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Inhibition by caltrin of calcium transport into spermatozoa, liver and heart mitochondria

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The calcium-transport inhibitor, caltrin, isolated from bovine seminal fluid inhibits calcium accumulation by bovine epididymal spermatozoa, spermatozoal mitochondria, rat liver mitochondria and beef heart mitochondria. Respiration studies demonstrate a marked stimulation of oxygen consumption by caltrin in filipin-treated spermatozoa and rat liver mitochondria. A biphasic effect of caltrin on rat liver mitochondrial respiration was noted, with stimulation at low caltrin concentrations and inhibition as the concentration of caltrin is increased. The ability of caltrin to uncouple and/or inhibit respiration in filipin-treated spermatozoa and isolated liver mitochondria indicates that inhibition of mitochondrial calcium accumulation by caltrin results from one of these mechanisms. Only a marginal effect of caltrin on respiration of intact spermatozoa was observed; indicating that the plasma membrane is impermeable to this protein. The differential effect of caltrin on respiration of intact and permeabilized spermatozoa indicates that caltrin inhibition of Ca^{2+} uptake into spermatozoa in vivo occurs at the level of the plasma membrane.

Introduction

Caltrin is a 5411 dalton basic protein from bovine seminal plasma that inhibits bovine epididymal spermatozoa calcium accumulation [1–3]. Previously, we proposed that caltrin inhibits spermatozoa calcium uptake by a mechanism which involves the plasma membrane Na^+/Ca^{2+} exchanger [4]. An integral aspect of this proposal is that caltrin exert its inhibitory effect at the level of the plasma membrane. Recent work from our laboratory demonstrated that ejaculated spermatozoa bind anticaltrin IgG over the acrosome and principal tail regions, but not to the midpiece [5].

In contrast to ejaculated spermatozoa, epididymal spermatozoa readily accumulate calcium and contain no caltrin as is demonstrated by their inability to bind anticaltrin IgG [5]. However, upon incubation with caltrin these cells adopt the regional acrosome/tail distribution of caltrin and calcium transport in inhibited [5].

Babcock et al. [6] have demonstrated that the majority of calcium accumulated by bovine spermatozoa is taken up by the mitochondria of the midpiece structure. Thus, the plasma membrane calcium flux inhibited by caltrin apparently operates in the acrosome and/or tail domains, and supplies the midpiece cytosol with calcium.

In the present work, we provide further evidence that caltrin inhibits spermatozoa calcium accumulation by interacting with the plasma membrane. In addition, we demonstrate that in permeabilized spermatozoa and in mitochondria from several sources, caltrin inhibits mitochondrial calcium accumulation by uncoupling and/or inhibiting the mitochondrial electron transport chain.

Materials and Methods

Purification of caltrin. Bovine semen was the generous gift of American Breeder's Service, DeForest, WI. Caltrin was purified as described earlier [3]. The purified caltrin was stored in a solution composed of 35% (v/v) glycerol, 90 mM NaCl and 10 mM sodium morpholinepropane sulfonate (pH 7.4) (Caltrin Storage Buffer). All experiments reported in this paper were conducted with the inhibitory form of caltrin [3].

Preparation of spermatozoa. Bovine epididymides were obtained from local slaughter houses. Epididymal spermatozoa were collected and washed as described previoulsy [7] in a solution (NKM) of 110 mM NaCl, 5

Abbreviation: CCCP, carbonyl cyanide *M*-chlorophenylhydrazone.

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mM KCl, 10 mM sodium morpholinepropane sulfonate (pH 7.4).

Preparation of filipin-treated cells. Filipin-treated cells were prepared as described by Morton and Lardy [8] with minor modifications. Washed epididymal spermatozoa were incubated in NKM for 6 min at 25 °C with 80 nmol filipin/10^8 cells. These cells were then washed twice with ice-cold 0.25 M mannitol, 70 mM sucrose and 10 mM Hepes-triethanolamine (pH 7.4) (Buffer M). The final pellet was resuspended in Buffer M to achieve a concentration of $(1.5–2.0) \cdot 10^9$ cells/ml, and kept on ice until use.

Preparation of rat hepatocytes. Isolated hepatocytes were prepared from livers of male rats, fasted 24 h as described earlier [9,10]. These cells were suspended in Ca^{2+}-free Krebs-Henseleit bicarbonate buffer [11].

Preparation of isolated mitochondria. Rat liver mitochondria were isolated according to the procedure of Johnson and Lardy [12] in Buffer M with the addition of 1.0 mM EGTA during the initial steps of isolation. Heavy beef heart mitochondria were prepared by the method of Blair [13].

Mitochondrial protein concentrations were determined by the biuret reaction on deoxycholate solublized mitochondrial samples [14], with bovine serum albumin as protein standard.

Preparation of freeze-thawed mitochondria. Freeze-thawed mitochondria were prepared by subjecting mitochondria (approx. 20 mg protein/ml) in a glass vial to repeated cycles of freezing in liquid nitrogen and thawing in a 25 °C water bath. The absence of a respiratory response to CCCP was taken as evidence of complete uncoupling. Three or four freeze-thaw cycles were required.

Assay of calcium uptake. Uptake of $^{45}Ca^{2+}$ by cell suspensions (whole spermatozoa and hepatocytes) and mitochondria (filipin-treated spermatozoa and isolated mitochondria) was determined by a filtration techique. Cells or mitochondria were preincubated for 10 min in a given medium (see table or figure legend for each experiment). Reactions were initiated by the addition of 0.5 μCi $^{45}CaCl_2$ to a final concentration of 0.2 mM $CaCl_2$ in a total volume of 125 μl. After a given reaction time, a 100 μl aliquot was quickly removed from each sample and immediately vacuum filtered on either GF/C (cells) or Millipore filters 0.45 μm (mitochondria). The filters were then washed three times with a 5-ml aliquot of 10 mM Tris (pH 7.4) plus either 150 mM NaCl/2 mM EGTA (cells) or 150 mM KCl/1 mM K-EGTA (isolated mitochondria). These filters were dried, added to 5 ml of Aquasol, and their radioactivity determined.

Respiration determinations. Oxygen consumption was monitored using a Clark oxygen electrode. Experimental conditions were as described in the figure legends.

Materials. Filipin, CCCP, glycerol 3-phosphate, Ruthenium red, antimycin A, cytochrome *c* and ascorbate were obtained from Sigma. Durohydroquinone was obtained from Pfaltz Bauer, Inc.

Results

Effect of caltrin on spermatozoa

As shown in Fig. 1, caltrin markedly inhibits calcium accumulation by both intact and filipin-permeabilized spermatozoa. In these experiments, the polyene antibiotic filipin was employed at concentrations which selectively permeabilize the sperm plasma membrane and leave the mitochondria functionally intact [8,15]. The I_{50} values for caltrin with intact and permeabilized cells are similar, 5.5 and 6.3 μg caltrin/$2.8 \cdot 10^7$ cells, respectively. It should be mentioned here that sperm mitochondria contain LDHx activity, thus we could use L-lactate as a mitochondrial substrate.

It is well established that respiratory uncouplers, such as CCCP, are potent inhibitors of calcium uptake into many systems including both intact and filipin-treated spermatozoa [16]. Therefore, an investigation of the effect of caltrin on spermatozoal respiration seemed prudent. As shown in Fig. 2, the addition of 1 μM CCCP results in a 6-fold stimulation of respiration. Such a concentration of CCCP has previously been shown to inhibit calcium accumulation in these cells [16]. In contrast, the addition of caltrin in an amount several-fold in excess of the I_{50} for calcium uptake has only a marginal stimulatory effect on respiration in intact cells (Fig. 2). However, caltrin added to filipin-

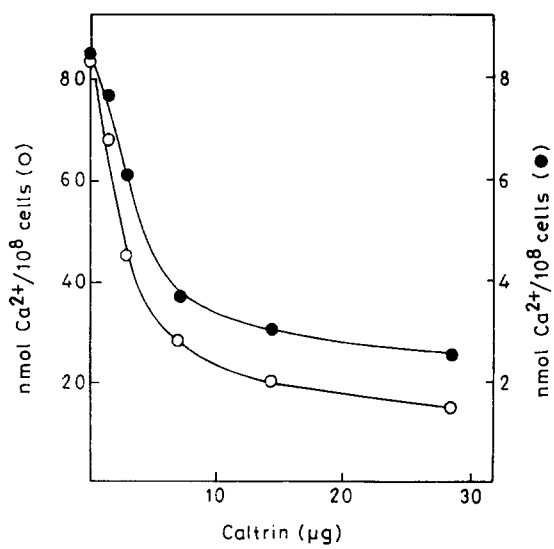

Fig. 1. Effect of caltrin on Ca^+ uptake into intact and filipin-treated spermatozoa. Intact (●) or filipin-treated spermatozoa (•) ($2.8 \cdot 10^7$ cells) were preincubated for 10 min at 37 °C in NKM plus 1.25 mM P_i, 10 mM L-lactate and a constant volume of Caltrin Storage Buffer containing increasing concentrations of caltrin. Reactions were initiated as described in Materials and Methods by the addition of $^{45}Ca^{2+}$ and terminated after 10 min by filtration.

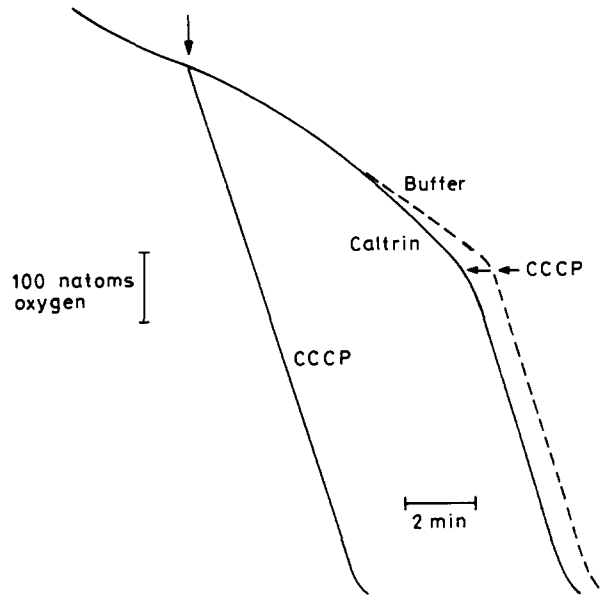

Fig. 2. Effect of caltrin on sperm respiration. Washed spermatozoa ($1.3 \cdot 10^8$ cells) were incubated in 2 ml of NKM at 25 °C in the presence of 5 mM durohydroquinone. Caltrin (143 μg), caltrin storage buffer and CCCP (1.0 μM) were added at the first arrow. Tracings obtaind with and without Caltrin Storage Buffer were identical.

treated cells stimulates respiration to a level comparable to that with CCCP (Fig. 3).

Effect of caltrin on hepatocyte calcium uptake

Caltrin has only a marginal inhibitory effect (5%) on calcium uptake into hepatocytes (Tabel I). However, CCCP remains effective; inhibiting hepatocyte calcium uptake by 42%. Higher concentrations of CCCP (> 0.4 nmol/$5.5 \cdot 10^7$ cells) did not further inhibit calcium uptake (data not shown).

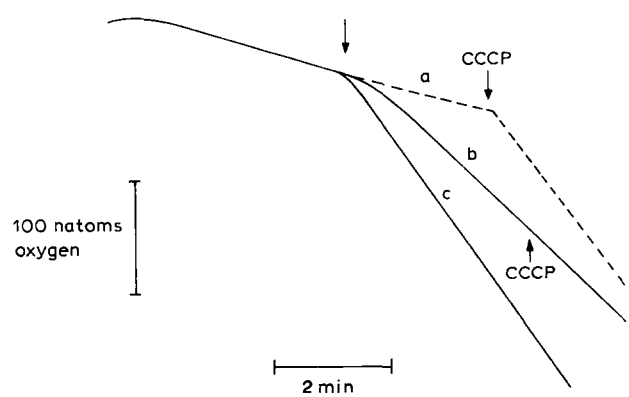

Fig. 3. Effect of caltrin on the respiraton of filipin-treated spermatozoa. Filipin-treated sperm ($1.3 \cdot 10^8$ cells) were incubated in 2 ml of Buffer M at 25 °C in the presence of 10 mM glycerol 3-phosphate and 0.2 mM $CaCl_2$. Caltrin Storage Buffer (a), 143 μg caltrin (b), and CCCP to 1.0 μM (c), respectively, were added at the first arrow. Subsequent additions of CCCP to 1 μM were made at the second arrows.

TABLE I

Effect of caltrin and CCCP on calcium uptake into rat hepatocytes

Rat hepatocytes ($5.5 \cdot 10^5$ cells) were suspended in 0.18 ml medium containing 119 mM NaCl, 4.7 mM KCl, 1.2 mM potassium phosphate, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$ (pH 7.4) which had been equilibrated with 95% O_2 5% CO_2. The suspensions were preincubated with Caltrin Storage Buffer (control), caltrin, or CCCP for 10 min at 37 °C in an atmosphere of 95% O_2 5% CO_2. Reactions were then initiated as described in Materials and Methods by the addition of $^{45}Ca^{2+}$ and terminated after 2 min by filtration.

Addition	Calcium uptake (nmol Ca^{2+}/min per 10^6 cells)	% Inhibition
Control	0.60	–
Caltrin (46 μg)	0.57	5
CCCP (0.4 nmol)	0.35	42

Effect of caltrin on mitochondrial calcium uptake

To investigate further the effect of caltrin on mitochondria, we studied the effect of this protein on both rat liver and beef heart mitochondrial calcium transport and/or respiration.

As shown in Fig. 4, low concentrations of caltrin greatly attenuate calcium uptake into isolated rat liver mitochondria, with the maximal extent of inhibition being equal to that of both CCCP and the specific mitochondrial calcium uptake inhibitor ruthenium red. In these experiments, an I_{50} of 30 μg caltrin/mg mitochondrial protein was determined. Similarly, caltrin in-

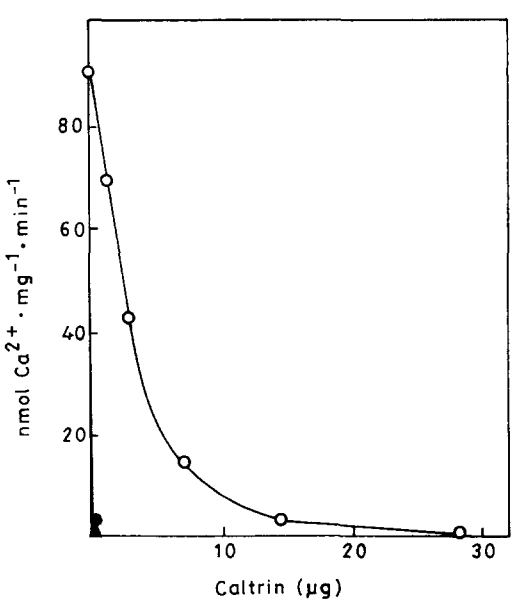

Fig. 4. Effect of caltrin on Ca^{2+} uptake into isolated rat liver mitochondria. Mitochondria (0.1 mg protein) were preincubated for 10 min at 25 °C in Buffer M containing 5 mM potassium succinate, 2 mM KP_i, 1 mM ADP, 5 mM $MgCl_2$, and either 2 μM CCCP (●), 2 μM Ruthenium red (▲), or a constant volume of Caltrin Storage Buffer containing increasing concentrations of caltrin (○). Reactions were initiated as described in Materials and Methods by the addition of $^{45}Ca^{2+}$ and terminated after 2 min by filtration.

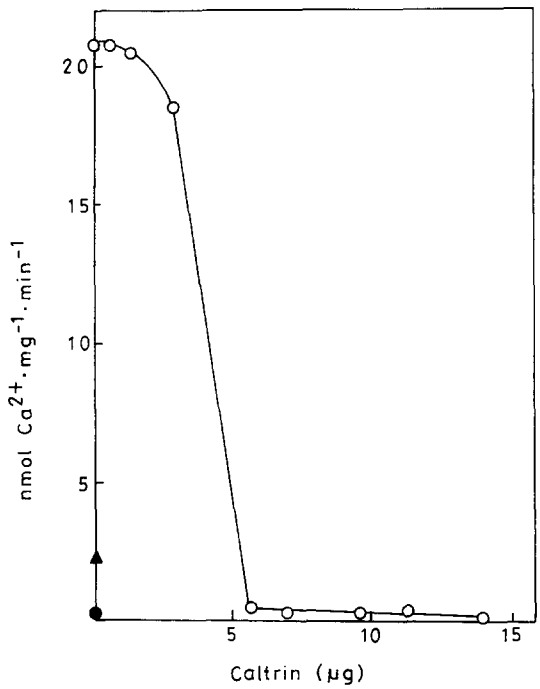

Fig. 5. Effect of caltrin Ca^{2+} uptake into isolated beef heart mitochondria. Mitochondria (0.1 mg protein) were preincubated for 10 min at 25 °C in Buffer M containing 5 mM potassium succinate, 0.15 mM KP_i, 0.1 mM ADP, 0.12 mM $MgCl_2$ and either 1.6 μM CCCP (●), 1.6 μM Ruthenium red (▲), or a constant volume of Caltrin Storage Buffer containing increasing concentrations of caltrin (○). Reactions were initiated as described in Materials and Methods by the addition of $^{45}Ca^{2+}$ and terminated after 2 min by filtration.

hibits calcium accumulaiton by beef heart mitochondria, with an I_{50} of about 40 μg caltrin/mg mitochondrial protein (Fig. 5). However, though the I_{50} for caltrin in both systems is similar, at the lower caltrin concentrations only a slight inhibition of calcium uptake into heart mitochondria was observed.

The effects of caltrin on rat liver mitochondrial respiration are portrayed in Fig. 6. Addition of caltrin in amounts up to 150 μg/mg mitochondrial protein result in proportional increases in the respiratory rate. Addition of 300 μg caltrin has no stimulatory effect on respiration. Interestingly, as the amount of caltrin increases, a progressive decrease in the post-caltrin CCCP-uncoupled respiratory rate is noted. Thus, it appears that caltrin both stimulates and inhibits rat liver mitochondrial respiration.

The inhibitory effect of caltrin on rat liver mitochondrial respiration was further investigated by utilizing permeabilized mitochondria and substrates whose oxidation occurs at different sites in the electron transport chain. Mitochondria subjected to freeze-thaw cycles become maximally uncoupled and are thus not affected by uncoupling agents. Both succinate and durohydroquinone yield electrons at site II. Ascorbate donates electrons through the catalytic action of cytochrome *C* [17]. As shown in Fig. 7, 300 μg caltrin/mg mitochon-

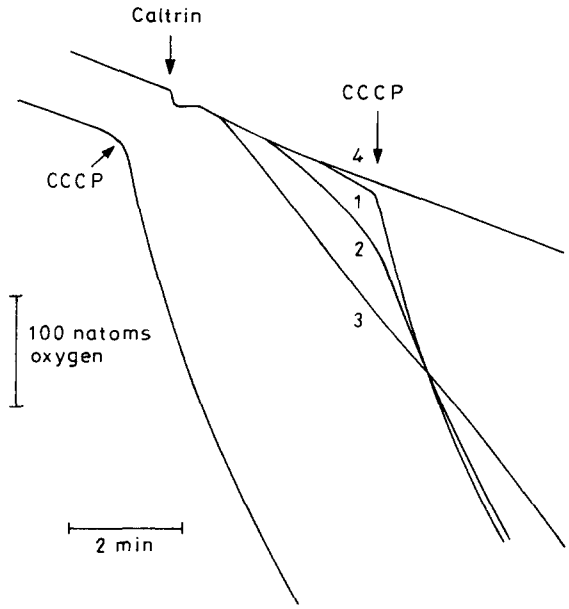

Fig. 6. Effect of caltrin on the respiration of rat liver mitochondria. Isolated rat liver mitochondria (1.0 mg protein) were incubated in 2 ml of Buffer M with 5 mM potassium succinate and 0.2 mM $CaCl_2$ at 25 °C. CCCP (1.0 μM) and caltrin were added as indicated by the arrows. Tracings 1 through 4 contained 40, 80, 150, and 300 μg caltrin, respectively.

drial protein inhibits respiration supported by the site II substrates. However, respiration supported by the ascorbate/cytochrome *c* pair is not affected by caltrin. As has been well documented, CN^- inhibition of cytochrome oxidase inhibits the ascorbate/cytochrome supported respiration, while antimycin A inhibition of site III is without effect (Ref. 17 and Fig. 7).

Discussion

In the present work we have demonstrated that caltrin inhibits calcium accumulation by intact bovine sperma-

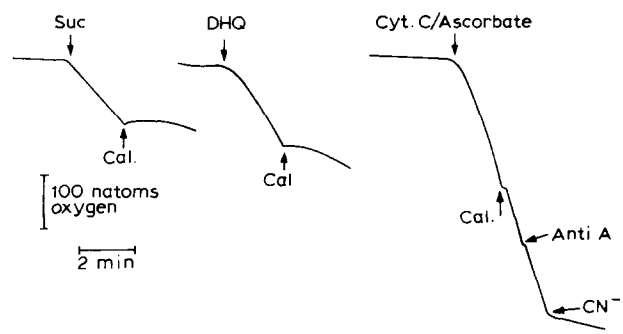

Fig. 7. Effect of caltrin on the respiration of freeze-thawed mitochondria. Freeze-thawed rat liver mitochondria (1.0 mg protein) were incubated in 2 ml of Buffer M with additions as indicated by the arrows. Final concentrations were potassium succinate 5 mM, durohydroquinone 5 mM, ascorbate 2.5 mM, cytochrome *C* 1.25 mg/ml, caltrin 150 μg/ml, antimycin A 30 μM, and KCN 0.5 mM.

tozoa, spermatozoa whose plasma membrane has been permeabilized with filipin, and isolated rat liver and beef heart mitochondria. Calcium uptake into rat hepatocytes was not affected by caltrin. Alone, these data indicate that caltrin is either an universal inhibitor of mitochondrial calcium accumulation with the ability to traverse the sperm plasma membrane, or a protein with the ability to inhibit both sperm plasma membrane and mitochondrial membrane calcium flux. The latter data interpretation assumes that the plasma membrane is impermeable to caltrin and that filipin treatment of spermatozoa allows interaction of caltrin with the mitochondria.

The inability of caltrin to alter mitochondrial respiration in intact spermatozoa or affect hepatocyte calcium uptake indicates that the plasma membrane in both cell types is impermeable to caltrin. Thus, caltrin specifically inhibits bovine spermatozoa plasma membrane calcium flux and generally inhibits calcium accumulation by exposed or isolated mitochondria. These findings are consistent with our previous report implicating the spermatozoa plasma membrane Na^+/Ca^{2+} exchanger as the in vivo site of caltrin inhibition [4].

Both spermatozoa mitochondrial and rat liver mitochondrial respirations are clearly enhanced by caltrin (Figs. 3 and 6). In addition, a biphasic effect of caltrin on rat liver mitochondrial respiration is evident, with stimulation at low caltrin concentrations and inhibition as the concentrion of caltrin is increased (Fig. 6). Further investigation of this phenomena using physically-uncoupled mitochondria indicates that caltrin inhibits the electron transport chain at a point(s) between sites II and cytochrome *C* (Fig. 7). Thus, caltrin has the ability to both enhance and inhibit mitochondrial respiration.

Inhibition of the electron transport chain by caltrin would decrease the membrane potential and result in diminished calcium accumulation. In addition, if caltrin stimulates mitochondrial respiration through an uncoupling mechanism, calcium accumulation will be inhibited.

Caltrin is a basic protein with an isoelectric point of approximately 10 [2,10]. Other basic proteins have long been known to inhibit and/or uncouple mitochondrial respiration. Protamines are 5000–10 000 dalton proteins with isoelectric points of approximately 12 [19]. Protamines in amounts of approx. 40 μg/mg mitochondrial protein inhibit the state 3 respiration of succinate by rat liver mitochondria [20,21]. Interestingly, protamines do not inhibit succinate oxidation in submitochondrial particles prepared by sonication [20]. Arginine-rich histones, H_3 and H_4, are 12 000–15 000 dalton basic proteins. In contrast to the protamines, arginine-rich histones uncouple the succinate-supported respiration of rat liver mitochondria, with maximal uncoupling occurring at 30 μg/mg mitochondrial protein [22]. Neither protamines nor histones H_3 and H_4 share obvious sequence similarity with caltrin.

The mitochondrial effects of protamines and arginine-rich histones have been attributed to their interaction with cytochrome oxidase [23]. However, the inability of caltrin to inhibit ascorbate/cytochrome *C* supported respiration in freeze-thawed mitochondria effectively rules out this terminal electron transport chain component as a site of caltrin inhibition.

Mitochondria obtained from brown fat are uncoupled [24]. The inner membrane of these mitochondria contains an integral 32 000 dalton protein which in dimer form functions as a proton channel, and thereby uncouples oxidative phosphorylation [25–29]. This protein and caltrin share no obvious sequence homology.

Electron transport chain inhibition by caltrin is reminiscent of the site-specific inhibition found with the guanidines [30]. These small molecular weight basic compounds inhibit energy transduction at site I, II or III, depending on the specific guanidine employed [30]. *n*-Octylbiguanide inhibits site I directly, while the other guanidines are believed to inhibit the respiration-linked transfer of protons across the mitochondrial membrane, likely through an alteration in surface charge [30]. Uncoupling agents release the respiratory inhibition induced by the 'surface charge' altering guanidines [30]. In this regard, the ability of caltrin to inhibit respiration in physically-uncoupled mitochondria indicates that this protein directly inhibits an electron transport chain component(s).

In summary, caltrin inhibits both bovine spermatozoa calcium accumulation at the level of the plasma membrane and inhibits calcium accumulation by mitochondria from several sources. Inhibition of mitochondrial calcium accumulation by caltrin may be due to the ability of this protein to inhibit and/or uncouple the electron transport chain. Further work is required to define more completely the complex interaction of caltrin with mitochondria.

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