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Calcium transport in bovine sperm mitochondria: effect of substrates and phosphate

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Calcium uptake into filipin-treated bovine spermatozoa is completely inhibited by the uncoupler CCCP or by ruthenium red. Both P_i and mitochondrial substrates are required to obtain the maximal rate of calcium uptake into the sperm mitochondria. Bicarbonate and other anions such as lactate, acetate or β-hydroxybutyrate do not support a high rate of calcium uptake. There are significant differences among various mitochondrial substrates in supporting calcium uptake. The best substrates are durohydroquinone, α-glycerophosphate and lactate. Pyruvate is a relatively poor substrate, and its rate can be greatly enhanced by malate or succinate but not by oxalacetate or lactate. This stimulation is blocked by the dicarboxylate translocase inhibitor, butylmalonate and can be mimiced by the non-metabolized substrate D-malate. The K_a for pyruvate was found to be 17 μM and 67 μM in the presence and absence of L-malate, respectively. The K_a for L-malate is 0.12 mM. It is suggested that in addition to the known pyruvate/lactate translocase there is a second translocase for pyruvate which is malate/succinate-dependent and does not transport lactate. In the presence of succinate, glutamate stimulates calcium uptake 3-fold, and this effect is not inhibited by rotenone. In the presence of glutamate plus malate or oxalacetate there is only an additive effect. It is suggested that glutamate stimulates succinate transport and/or oxidation in bovine sperm mitochondria. The α-hydroxybutyrate is almost as good as lactate in supporting calcium uptake. Since the α-keto product is not further metabolized in the citric acid cycle, it is suggested that lactate can supply the mitochondrial needs for NADH from its oxidation to pyruvate by the sperm lactate dehydrogenase x. Thus, when there is sufficient lactate in the sperm mitochondria, pyruvate need not be further metabolized in the citric acid cycle in order to supply more NADH.

Introduction

Mitochondria isolated from various tissues have the capacity to accumulate large amounts of Ca^{2+} in the matrix compartment by a transport process energetically coupled to electron transport [1,2] It is generally accepted that Ca^{2+} transport, as the primary process, is coupled to the uptake of a permeant anion such as phosphate [3,4] The active anions share a common property, the potential ability to carry protons to the intramitochondrial matrix [5]

The intracellular calcium concentration in spermatozoa has a regulatory role in the control of motility and capacitation [6–11] In mammalian spermatozoa, the systems that regulate intracellular Ca^{2+} concentration involve the mitochondria [12] and the plasma membrane ATP-dependent Ca^{2+} pump [13–15] and Na^+/Ca^{2+} antiporter [16,17] Approx 90% of the Ca^{2+} that is taken up by ram or bull spermatozoa is accumulated in the mitochondria [7,12] The transport of Ca^{2+} into sperm mitochondria is inhibited by the sulfhydryl reagents NEM and mersalyl [18] and by ruthenium red [19] We have demonstrated the effect of phosphate, bicarbonate and various mitochondrial substrates on Ca^{2+} transport into intact bovine sperm [20] In the present paper, we examine these factors with regard to calcium transport into sperm mitochondria

Abbreviations NEM, *N*-ethylmaleimide, TEA, triethanolamine, OAA, oxaloacetate, CHC, α-cyanohydroxycinnamic acid, LDH, lactate dehydrogenase

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Material and Methods

Bovine epididymides were obtained from local slaughter houses Epididymal spermatozoa were collected and washed as described previously [12] The

standard medium used contains 110 mM NaCl, 5 mM KCl, 10 mM sodium morpholinopropanesulfonate (pH 7 4) (medium A) The cells were washed twice with medium A by centrifugation at $600 \times g$, 10 min at 25 °C

Filipin treatment A suspension of 2 25 10^8 cells/ml in 15 ml medium A was mixed with 30 μl of a fresh solution of 0 1 M filipin in DMSO for 6 min at 25 °C To this suspension 15 ml of cold (4 °C) buffer M composed of 250 mM mannitol, 70 mM sucrose, 10 mM TEA-Hepes (pH 7 4) were added, and the suspension was centrifuged at 4 °C for 10 min, at $600 \times g$ The pellet was resuspended with 15 ml of cold buffer M, centrifuged as above and the sperm pellet was suspended in 1 8 ml cold buffer M and kept on ice

Calcium uptake Uptake of ^{45}Ca by filipin-treated cells was determined by the filtration technique Cells were incubated in a final volume of 125 μl medium A (2 5 10^8/ml) containing 0 2 mM $CaCl_2$ and 0 5 μCi $^{45}CaCl_2$ The concentration of phosphate when used was 1 25 mM The cells were held for 10 min at 37 °C and the substrate was added 10 s before starting the reaction by the addition of calcium After 2 min of incubation 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 150 mM NaCl, 10 mM Tris (pH 7 4) and 2 mM EGTA The dry filters were counted in scintillation vials with 5 ml Aquasol (DuPont) The 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 using a Clark oxygen electrode

Except where noted, the experiments shown were performed on a single preparation of sperm pooled from the epididymides of two or three bulls and are representative of three or more experiments performed with other such pools

Materials Chemicals were obtained from standard commercial sources Filipin, rotenone, and D-malate were obtained from Sigma, durohydroquinone from Pfaltz-Bauer, Inc and α-cyano-3-hydroxycinnamic acid from Aldrich

Results

Effect of phosphate and bicarbonate on calcium uptake

The effect of phosphate and bicarbonate on calcium uptake into filipin-treated sperm can be seen in Fig 1 Calcium uptake into filipin-treated sperm is completely inhibited by the uncoupler CCCP or by ruthenium red (data not shown) thus we can consider this calcium uptake as an accumulation of calcium in the sperm mitochondria Calcium uptake by sperm mitochondria is absolutely dependent upon the presence of mitochondrial substrate and phosphate With L-lactate as the substrate, a small but significant degree of calcium uptake (2 1 nmol/10^8 cells) occurs without phosphate, but almost no uptake (0 5 nmol/10^8 cells) occurs with the non-ionic substrate durohydroquinone in the absence of phosphate Bicarbonate, which supports calcium uptake in liver mitochondria [21], alone has no effect on calcium uptake by bovine sperm mitochondria Other proton-donating acids such as acetic and β-hydroxybutyric which support calcium transport in liver mitochondria [5], have no effect on sperm mitochondria (data not shown)

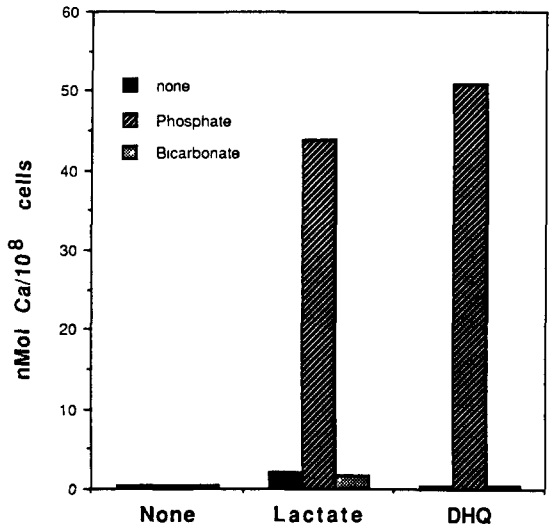

Fig 1 The effect of phosphate and bicarbonate on calcium uptake into filipin-treated cells Calcium uptake during 10 min was determined as described in Materials and Methods The substrates used are 10 mM lactate or durohydroquinone (DHQ) The concentration of P_i is 1 25 mM and that of $NaHCO_3$ is 2 mM

Effect of substrate on calcium uptake and respiration

The effect of various substrates on calcium uptake into filipin-treated sperm is shown in Fig 2 Three substrate groups are designated (1) Good – durohydroquinone (DHQ), α-glycerophosphate (α-GP), lactate, α-hydroxybutyrate (α-HB), oxaloacetate and glutamate (2) Fair – β-hydroxybutyrate (β-HB), pyruvate, succinate and malate (3) Poor – acetate and acetoacetate To further investigate these results we measured the respiratory rate with the various substrates The data in Table I show that respiration rates correlate fairly well with the data on calcium uptake However, the lack of strict correlation indicate that factors other than the energy requirement for calcium transport influence respiratory rate Substrates such as succinate, α-glycerophosphate and durohydroquinone, which provide electrons at site II of the electron transport chain, support higher O_2 consumption per unit of calcium transported than do NADH-generating substrates In fact, the respiration is higher than can be

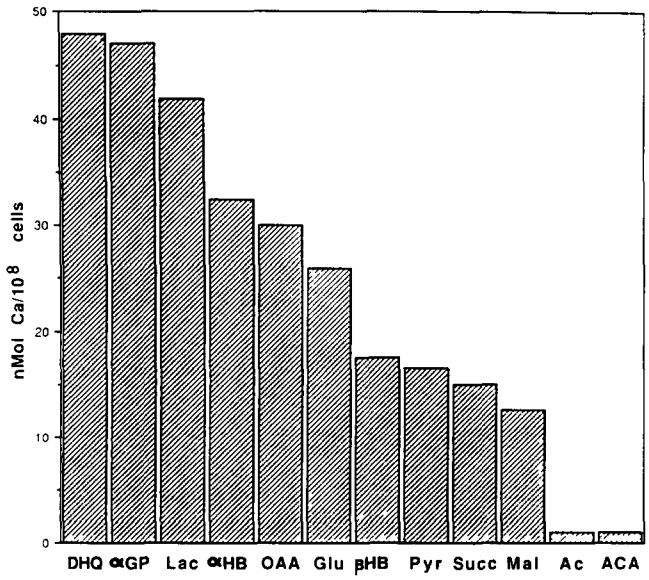

Fig 2 The effects of various substrates on calcium uptake into filipin-treated cells Calcium uptake was determined in the presence of 10 mM substrate and 1 25 mM P_i The substrates are durohydroquinone (DHQ), α-glycerophosphate (α-GP), lactate (Lac), α-hydroxybutyrate (α-HB), oxaloacetate (OAA), glutamate (Glu), β-hydroxybutyrate (β-HB), pyruvate (Pyr), succinate (Succ), malate (Mal), acetate (Ac), acetoacetate (ACA)

accounted for by the difference in P/O ratios for these two classes of substrates We have shown in Fig 2 that pyruvate, succinate or malate are not as effective as lactate in supporting calcium transport into the mitochondria However, when pyruvate was added in the presence of malate or succinate a 3-fold enhancement of calcium uptake occurred (see Fig 3) In contrast, when pyruvate was added in the presences of β-hydroxybutyrate, only an additive effect occurred The respiratory rate with pyruvate was stimulated by malate or succinate, but not with β-hydroxybutyrate or OAA (Table I) The respiratory rate with L-lactate is inhibited by adding pyruvate Calcium uptake in the presence of L-lactate and other substrates is approximately the sum of the activities of the two substrates, lactate does not increase calcium uptake in the presence of pyruvate (Fig 4) Thus, pyruvate inhibition of lactate oxidation

TABLE I

Respiration of filipin-treated cells in the presence of various substrates

Respiration rate was determined in 2 ml medium A (1 46 10^8 cells) which contained 1 25 mM P_i and 0 2 mM $CaCl_2$ The final concentration of CCCP was 1 μm $n = 2$

5 mM substrate	0 5 mM addition	ng atom O/min per 10^8 cells		
		State 4	+0 5 mM Pyr	+CCCP
None		4 4	8 9	11
Durohydroquinone		48	–	184
α-Glycerophosphate		33	–	72
Lactate		15	11	33
Oxalacetate		9 2	8 7	–
β-Hydroxybutyrate		9 7	12	–
Pyruvate		9 2	–	–
Succinate		18	29	–
L-Malate		8 9	16	29
Acetate		2 0	–	–
Citrate		3 6	–	–
Acetate	malate	2 6	–	–
Pyruvate	malate	15	–	29
Pyruvate	oxaloacetate	6 6	–	8 2

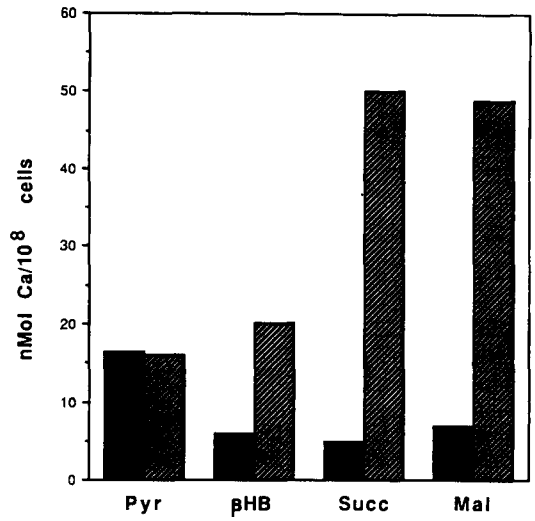

Fig 3 The effect of pyruvate in the presence of other substrates on calcium uptake into filipin-treated cells Calcium uptake was determined with 5 and 0 5 mM pyruvate (Pyr) and in the presence of β-hydroxybutyrate (β-HB), succinate (Succ), or malate (Mal) alone (black bars) or plus 0 5 mM pyruvate (hatched bars) P_i (1 25 mM) was present

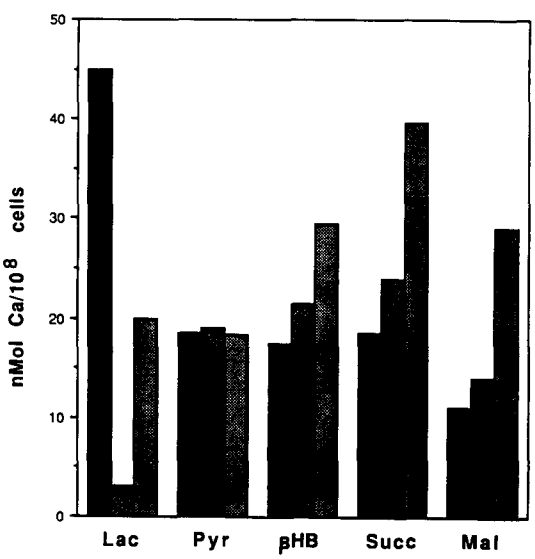

Fig 4 The effect of lactate in the presence of other substrates on calcium uptake into filipin-treated cells Calcium uptake was measured with 10, 0 05 and 0 5 mM lactate (Lac) alone, (black, hatched and dotted bars, respectively) or in the presence of 10 mM pyruvate (Pyr), β-hydroxybutyrate (β-HB), succinate (Succ), or malate (Mal) alone (black bars) or plus 0 05 (hatched bars) or 0 5 mM lactate (dotted bars)

TABLE II

The effect of glutamate, pyruvate and rotenone on calcium uptake

Calcium uptake was determined in the presence of various substrate combinations P_i was present at 1 25 mM

Substrate	Calcium uptake (nmol/10^8 cells per 2 min)				
	None	0 5 mM glutamate		0 5 mM pyruvate	
		control	10 μM rotenone	control	10 μM rotenone
5 0 mM L-malate	7 6	11	–	58	0 7
0 5 mM succinate	0 5	11	–	41	5 4
5 0 mM succinate	5 2	21	22	63	34
0 5 mM oxaloacetate	22	25	–	21	–
5 0 mM oxaloacetate	26	30	1 0	27	0 2
0 5 mM glutamate	3 1	–	–	–	–
5 0 mM glutamate	27	–	–	25	–
0 5 mM pyruvate	16	–	–	16	0 14

(Table I) is reflected in the failure of lactate to stimulate calcium uptake in the presence of pyruvate This behavior is a reflection of the known inhibition of lactate dehydrogenase by pyruvate [22]

The effect of glutamate on calcium uptake can be seen in Table II As with β-hydroxybutyrate and pyruvate, the addition of glutamate to malate or oxaloacetate resulted in only an additive effect and there is no effect of pyruvate added to oxaloacetate However, glutamate added in the presence of succinate enhanced calcium uptake 3-fold This synergistic effect of succinate and glutamate was not inhibited by rotenone In contrast, the effect of malate or oxalacetate in the presence of pyruvate was completely inhibited by rotenone In the presence of pyruvate and succinate, rotenone inhibition was greater with 0 5 mM succinate than with 5 0 mM succinate

We next attempted to determine if the effect of malate is due to the operation of the citric acid cycle When acetate or acetyl carnitine was added in the presence of malate, only a small increase in calcium uptake occurred, in contrast to the synergistic effect of malate plus pyruvate (see Fig 3) Similarly, the addition of pyruvate, acetate or acetyl carnitine to oxalacetate and the addition of pyruvate in the presence of glutamate, had no effect on the degree of calcium uptake (see Tables II and III) Likewise, there was no effect on the respiration supported by the oxalacetate or acetate, by adding pyruvate or malate, respectively (Table I)

To investigate further the mechanisms by which malate or succinate enhance calcium uptake and respiration in the presence of pyruvate, we attempted to inhibit the citrate synthase by the addition of citrate [23] The results in Table IV show that citrate caused only 6% inhibition of respiration in the presence of pyruvate, but in the presence of malate or succinate plus pyruvate it inhibited 23% and 32%, respectively

TABLE III

The effect of acetate and acetyl carnitine on calcium uptake in the presence of malate or oxaloacetate

Calcium uptake was determined as described in Material and Methods

Substrate	Calcium uptake (nmol Ca/10^8 cells per 2 min)		
	None	0 5 mM acetate	0 5 mM acetyl carnitine
10 mM L-malate	11	12	19
5 0 mM oxaloacetate	30	30	31
Medium A	–	0 9	7 5

(compare Table I to Table IV) Citrate also causes 15% inhibition with α-glycerol phosphate as substrate In contrast there is no effect of citrate on respiration when β-hydroxybutyrate is the substrate

The greater inhibition by citrate of pyruvate oxidation in the presence of malate or succinate indicates that malate and succinate stimulate oxidation of pyruvate via the trichloroacetate cycle The fact that succinate is metabolized to malate, together with the well-recognized ability of malate to enhance mitochondrial pyruvate uptake by bull sperm [24] indicate that this effect results from the stimulation of pyruvate transport by malate The results in Table V show that butylmalonate, an inhibitor of the dicarboxylate transporter, causes 90% inhibition of the L-malate effect on pyruvate supported calcium uptake α-Cyanohydroxycinnamic acid (CHC), which inhibits the pyruvate/lactate carrier in bull sperm mitochondria [25] results in an even higher inhibition of calcium uptake CHC causes 90% inhibition in the presence of pyruvate alone, and 74% inhibition when pyruvate plus malate are present If only the pyruvate/lactate translocase is inhibited by CHC, we expected to see only about 30% inhibition, it

TABLE IV

Respiration of filipin-treated cells in the presence of 5 mM citrate

The respiratory rate was measured as described in Table I In all samples 5 mM citrate was present $n = 2$

5 mM substrate	ng atom O/min per 10^8 cells			
	0 5 mM pyruvate	state 4	+CCCP	% inhibition by citrate [a]
Pyruvate	–	8 7	11	6
L-Malate	+	12	28	23
Succinate	+	19	29	32
α-Glycerophosphate	–	28	61	15
β-Hydroxybutyrate	–	10	16	0

[a] The experiments in Tables I and IV are comparable experiments and percentage inhibition is calculated in comparison to the control (without citrate) in Table I

TABLE V

The effect of D-malate, malonate, α-cyano-3-hydroxycinnamate (CHC) and butyl malonate on calcium uptake into filipin-treated spermatozoa

Calcium uptake was determined as described in Material and Methods

Substrate	Calcium uptake (nmol Ca/10^8 cells per 2 min)		
	None	10 μM CHC	10 mM butyl malonate
0 5 mM L-malate	1 3	1 6	0 06
0 5 mM pyruvate	16	1 4	12
0 5 mM pyruvate +0 5 mM L-malate	49	13	19
0 5 mM pyruvate +0 5 mM D-malate	20	–	–
0 5 mM pyruvate +5 mM D-malate	29	–	12
0 5 mM pyruvate +10 mM malonate	21	–	9 9
0 5 mM D-malate	0 01	–	–
10 mM D-malate	0 02	–	–

is possible that the malate-dependent pyruvate transport is also partially inhibited by CHC In spite of this, we can see that in the presence of CHC malate causes a 9-fold enhancement, whereas in the absence of CHC the enhanced effect of malate is only 3-times It is also shown in Table V that D-malate, which is a non-metabolized substrate, stimulates Ca uptake in the presence of pyruvate, and this effect is inhibited by butyl malonate We also found that 5 mM D-malate causes

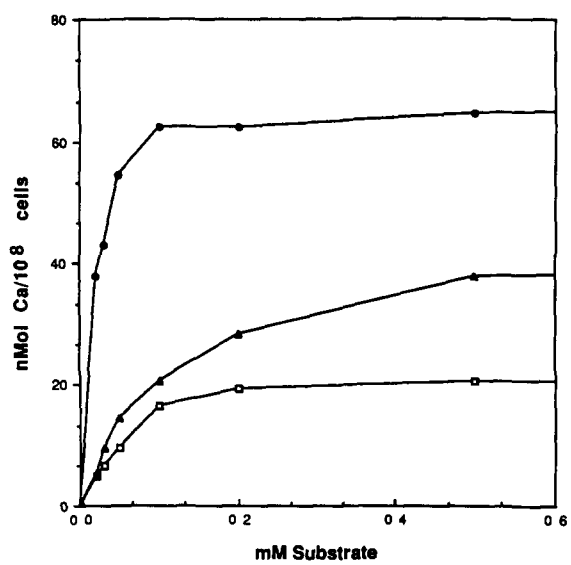

Fig 5 Effect of pyruvate and malate concentration on calcium uptake Calcium uptake was determined under the conditions that have been described in Materials and Methods The symbols are □, pyruvate, ●, pyruvate in the presence of 0 5 mM malate, and ▲, L-malate in the presence of 0 5 mM pyruvate The values for 1 mM substrate are identical to those with 0 5 mM (not shown)

35% and 66% inhibition of calcium uptake in the presence of 5 mM L-malate or succinate, respectively (data not shown) These results indicate competition of L-malate for the C_4 dicarboxylate translocase It was also found that 10 mM methyl succinate causes 46% inhibition of the succinate stimulatory effect in the presence of pyruvate, but is without effect in the presence of pyruvate alone (data not shown) These findings indicate competition between methylsuccinate and succinate for the translocase site Because methyl succinate cannot stimulate pyruvate-supported calcium uptake it is suggested that this compound is not transported into the mitochondria as is succinate, but binds to the succinate translocase to inhibit succinate uptake The data in Fig 5 show the effect of L-malate concentrations on calcium uptake in the presence of pyruvate The K_a for L-malate was calculated from a Lineweaver-Burk reciprocal plot and found to be 0 12 mM The K_a for malate, for its stimulatory effect on isocitrate metabolism in rat liver mitochondria was found to be 0 18 to 0 24 mM [26] or 0 16 mM [27] When the effect of pyruvate concentration on calcium uptake was determined, K_a values of 17 μM and 67 μM for pyruvate in the presence and absence of L-malate, respectively, were determined

Discussion

The object of the present paper was to investigate the role of different substrates on Ca^{2+} transport into the sperm mitochondria The isolation of coupled sperm mitochondria is difficult because their separation requires the breakage of disulphide bridges [28] Filipin treatment disrupts the sperm plasma membrane while leaving the mitochondria functionally intact [29] When filipin-treated cells were washed free of P_i and mitochondrial substrate, little calcium uptake occurs However, a high degree of calcium uptake results with the addition of mitochondrial substrate and P_i In contrast to their effect in mitochondria from somatic cells [5], bicarbonate and other proton carrying anions do not support high calcium uptake by bull sperm mitochondria We suggest that the requirement of P_i for high calcium uptake is probably due to the precipitation of calcium phosphate in the mitochondrial matrix

Maximal calcium uptake was achieved when the respiratory rate reached about 15 or 30 ng atoms O/min per 10^8 cells for site I or site II substrates, respectively These rates can be achieved with lactate or α-glycerophosphate or with the combination of pyruvate and either malate or succinate The additive effect on calcium uptake shown by combination of various substrates (Fig 4 and Tables II, III) is further support the assumption that the rate of electron transport to oxygen is the rate limiting factor in supporting calcium transport into the mitochondria Thus, in order to create a given $\Delta\psi$,

several substrates can be oxidized at the same time to produce NADH

It was interesting to find that α-hydroxybutyrate is a good substrate to support calcium uptake (Fig 2) for it can be oxidized by the mitochondrial LDHx, but the α-keto product is not further metabolized in the citric acid cycle Thus, the NADH produced is derived only from the LDHx activity The fact that α-hydroxybutyrate is almost as effective as lactate in supporting calcium uptake indicates that with lactate the majority of the NADH produced is derived from the LDHx reaction, and the pyruvate produced need not be further metabolized in the citric acid cycle This is a unique system for sperm, since mitochondrial LDH has been found only in spermatozoa [30–32] Thus, under conditions where the spermatozoa have sufficient lactate, there is no need to operate the citric acid cycle to produce additional NADH It has been found that LDHx is inhibited by high concentrations of pyruvate [22] Thus, when the cells have enough lactate, removal of pyruvate via the citric acid cycle may prevent inhibition of the LDHx Our data (Fig 4), show that calcium uptake supported by pyruvate cannot be stimulated by adding lactate, also indicates the inhibition of LDHx by pyruvate It is not likely that the pyruvate-lactate translocase may have become rate-limiting under these conditions, because with lactate alone we can see very high calcium uptake which is completely blocked by α-cyano-3-hydroxycinnamate, a pyruvate translocase inhibitor [24,33], (data not shown) thus indicating that lactate is not transported via another carrier It is also possible that high pyruvate concentration inhibits the pyruvate-lactate translocase since the V_{max} for calcium uptake is reached at 0 1 mM pyruvate (Fig 5), which is a very low concentration in comparison to lactate (see Fig 4)

The stimulation of pyruvate-dependent calcium uptake by malate or succinate (Fig 3) can be blocked partially by adding butyl malonate (Table V) which is a dicarboxylate transport inhibitor [34] In addition, the non-metabolized D-malate and malonate (Table V) can stimulate calcium uptake in the presence of pyruvate These data indicate the existence of a malate-dependent pyruvate transporter

Malate could possibly stimulate pyruvate-dependent calcium uptake by supplying oxalacetate, since pyruvate dehydrogenase may be inhibited by an increased concentration of acetyl-CoA [35] However, if this were the case we would expect to find stimulation of pyruvate supported calcium uptake by adding oxalacetate, which we do not see (Table II) Because of this, we can exclude the possibility that pyruvate enhances malate oxidation by decreasing the OAA concentration which would inhibit the malate dehydrogenase It has been shown in bovine sperm [24], that in the presence of malate, 57% of the pyruvate is converted to lactate, while in the absence of malate only 20–30% is converted This diversion of electrons to pyruvate reduction cannot explain the higher rate of calcium uptake that we see in the presence of malate plus pyruvate It is likely that more pyruvate is transported into the mitochondria in the presence of malate, resulting in more pyruvate converted to lactate and more pyruvate oxidized via the citric acid cycle Bovine sperm mitochondria accumulate a significant amount of citrate in the presence of pyruvate plus malate [24] The suggestion that pyruvate oxidation via the citric acid cycle is stimulated by malate is supported by the data (Table IV) which show that citrate causes greater inhibition of respiration when pyruvate and malate are present (23%) in comparison to pyruvate alone (6%) We have shown (Table II) that in the presence of pyruvate calcium uptake is inhibited 87 and 46% by 0 5 or 5 0 mM succinate, respectively, by rotenone These data indicate, in addition to the stimulatory effect of succinate on pyruvate oxidation, that pyruvate stimulates the oxidation of succinate Thus, not only more pyruvate but also more succinate is transported into the mitochondria

It has been shown that the malate-aspartate cycle occurs in boar sperm mitochondria [36] When the effect of glutamate was determined in the presence of malate or oxalacetate, we found only an additive effect on calcium uptake (Table II) But when glutamate was added with succinate we saw about 3 fold stimulation above the additive effect Since this stimulation is not inhibited by rotenone, the $\Delta\psi$ for calcium uptake under these conditions must come from $FADH_2$ oxidation Thus, glutamate may stimulate succinate transport or oxidation (or both) in the mitochondria Because glutamate has no stimulatory effect with malate or oxalacetate, we suggest that the effect of glutamate does not result from supplying α-ketoglutarate to the citric acid cycle Since we see an additive effect in the presence of malate plus glutamate, we suggest that the α-ketoglutarate, produced from glutamate, enters the citric acid cycle rather than exchanging for malate In the presence of succinate plus glutamate the intramitochondrial malate, which has been produced from succinate, is not further metabolized, since there is no inhibition by rotenone However, the malate produced may be exchanging for succinate It has been found that the dicarboxylate transport inhibitor butyl malonate inhibits the glutamate-dependent NADH oxidation in boar sperm mitochondria [36] Thus it is possible that sperm mitochondria contain a glutamate-malate transporter as well

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