondrial substrate supply, the rate of calcium uptake into the cell is dependent upon the rate of mitochondrial respiration.

Materials and Methods

Bovine epididymides were obtained from local slaughter houses. Epididymal spermatozoa were collected and washed as described previously [13], using a standard medium A which contained 110 mM NaCl, 5 mM KCl, 10 mM sodium morpholinopropane sulfonate, (pH 7.4). The collected cells were washed twice with medium A by centrifugation at $600 \times g$ for 10 min at 25° C. All incubations were carried out in closed tubes containing CO_2.

Filipin treatment. A fresh solution of 0.1 M filipin in DMSO was added to 15 ml of medium A containing $2.25 \cdot 10^8$ cells/ml to provide a final concentration of 0.2 mM filipin. After 6 min at 25° C, 15 ml of cold (4° C) 250 mM mannitol, 70 mM sucrose, 10 mM TEA-Hepes (pH 7.4) (Buffer M) were added, and the cell suspension was centrifuged for 10 min at $600 \times g$ and 4°C. The pellet was resuspended with 15 ml of cold buffer M and centrifuged at $600 \times g$ for 10 min at 4°C. The resulting pellet was then resuspended in 1.8 ml cold buffer M and kept in ice until use.

Calcium uptake. Uptake of ^{45}Ca by cells was determined by a filtration technique. Cells ($2.5 \cdot 10^8$/ml) were incubated in a final volume of 125 µl in medium A plus 0.2 mM $CaCl_2$ (0.5 µCi ^{45}Ca) and 1.25 mM phosphate. Prior to the addition of calcium, the cells were incubated for 10 min at 37°C. The substrate being tested was added 10 s before initiating the reaction by the addition of calcium. After 2 min reaction time at 37° C, 0.1 ml was quickly removed and immediately vacuum filtered on GF/C filters. The filters were then washed three times with 5 ml of a solution composed of 150 mM NaCl, 10 mM Tris (pH 7.4) and 2 mM EGTA. The filters were dried and counted in scintillation vials with 5 ml Aquasol (DuPont). Data are expressed as the experimental value corrected for the calcium bound in the presence of the mitochondrial uncoupler CCCP.

Respiration. Mitochondrial respiration was monitored at 37° C using a Clark oxygen electrode.

ATP determination. Cells ($2.5 \cdot 10^8$/ml) were incubated in a final volume of 0.25 ml in medium A with 1.25 mM P_i, 0.2 mM $CaCl_2$ and 10 mM substrate. After 10 min at 37° C the incubation was terminated by adding 0.25 ml ice cold 20% TCA. The TCA was then extracted with a two volume portion of Freon/tri-*n*-octylamine (3 : 1, v/v) [17] and the two phases separated by centrifugation. In this procedure the precipitated protein appeared as a thin layer between the two phases. An aliquot of the aqueous phase was removed and assayed for ATP using the luciferase method [18].

Materials. Chemicals were obtained from standard commercial sources. Filipin, Mersalyl-Acid, Oligomycin, Rotenone, Luciferase and Luciferin were from Sigma. Durohydroquinone was from Pfaltz Bauer, Inc.

In most cases the data shown are from typical experiments, the results of which have been verified repeatedly. Averaging data gathered with epididymal spermatozoa introduces wide variations in absolute values because of differing properties of sperm from different bulls, some of which may be marketed because of infertility. Except where noted, the experiments shown were performed in duplicate on the preparation of sperm pooled from the epididymides of two or three animals and are representative of three or more experiments performed with other such pools.

Results

Effect of phosphate and bicarbonate

Calcium uptake into epididymal bovine spermatozoa is highly dependent on the concentration of added phosphate. As shown in Fig. 1, a maximum 7-fold stimulation of calcium uptake occurred with 1 mM phosphate. Phosphate is not released from the cells during the time course of these incubations for, in the absence of added phosphate, the cell suspensions contain no detectable extracellular phosphate (data not shown).

The effect of phosphate and bicarbonate on calcium uptake into spermatozoa at various calcium concentrations is shown in Fig. 2. Enhancement of calcium uptake by phosphate was observed at all concentrations of calcium tested. Unlike phosphate, bicarbonate (2 mM) had only a small stimulatory effect on calcium uptake. Furthermore, when the bicarbonate concentration was

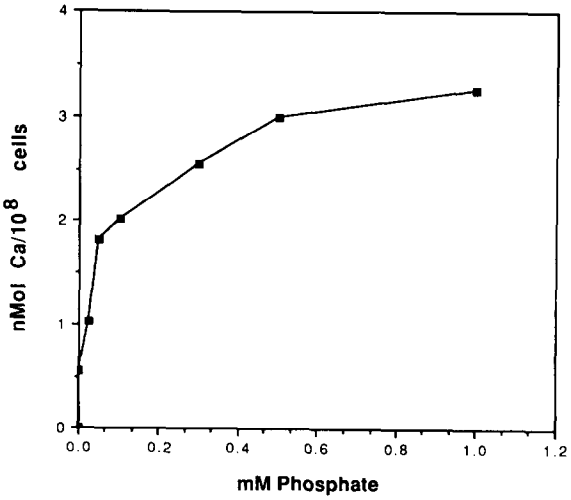

Fig 1. Effect of phosphate concentrations on calcium uptake into epididymal sperm: washed cells ($2.5 \cdot 10^7$) were incubated in medium A with 10 mM fructose, 0.2 mM $CaCl_2$. The $^{45}Ca^{2+}$ uptake was determined after 2 min at 37° C.

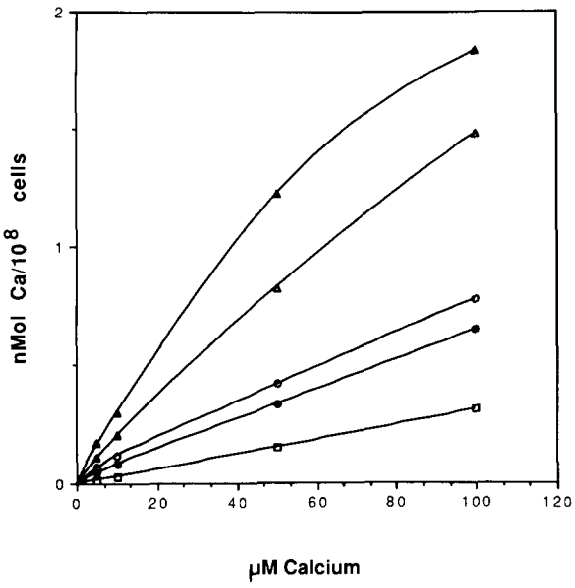

Fig. 2. Effect of phosphate and bicarbonate on calcium uptake. Washed epididymal sperm ($2.5 \cdot 10^7$) were incubated in medium A with 10 mM fructose, and the $^{45}Ca^{2+}$ uptake was determined after 2 min at 37°C. Symbols: control (●———●), 2 mM bicarbonate (○———○), 20 mM bicarbonate (□———□), 2 mM phosphate (△———△), 2 mM phosphate + 20 mM bicarbonate (▲———▲).

increased to 20 mM, a 50% inhibition of calcium uptake resulted (Fig. 2). In the presence of phosphate, 20 mM bicarbonate caused a 40% enhancement of calcium uptake (see also Figs. 3 and 4).

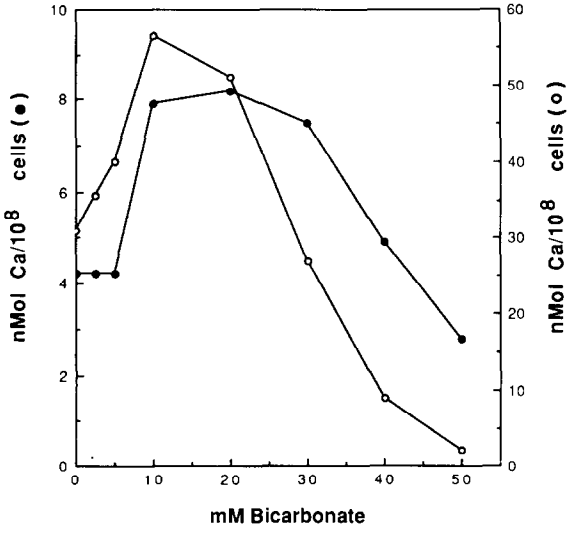

Fig. 3. Effect of HCO_3^- concentration on calcium uptake into intact and filipin-treated cells: washed epididymal sperm ($2.8 \cdot 10^7$) were incubated in medium A with 10 mM fructose (intact cells) or 10 mM L-lactate (filipin-treated cells), 1 mM P_i and 0.2 mM $CaCl_2$. For filipin treatment, 0.2 mM filipin (in DMSO) was added and the cells were incuabted 6 min at 37°C before starting the reaction with $^{45}CaCl_2$. The ^{45}Ca uptake was determined after 10 min at 37°C. Closed circles represent intact sperm, and open circles filipin-treated cells.

As shown in Fig. 3, bicarbonate has a marked effect on calcium uptake into both intact and filipin-treated spermatozoa incubated in the presence of 1.0 mM phosphate. In contrast to filipin-treated cells, no effect of bicarbonate on intact cells was noted until bicarbonate concentration exceeded 5 mM. With intact cells maximum calcium uptake was observed from 10 to 30 mM bicarbonate; and further increases produced inhibition. In filipin-treated cells the situation is similar, except that both the maximum stimulatory effect and the inhibitory effect of bicarbonate occurs at somewhat lower bicarbonate concentrations. With filipin-treated cells calcium uptake occurs directly into the mitochondria, and the effect of bicarbonate is therefore mediated via sperm mitochondrial transport or retention.

Washing of filipin-treated cells has a marked effect on their ability to accumulate calcium (Table I). In the presence of phosphate and bicarbonate no significant calcium uptake occurs without the addition of an oxidizable substrate. Sperm mitochondria contain lactate dehydrogenase, thus we could use L-lactate as substrate. The addition of phosphate, but not bicarbonate, dramatically increases the degree of calcium uptake in the presence of L-lactate. In contrast, the addition of L-lactate to unwashed filipin-treated cells dramatically increases calcium uptake in the absence of added phosphate. These cells release enough phosphate (0.1 mM phosphate final) as a result of filipin treatment to enable a calcium uptake of 40 nmol calcium/10^8 cells. These same cells when washed contain no detectable extracellular phosphate and accumulate only 0.5 nmol calcium/10^8 cells.

The effect of 20 mM bicarbonate on calcium uptake in intact and filipin-treated cells in the presence of 0, 1.0 and 10 mM phosphate is shown in Fig. 4. Calcium uptake into intact cells is inhibited by bicarbonate in the absence of phosphate; however, as also shown in Fig. 2, a high enhancement of calcium uptake occurs in the presence of bicarbonate plus 1.0 or 10 mM phosphate. In contrast, in filipin-treated cells bicarbonate has no significant effect in the absence of phosphate,

TABLE I

Effect of P_i and HCO_3^- on calcium uptake into washed filipin-treated cells

Washed epididymal cells were treated with filipin then washed twice as described in Methods. The cells ($2.5 \cdot 10^7$) were incubated in medium A for 2 min at 37°C for ^{45}Ca uptake determination. The L-lactate concentration was 10 mM and $CaCl_2$ 0.2 mM.

	nmol Ca/10^8 cells	
	no substrate added	L-lactate
None	0.49 ± 0.10	2.1 ± 0.2
2 mM phosphate	0.51 ± 0.12	44.0 ± 5.8
2 mM bicarbonate	0.49 ± 0.10	1.6 ± 0.2

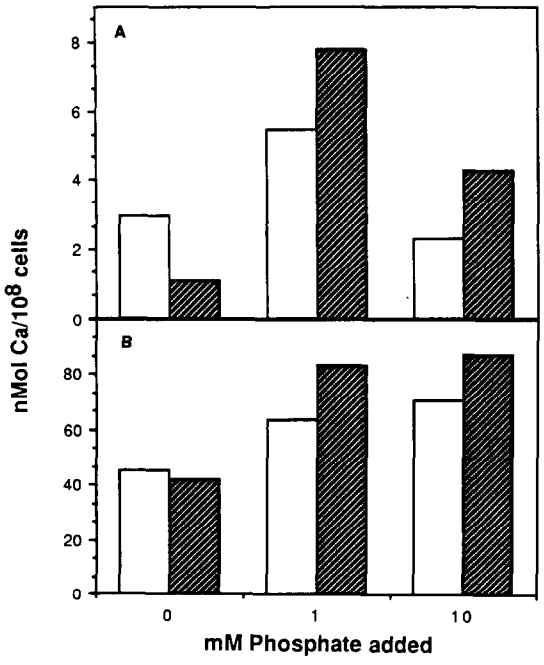

Fig. 4. Effect of HCO_3^- with thee concentrations of P_i on calcium uptake into intact and filipin-treated cells: washed epididymal cells ($2.5 \cdot 10^7$) were incubated in medium A with 10 mM fructose, 0.2 mM $CaCl_2$ and 0, 1.0 and 10.0 mM P_i. For filipin treatment see legend to Fig. 3. Since the cells were not washed after filipin treatment, the final P_i concentrations for the filipin-treated cells are 0.1, 1.1 and 10.1 mM P_i, respecitively. The ^{45}Ca utpake was determined after 10 min at 37°C. (A) Intact cells. (B) Filipin-treated cells. Open bars are controls and closed bars contain 20 mM $NaHCO_3$. To the control tubes 20 mM NaCl was added.

while the stimulatory effect of bicarbonate in the presence of phosphate is maintained. In addition, 10 mM phosphate inhibits calcium uptake into intact cells (Breitbart and Lardy, 1987 and Fig. 4) but has no significant effect on calcium uptake into filipin-treated cells. These results indicate that the inhibitory effect of high extracellular phosphate is on calcium transport via the plasma membrane and not into the mitochondria.

We have recently shown that the inhibitory effect of 10 mM phosphate on calcium uptake into intact cells does not occur in the presence of mersalyl (Breitbart and Lardy, 1987 and Table II). The effect of mersalyl on the bicarbonate effect of calcium uptake at various phosphate concentrations is shown in Table II. In the presence of 10 mM P_i there is no inhibition by mersalyl in the absence of HCO_3^-, whereas in its presence there is 32% inhibition (to the level without HCO_3^-). In addition, the rate of calcium uptake in the presence of 1 mM phosphate is enhanced 97% (from 5.1 to 10.1 nmol $Ca/10^8$ cells) by the inclusion of HCO_3^-, whereas in mersalyl-treated cells there is only a 39% enhancement (from 3.9 to 5.5 nmol $Ca/10^8$ cells). The enhanced effect of 1 mM P_i on calcium uptake in the presence of HCO_3^- plus mersalyl (5.5 nmol $Ca/10^8$ cells) is similar to the stimulation of calcium uptake in the absence of HCO_3^- plus mersalyl (5.1 nmol $Ca/10^8$ cells). Thus, the effect of P_i is substantially increased by HCO_3^- and this augmentation by HCO_3^- is completely inhibited by mersalyl. Finally, it is shown in Table II that the enhanced effect of 10 mM P_i in the presence of HCO_3^- plus mersalyl (4.4 nmol $Ca/10^8$ cells) is similar to its effect in the absence of HCO_3^- plus mersalyl (4.1 nmol $Ca/10^8$ cells). We conclude that the stimulation by HCO_3^- of Ca uptake is inhibited by mersalyl.

Effect of aging. To investigate the possibility that two separate carriers may be involved in calcium transport and that the putative carriers have differing stabilities, cells were aged and their phosphate-dependent and phosphate-independent calcium uptake monitored. Phosphate-dependent calcium transport activity is decreased with time (Fig. 5) while no change in phosphate-independent activity was observed. To test whether the response reflects inhibition of mitochondrial calcium accumulation with cell aging, cells were aged and then treated with filipin to assess mitochondrial calcium uptake. Treatment of the aged cells with filipin, just prior to the assay of calcium uptake, dis-

TABLE II

Effect of mersalyl on Ca uptake into intact cells in the presence of HCO_3^- and P_i

Washed epididymal cells ($2.5 \cdot 10^7$) were incubated in medium A with 10 mM fructose and the ^{45}Ca uptake was determined after 10 min at 37°C. The concentration of mersalyl was 20 μM. The Δ represents the effect of P_i or HCO_3^- after substracting the Ca uptake in controls without P_i.

mM P_i	No HCO_3^-			Plus 20 mM HCO_3^-		
	nmol $Ca/10^8$ cells		% effect of mersalyl	nmol $Ca/10^8$ cells		% inhibition by mersalyl
	control	mersalyl		control	mersalyl	
0	3.2±0.3	0.8±0.1	−76	1.2±0.1	0.6±0.1	54
1	8.3±0.7	4.7±0.5	−43	11.3±1.2	6.0±0.7	47
10	4.7±0.5	4.9±0.6	0	7.3±0.6	4.9±0.2	32
Δ P_i (1 mM)	5.1	3.9	−23	10.1	5.5	46
Δ P_i (10 mM)	1.5	4.1	178	6.1	4.4	28
Δ HCO_3^- (1 mM P_i)	–	–		3.0	1.3	56
Δ HCO_3^- (10 mM P_i)	–	–		2.5	0.04	98

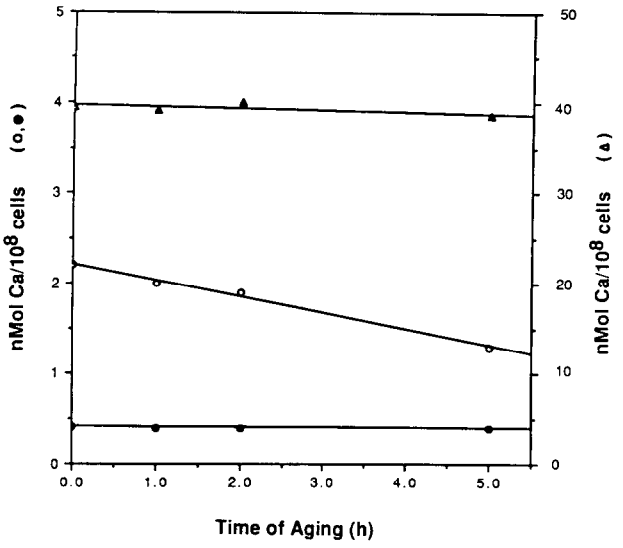

Fig. 5. Calcium uptake into aged sperm: washed epididymal cells ($1.7 \cdot 10^9$/ml) were incubated in medium A at 25°C for 1–5 h. At the end of the incubation, thee aliquots (in duplicate) were taken, diluted to $2 \cdot 10^8$ cells/ml in medium A, 10 mM fructose. The reaction has been started with $CaCl_2$ and the ^{45}Ca uptake was determined after 2 min at 37°C. Symbols: no P_i (●———●), 1.0 mM P_i (○———○), 1.0 mM P_i + filipin (△———△) (see legend to Fig. 3).

closed no decrease in mitochondrial calcium uptake with cell aging.

Effect of substrates. In the early stages of this research we discovered that extracellular fructose enhances calcium uptake into bovine sperm. It can be seen in Table III that fructose increases calcium uptake three fold above the rate with endogenous substrate. Deoxyglucose completely inhibits the effect of fructose, thus it is evident that metabolism of fructose is required for its stimulation of calcium uptake. L-Lactate alone produced a 4-fold increase above the endogenous rate and its effect is completely abolished by rotenone, indicating that oxidation of NADH via the mitochondrial electron transport chain is the source of energy for Ca uptake into these cells.

As a further investigation into factors which influence calcium uptake into spermatozoa, cellular ATP levels were determined and compared to the degree of

TABLE III

Effect of substrates on Ca uptake into intact cells

Washed epididymal cells (2.5·107) were incubated in medium A in the presence of various substrates. The uptake of ^{45}Ca was determined after 10 min at 37°C. The concentration of rotenone was 8 μM.

Substrate	nmol Ca/10^8 cells
None	1.8 ± 0.2
10 mM fructose	5.4 ± 1.4
10 mM fructose + 20 mM Deoxyglucose	2.0 ± 0.3
10 mM L-lactate	7.2 ± 1.5
10 mM L-lactate + rotenone	0.8 ± 0.1

TABLE IV

Effect of substrates on Ca uptake and ATP-levels in intact cells

Washed epididymal cells ($2.5 \cdot 10^7$) were incubated in medium A, 1.0 mM P_i and 10 mM substrate. Half of the cells were taken for ^{45}Ca uptake and the other half for ATP determination after 10 min at 37°C. All substrate solutions had been prepared in medium A, (pH 7.4). The concentration of oligomycin was 50 μM.

Substrate	nmol Ca/10^8 cells ($n = 4$)		nmol ATP/10^8 cells ($n = 2$)	
	control	oligomycin	control	oligomycin
None	1.7	1.7	32.4	1.5
Durohydroquinone	17.1	13.5	17.9	0.4
L-Lactate	6.8	7.4	36.1	1.5
Glycerol	6.8	1.1	35.3	1.4
Fructose	6.2	6.8	32.5	27.7
Dihydroxacetone	6.9	8.1	32.1	24.8
β-Hydroxybutyrate	3.9	3.6	37.5	0.8
Pyruvate	2.1	1.8	33.9	14.4
Acetate	0.4	0.6	36.1	1.2
Acetoacetate	0.5	0.6	32.3	1.8

calcium uptake determined in parallel experiments. As shown in Table IV, no correlation was found between cellular ATP levels and calcium uptake. Moreover, oligomycin, which inhibits mitochondrial ATP synthesis, had no effect on the degree of calcium uptake, though this inhibitor greatly reduced ATP levels. No correlation was found between calcium uptake and ATP levels supported by various substrates, but the rate of respiration supported by given substrate correlated well with calcium uptake (Table V). Four groups of substrates can be recognized with regard to their ability to support calcium uptake: High uptake, durohydroquinone; moderate uptake, L-lactate, glycerol, fructose and

TABLE V

Effect of substrate on Ca uptake and repiration in washed filipin-treated cells

Epididymal cells were treated with filipin and washed twice as appeared in Methods. The ^{45}Ca uptake and respiration were determined in medium A which contains 1.0 mM P_i, 10 mM substrate and 0.2 mM $CaCl_2$.

Substrate	nmol Ca/2 min per 10^8 cells ($n = 4$)	nmol atom O/min per 10^8 cells ($n = 2$)
None	0.5	4.4
Durohdroquinone	40.1	48.5
α-Glycerol phosphate	37.4	33.2
L-Lactate	35.2	15.2
β-Hydroxybutyrate	17.5	9.7
Pyruvate	16.6	9.2
Acetate	0.6	2.0
Acetoacetate	0.6	–

dihydroxyacetone; fair uptake, β-hydroxybutyrate and pyruvate; and finally a poor uptake group, acetate and acetoacetate.

Discussion

The data presented in this paper demonstrate possible mechanisms for the enhanced effect of phosphate, bicarbonate and various substrates on calcium uptake into bovine spermatozoa. We have presented data which indicate the presence of a calcium-phosphate carrier in the spermatozoa plasma membrane [16]. In this paper we present data which further support this proposal.

In Figs. 2 and 3 the dependency of calcium uptake upon extracellular phosphate was clearly shown. Calcium uptake into intact epididymal spermatozoa occurs to a small degree in the absence of added phosphate (Fig. 1). Our finding that the spermatozoa release no detectable phosphate to the medium during the time course of these experiments indicates that this small degree of calcium uptake is independent of extracellular phosphate. Thus we suggest that the plasma membrane of bovine spermatozoa contains two carriers for calcium transport, i.e., a phosphate-dependent calcium transporter and a phosphate-independent transporter. Support for the phosphate-dependent calcium carrier can be found in previous papers from this laboratory. In this earlier work, phosphate transport into bull and ram spermatozoa was enhanced by calcium, and both calcium and P_i uptake were inhibited in parallel by mersalyl [19,20]. The finding that phosphate-dependent calcium uptake activity is decreased with time, whereas the phosphate-independent activity remains stable (Fig. 5) supports our proposal for two independent calcium transporters.

Bicarbonate was unable to substitute for phosphate to mediate calcium uptake, and high bicarbonate (20 mM) resulted in 50% inhibition of calcium uptake (Fig. 2). However, in the presence of P_i this concentration of bicarbonate enhanced calcium uptake into both intact and into filipin-treated cells (see Figs. 2, 3 and 4). It is possible that the enhanced effect of bicarbonate in the presence of phosphate is due to stimulation of mitochondrial calcium uptake. Such a stimulatory effect of bicarbonate on rat liver mitochondrial calcium uptake is well documented [21]. The similar effect of bicarbonate on calcium uptake into intact and filipin-treated cells (Fig. 3) supports this notion.

The inhibitory effect of 10 mM P_i on calcium uptake does not occur in the presence of mersalyl. From these data, we suggest that mersalyl mimics the inhibitory effects of high P_i by modification of the P_i inhibitory site. In Table II we show that in the presence of 10 mM P_i, the addition of mersalyl has no significant effect on calcium uptake into whole cells in the absence of HCO_3^-, while in the presence of HCO_3^- there is 32% inhibition. It is also shown that the stimulatory effect of HCO_3^- in the presence of 10 mM P_i is inhibited by mersalyl, the enhanced effect of 1 mM P_i in the presence of HCO_3^- is completely inhibited by mersalyl although there is small inhibition (23%) of the P_i effect itself, and that augmentation of the enhanced effect of 10 mM P ion Ca uptake by HCO_3^- is completely blocked by mersalyl. Because mersalyl affects only the plasma membrane of intact cells [16], and the stimulatory effect of HCO_3^- is inhibited by mersalyl, we suggest that HCO_3^- has a direct stimulatory effect on the calcium-phosphate carrier of the plasma membrane in addition to the previously proposed effect of HCO_3^- on the mitochondria. In the absence of external P_i, HCO_3^- causes high inhibition of Ca uptake into the cells, but has no significant effect on calcium uptake into filipin-treated cells (Fig. 4). Taken together, these data further support our contention that the plasma membrane contains a second carrier for Ca which is phosphate independent, and indicate that this carrier is inhibitable by bicarbonate.

As previously mentioned, the majority of the calcium transported into the spermatozoa is accumulated by the mitochondria. Thus, the question arises as to the possible mechanisms by which a mitochondrial substrate can affect calcium transport via the plasma membrane? Initially, we speculated that ATP may be involved in the process of calcium transport via the plasma membrane. However, the data in Table IV show that the degree of calcium uptake supported by various substrates differs significantly, while the cellular ATP level remains constant. In addition, oligomycin, which inhibits mitochondrial ATP synthesis, does not affect calcium uptake. Thus, variations of ATP level are not the answer.

Although we found no correlation between calcium uptake and ATP concentrations in intact cells, we did find a correlation between calcium uptake into both intact and filipin-treated cells with regard to the given substrate employed (Tables IV, V). In addition we found a correlation between calcium uptake and respiration in the filipin-treated cells (Table V). These data indicate that the rate of calcium uptake into bovine spermatozoa is determined primarily by the rate of mitochondrial respiration.

This conclusion is supported by others which showed that calcium uptake by bovine spermatozoa is regulated by the redox state of the mitochondrial pyridine nucleotides [23].

In conclusion, we have suggested that the involvement of two carriers for calcium transport via the plasma membrane. One carrier is phosphate-independent and likely inhibitable by HCO_3^-. The second carrier is phosphate-dependent and can be activated by HCO_3^-. Given the finding that calcium transport via the plasma membrane is highly enhanced by mitochondrial substrates, it may be that mitochondrial respiration

affects the activity of these plasma membrane calcium transporters directly by inducing changes in the polarization state of the plasma membrane. It has been described that the plasma membrane of mammalian spermatozoa contains a voltage-dependent calcium channel, which can be activated though depolarization of the membrane by high K^+ concentrations [22]. Our data, which show that high concentrations of the anions HCO_3^- or P_i (10 mM) cause inhibition of calcium transport, can be explained by suggesting that this putative depolarization of the plasma membrane is inhibited by high HCO_3^- or P_i. Further research with isolated plasma membranes may provide direct evidence for existence of the calcium transporters discussed in this work, and further qualify the influence of HCO_3^- and P_i on their respective activities.

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