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RELATIONSHIP BETWEEN CALCIUM ION TRANSPORT AND $(Ca^{2+} + Mg^{2+})$-ATPase ACTIVITY IN ADIPOCYTE ENDOPLASMIC RETICULUM

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Summary

Calcium uptake by adipocyte endoplasmic reticulum was studied in a rapidly obtained microsomal fraction. The kinetics and ionic requirements of Ca^{2+} transport in this preparation were characterized and compared to those of $(Ca^{2+} + Mg^{2+})$-ATPase activity. The time course of Ca^{2+} uptake in the presence of 5 mM oxalate was nonlinear, approaching a steady-state level of 10.8—11.5 nmol Ca^{2+}/mg protein after 3—4 min of incubation. The rate of Ca^{2+} transport was increased by higher oxalate concentrations with a near linear rate of uptake at 20 mM oxalate. The calculated initial rate of calcium uptake was 18.5 nmol Ca^{2+}/mg protein per min. The double reciprocal plot of ATP concentration against transport rate was nonlinear, with apparent K_m values of 100 μM and 7 μM for ATP concentration ranges above and below 50 μM, respectively. The apparent K_m values for Mg^{2+} and Ca^{2+} were 132 μM and 0.36—0.67 μM, respectively. The energy of activation was 23.4 kcal/mol. These kinetic properties were strikingly similar to those of the microsomal $(Ca^{2+} + Mg^{2+})$-ATPase. The presence of potassium was required for maximum Ca^{2+} transport activity. The order of effectiveness of monovalent cations in stimulating both Ca^{2+} transport and $(Ca^{2+} + Mg^{2+})$-ATPase activity was $K^+ > Na^+ = NH_4^+ > Li^+$. Ca^{2+} transport and $(Ca^{2+} + Mg^{2+})$-ATPase activity were both inhibited 10—20% by 6 mM procaine and less than 10% by 10 mM sodium azide. Both processes were completely inhibited by 3 mM dibucaine or 50 μM *p*-chloromercuribenzene

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Abbreviation: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

sulfonate. The results indicate that Ca^{2+} transport in adipocyte endoplasmic reticulum is mediated by a (Ca^{2+} + Mg^{2+})-ATPase and suggest an important role for endoplasmic reticulum in control of intracellular Ca^{2+} distribution.

Introduction

Calcium ions are known to participate in a variety of cellular activities including modulation of contractile processes [1], nerve action potentials [2], secretory processes [3], cell division [4], and activity of various enzymes [5]. Regulation of intracellular calcium concentration thus appears to be crucial for the maintenance of normal cell function. Both mitochondria and the plasma membrane are believed to play a role in controlling the cytoplasmic calcium concentration of non-contractile tissues [6,7]. Recently, a regulatory role for the endoplasmic reticulum has been proposed, analogous to the well known Ca^{2+}-sequestering function of sarcoplasmic reticulum in muscle [8,9].

Ca^{2+} transport activity has now been described in microsomal or endoplasmic reticulum preparations from liver [8,10,11], fibroblasts [12,13], kidney cells [14], brain tissue [15], platelets [16], and adipocytes [9]. However, the mechanism by which the endoplasmic reticulum accumulates Ca^{2+} has not been determined. The Mg^{2+}-ATP-dependent nature of microsomal Ca^{2+} accumulation has led to the proposal that transport may be linked to (Ca^{2+} + Mg^{2+})-ATPase activity as in the sarcoplasmic reticulum system. Although Ca^{2+}-stimulated ATPase activity was detected in several of the above preparations, a relationship between enzyme activity and Ca^{2+} transport could not be established. Alternatively, it has been suggested that Ca^{2+} transport may be related to establishment of ion and proton gradients [11].

The present study is part of a series of investigations designed to elucidate the mechanism and regulation of Ca^{2+} transport in adipocyte endoplasmic reticulum. Previous studies of this system indicated a requirement for ATP and Mg^{2+}, a low K_m for Ca^{2+} (1—4 μM), and an enhancement of Ca^{2+} accumulation in the presence of oxalate [9]. These studies utilized endoplasmic reticulum vesicles suspended in the 20 000 $\times g$ supernatant (S_1) of the total cell homogenate, were performed at 24°C and did not employ initial rate measurements. The Ca^{2+} transport activity of the preparation was extremely labile and the endoplasmic reticulum vesicles lost their ability to accumulate Ca^{2+} when isolated from the supernatant by the standard 60-min centrifugation. The present work describes a rapid method of obtaining endoplasmic reticulum vesicles free of cytosol which retain both Ca^{2+} transport and (Ca^{2+} + Mg^{2+})-ATPase activity. The kinetics and ionic requirements of Ca^{2+} transport are characterized in this preparation at 37°C utilizing initial rate measurements and are compared to those of the endoplasmic reticulum (Ca^{2+} + Mg^{2+})-ATPase. The results indicate that Ca^{2+} transport by adipocyte endoplasmic reticulum is mediated by Ca^{2+}-stimulated ATP hydrolysis and suggest that this mechanism of Ca^{2+} regulation is common to both contractile and non-contractile cells.

Materials and Methods

Materials. Male Wistar rats of 120 g were purchased from National Laboratory Animal Co., O'Fallon, MO. Collagenase (Type I) from *Clostridium*

histolyticum, bovine serum albumin (fraction V), and ATP (disodium or Tris salt) were purchased from Sigma Chemical Co., St. Louis, MO. Type HAWP membrane filters with a 0.45 μM pore size were obtained from Millipore Corp., Bedford, MA, and were soaked in 0.25 M KCl prior to use. $^{45}CaCl_2$ was obtained from New England Nuclear, Boston, MA. [γ-^{32}P]ATP was obtained from Amersham/Searle, Arlington Heights, IL., or was synthesized according to the method of Beutler and Guinto [17] from $^{32}P_i$ purchased from New England Nuclear. All other reagents were of reagent grade quality and were purchased from standard sources. All reagents were prepared with water deionized by a double-chambered mixed bed ion exchange resin system (Culligan, Inc., Northbrook, IL.) which was filtered prior to use with a 0.25 μm pore size filter (Ultipor, PTM Corp., Cortland, NY).

Adipocyte fractionation. Adipocytes were isolated from rat epididymal fat pads as previously described [18]. Isolated adipocytes were homogenized in 0.25 M sucrose with 10 mM Tris-HCl, pH 7.4 at 4°C (Tris/sucrose). The homogenate was centrifuged at 20 000 × *g* for 15 min. The rapidly isolated microsomal preparation was obtained by adding ATP (0.5 mM) and $MgCl_2$ (0.25 mM) to the resulting supernatant (S_1) and centrifuging at 160 000 × *g* for 10 min to obtain a microsomal pellet. The pellet was resuspended in Tris/sucrose by brief homogenization. Previous characterization of microsomes isolated by a 60 min centrifugation of S_1 indicated that the preparation was highly enriched in endoplasmic reticulum [19,20]. The rapidly isolated microsomal preparation was found to be equally enriched in endoplasmic reticulum by assays of cytochrome *c* reductase activity which indicated an 8.3-fold enhancement of activity in the microsomes as compared to values in the homogenate. Based on the activity of this marker enzyme, 30% of the endoplasmic reticulum present in the homogenate was recovered in the rapidly prepared microsomes, similar to a 35% recovery in microsomes prepared by the standard 60 min centrifugation (n = 5). Microsomes were either immediately treated with deoxycholate or rapidly frozen in small aliquots with solid CO_2/ethanol and stored at −70°C until used. Mitochondrial and plasma membrane fractions were obtained as previously described and characterized [21,22].

Deoxycholate-treated microsomes. Freshly isolated microsomes at a concentration of 1—2 mg protein/ml were added to an equal volume of deoxycholate in KCl giving a final concentration of 0.09% deoxycholate-0.15% KCl. The deoxycholate-microsomes solution was stirred for 5 min at 24°C, diluted 5—10-fold with Tris/sucrose plus 0.5 mM ATP and 0.25 mM $MgCl_2$, and centrifuged at 160 000 × *g* for 10 min. The resulting pellet was resuspended in Tris/sucrose, rapidly frozen and stored at −70°C until used. This preparation has been used previously to characterize the (Ca^{2+} + Mg^{2+})-ATPase activity [23].

Calcium transport assays. Ca^{2+} accumulation by isolated microsomal vesicles was assayed using a filtration technique [9]. Standard incubations were performed in duplicate in polystyrene tubes with constant shaking for 1 min at 37°C. The assay was initiated by addition of 20—30 μg of protein to a standard incubation medium containing 50 mM Tris-HCl, pH 6.8 at 37°C, 100 mM KCl, 1 mM $MgCl_2$, 5 mM K^+ or Tris-oxalate, 0.5 mM Na_2ATP or Tris-ATP, 0.2 mM EGTA, 0.18 mM $CaCl_2$ (approx. 10 μM free Ca^{2+}), and approx. 0.5 μCi of $^{45}CaCl_2$ in a total volume of 500 μl. The assay was terminated by membrane

filtration of 400-μl aliquots and immediate rinsing of the filters with 15 ml of 0.25 M sucrose. The filters were dried and $^{45}Ca^{2+}$ quantitated by liquid scintillation counting. All assays included appropriate control tubes without ATP and these values were subtracted from total Ca^{2+} accumulation in the presence of ATP to give a value for energy-dependent Ca^{2+} transport. Tubes without protein were also included to measure binding of Ca^{2+} to the filters (routinely less than 5% of the total counts associated with the protein).

ATPase assays. ATP hydrolysis by deoxycholate-treated microsomes was measured by an isotopic assay method [24]. Standard incubations were performed in duplicate with constant shaking at 37°C. The assay was initiated by addition of 10—20 μg of protein to a standard incubation buffer identical to that used for Ca^{2+} transport studies except that tracer [γ-^{32}P]ATP was substituted for $^{45}CaCl_2$. Routinely, the reaction was terminated after 20 min by addition of 1% sodium dodecyl sulfate. Under these assay conditions, the reaction is linear with time for 30 min [23]. Therefore, results obtained at 20 min incubation reflect the initial rate of the reaction. The hydrolyzed ^{32}P was extracted in xylene/isobutanol and quantitated by liquid scintillation counting in toluene/omnifluor/ethanol. In all assays, appropriate tubes without protein were included to correct the nonenzymatic ATP hydrolysis. To measure (Ca^{2+} + Mg^{2+})-ATPase activity, assays were performed with and without Ca^{2+}. Values obtained in the absence of Ca^{2+} (basal ATPase activity) were subtracted from the total ATPase activity in the presence of Ca^{2+} to give a value for (Ca^{2+} + Mg^{2+})-ATPase activity.

Other assays. Protein was measured by the method of Lowry et al. [25] using bovine serum albumin as a standard. NADH-cytochrome *c* reductase activity was measured as previously described [19].

Calculations. Free Ca^{2+} concentrations in the incubation buffer were calculated from the EGTA/calcium ratios as described by Schatzmann [7]. Initial rates of Ca^{2+} transport were calculated from time curves by the method of Guggenheim [26].

Results

Determination of optimal conditions for microsomal preparation

Initial attempts to measure Ca^{2+} transport activity in adipocyte microsomes revealed that vesicles isolated from the S_1 by a 10 min centrifugation gave 7—8-fold higher transport rates than vesicles isolated by the standard 60-min centrifugation. The rapid centrifugation method did not merely isolate a fraction of vesicles enriched in Ca^{2+} transport, since recovery of endoplasmic reticulum (based on cytochrome *c* reductase activity) was similar to that obtained by the 60-min centrifugation.

Ca^{2+} transport in the rapidly prepared microsomes could be further enhanced by adding ATP and Mg^{2+} to the S_1 prior to centrifugation. The addition of 0.5 mM ATP plus 0.25 mM $MgCl_2$ or 5.0 mM ATP plus 2.5 mM $MgCl_2$ increased Ca^{2+} transport in the subsequently isolated microsomes by 23 and 37%, respectively (n = 5) compared to microsomes prepared rapidly in the absence of ATP and magnesium. The lower ATP/Mg^{2+} concentration did not significantly alter control values (Ca^{2+} associated with microsomal vesicles in

the absence of ATP) indicating that the ATP added to the S_1 was not present in the resuspended microsomal vesicles. Since the use of 5 mM ATP during microsomal preparation yielded slightly elevated control values, the lower ATP/Mg^{2+} concentrations were used in the standard microsomal isolation procedure. The presence of 0.5 mM ATP plus 0.25 mM $MgCl_2$ during a 60-min centrifugation of S_1 resulted in microsomal Ca^{2+} transport values only 27.8% of those obtained in comparable 10-min centrifugations (n = 4). Thus, both rapid isolation of microsomal vesicles from the cytosolic factors and the presence of ATP and Mg^{2+} during this step were necessary for maximum retention of Ca^{2+} transport activity.

The Ca^{2+} transport system was relatively stable in the isolated microsomal preparation. Transport activity was reduced by only 5% in microsomes stored at 4°C for an hour, and by less than 20% in microsomes stored at —70°C for six weeks.

Ca^{2+} transport activity of endoplasmic reticulum suspended in S_1 was compared to that of microsomes prepared from S_1 by the rapid method, assuming that 8.8% of the S_1 protein represented endoplasmic reticulum protein as previously reported [9]. Parallel assays revealed that 77 ± 9.6% of the Ca^{2+} transport activity present in the S_1 was retained by the isolated microsomal preparations (n = 6).

Localization of Ca^{2+} transport to endoplasmic reticulum vesicles

Rapidly prepared microsomal vesicles accumulated an average of 8.5 ± 0.8 nmol Ca^{2+}/mg protein in 1 min (n = 24) under standard incubation conditions. The addition of the Ca^{2+} ionophore A23187 at various times after the initiation of calcium uptake caused the rapid release of all the Ca^{2+} which was accumulated, resulting in values similar to the amount of calcium bound in the absence of ATP. These data indicate that the Ca^{2+} is accumulated intravesicularly rather than simply being bound to the membranes. To determine whether plasma membrane vesicles present in the microsomal fraction might contribute to this Ca^{2+} uptake, Ca^{2+} accumulation by highly enriched plasma membrane preparations was assayed under identical incubation conditions. The Ca^{2+} transport rate of six plasma membrane preparations was only 1.1 ± 0.09 nmol Ca^{2+}/mg per min, indicating that plasma membrane contamination could not account for a significant portion of Ca^{2+} transport by the microsomal fraction. The extent to which mitochondria contributed to microsomal Ca^{2+} uptake was ascertained by adding azide to the standard incubation medium. Addition of 10 mM sodium azide, a concentration which inhibits adipocyte mitochondrial calcium transport by 99% [9], reduced transport in the microsomal fraction by less than 10% (from 10.8 ± 0.6 to 10.1 ± 0.6 nmol Ca^{2+}/mg per min, n = 3). Thus, Ca^{2+} transport is localized to the endoplasmic reticulum vesicles of the microsomal fraction.

Time course and oxalate dependency of Ca^{2+} transport

The effect of various oxalate concentrations (0, 10 mM, and 20 mM) on the time course of Ca^{2+} transport was determined (Fig. 1). Ca^{2+} accumulation was not linear for the first minute of incubation under any of these conditions, but approached linearity after 1—2 min in the presence of 20 mM oxalate. This

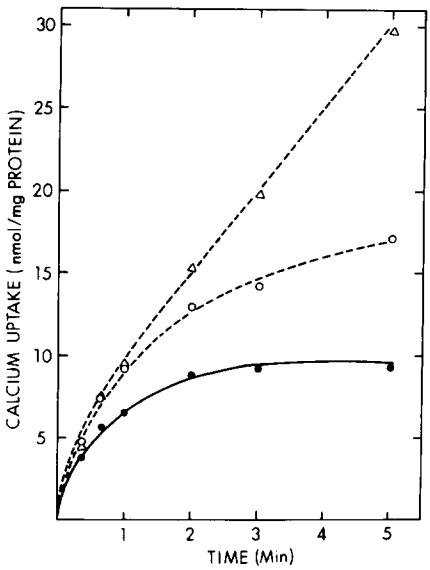

Fig. 1. Dependence of Ca^{2+} uptake on concentration of oxalate. Ca^{2+} uptake at 20 s to 5 min was determined using the standard assay system with no oxalate (●), 10 mM oxalate (○), or 20 mM oxalate (△). Each point represents the mean value obtained from three microsomal preparations, each assayed in duplicate.

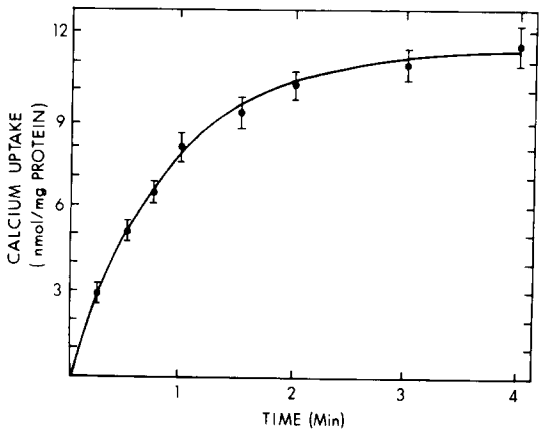

Fig. 2. Time course of Ca^{2+} uptake. Ca^{2+} uptake at 15 s to 4 min was determined using the standard assay system which contains 5 mM oxalate. Each point represents the mean value (±S.E.) obtained from six microsomal preparations, each assayed in duplicate. The exponential curve was generated by computer by an iterative nonlinear least-squares method using the equation $Y = A\ (1 - e^{-Kt})$ [52], where Y = uptake, A = asymptote, t = time, and K = rate constant.

linear phase of Ca^{2+} accumulation was maintained for at least 30 min in the presence of 20 mM oxalate, reaching values as high at 150 nmol Ca^{2+}/mg protein.

For subsequent studies of Ca^{2+} transport, it was desirable to use incubation conditions similar to those used to assay (Ca^{2+} + Mg^{2+})-ATPase activity [23,27]. Since oxalate concentrations greater than 5 mM interfered with ^{32}P extraction in the ATPase assay [24], 5 mM oxalate was used in the present studies for both (Ca^{2+} + Mg^{2+})-ATPase and Ca^{2+} transport assays. Under these conditions, the time course of Ca^{2+} uptake appeared to represent an exponential approach to a steady-state level, and a computer generated exponential curve fitted the experimental data extremely well (Fig. 2). Calculation of the initial rate of Ca^{2+} transport from these data by the method of Guggenheim (which assumes an exponential curve) gave a value of 18.5 nmol Ca^{2+}/mg per min.

The observed exponential time curve for Ca^{2+} uptake could have resulted from a decrease in Ca^{2+} transport rate during the incubation period. This was tested by pre-incubating vesicles for 3 min in the standard incubation buffer minus isotope. $^{45}CaCl_2$ was then added and Ca^{2+} accumulation measured after 0.5, 1.0, 1.5, 3.0, and 5.0 min of additional incubation. The Ca^{2+} transport values at these time points were within 15% of the values obtained in a parallel assay with no pre-incubation period (data not shown). This result suggests that

the transport of Ca^{2+} into microsomal vesicles remained relatively constant throughout the incubation period.

Further studies of Ca^{2+} transport were performed using a 1-min incubation period unless otherwise stated. At this time point, Ca^{2+} accumulation was a linear function of microsomal protein between protein concentrations of 20 and 100 μg/ml (data not shown).

ATP dependency of Ca^{2+} transport

Ca^{2+} transport required the presence of ATP. In the absence of ATP, a small amount of Ca^{2+} (1—1.5 nmol/mg protein) was associated with the vesicles, presumably due to passive binding of Ca^{2+} to the membranes and/or diffusion into the vesicles. This value was reached by 15—30 s and remained constant during further incubation.

Ca^{2+} transport was dose dependent and saturable as a function of ATP concentrations between 0.05 and 1.0 mM in the standard assay medium (data not shown). Analysis of the data ($n = 3$) by double reciprocal plots yielded a broken line with at least two components. In the ATP concentration range of 10—50 μM, the K_m was approx. 7 μM, whereas at higher ATP concentrations the apparent K_m was 100 μM. The nonlinear plot did not appear to be an artifact due to the nonlinearity of Ca^{2+} accumulation with time. This was ascertained by comparing time curves of transport at 0.01 mM, 0.1 mM, and 1.0 mM ATP. In each case, the Ca^{2+} accumulation at 1 min represented a constant percentage (62—69%) of that accumulated at steady state (3—5 min) and the shapes of the curves were identical. A Hill plot of the data was linear between ATP concentrations of 10—200 μM, with a slope (Hill coefficient) of 0.56.

Ca^{2+} and Mg^{2+} dependency of Ca^{2+} transport

Ca^{2+} transport required the presence of both Ca^{2+} and Mg^{2+}. Ca^{2+} accumulation was saturable, reaching a maximum at 1—3 μM free Ca^{2+} (data not shown). A double-reciprocal plot of the data indicated a K_m for free Ca^{2+} of 0.36 μM and a V of 11.0 nmol Ca^{2+}/mg per min. In one additional experiment, time curves of Ca^{2+} transport were obtained for Ca^{2+} concentrations between 0.3 and 10.0 μM, and the initial transport rate at each concentration of Ca^{2+} was calculated by the method of Guggenheim (not shown). The K_m for free Ca^{2+} obtained by using initial rate estimates of velocity was 0.42 μM, similar to the K_m obtained by measuring Ca^{2+} accumulation at 1 min. The free Ca^{2+} concentrations used in these studies were estimated from the Ca^{2+}/EGTA ratios in the incubation buffer as described by Schatzman [7]. A similar K_m for free Ca^{2+} of 0.67 μM was obtained by using free Ca^{2+} concentrations calculated by the method of Katz [28].

Ca^{2+} transport was dose dependent and saturable as a function of Mg^{2+} concentration (data not shown). Maximum rate of Ca^{2+} accumulation was reached at 0.5—2.0 mM Mg^{2+}; this rate was reduced by approx. 13% in the presence of 5 mM Mg^{2+}. Analysis of the data by double-reciprocal plots yielded a K_m for Mg^{2+} of 132 μM and a V of 10.6 nmol Ca^{2+}/mg per min.

Temperature dependency of Ca^{2+} transport

The rate of Ca^{2+} accumulation was highly dependent upon temperature

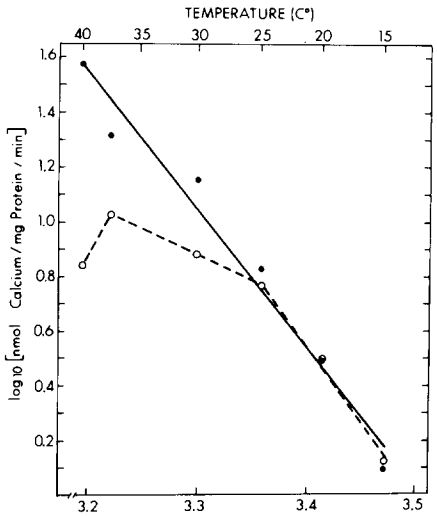

Fig. 3. Arrhenius plots of Ca^{2+} uptake. Ca^{2+} uptake was determined at temperatures of 15—40°C using the standard incubation system. The pH of the assay buffer was adjusted to 6.8 at each temperature. Each point designating net Ca^{2+} uptake at 1 min (○) represents the mean value obtained from a single microsomal preparation assayed in triplicate. Each point designating initial rate of Ca^{2+} uptake (●) represents the mean value calculated from time curves performed in triplicate at each temperature using the same preparation. The slope of the line depicting initial rate of calcium uptake was derived by linear regression and has a correlation coefficient of —0.987.

between 15—40°C. Time curves performed at various temperatures within this range revealed that calcium accumulation was linear for at least 1 min at 15°C and 20°C, became somewhat nonlinear at 25°C, and was increasingly nonlinear at higher temperatures (data not shown). Therefore, values of Ca^{2+} transport at 1-min time points from such studies gave nonlinear Arrhenius plots, but when initial rates of Ca^{2+} transport were calculated at each temperature, the resulting Arrhenius plot was linear (Fig. 3). The energy of activation and Q_{10} for initial rate of Ca^{2+} transport were 23.4 and 3.3 kcal/mol, respectively.

Comparison of Ca^{2+} transport and (Ca^{2+} + Mg^{2+})-ATPase activity

Ca^{2+} transport by microsomal vesicles was compared to the (Ca^{2+} + Mg^{2+})-ATPase activity of microsomal vesicles treated with deoxycholate. Deoxycholate-treated vesicles were required to accurately measure (Ca^{2+} + Mg^{2+})-ATPase activity. Deoxycholate treatment lowers the nonspecific (basal) ATPase activity and increases the (Ca^{2+} + Mg^{2+})-ATPase activity from 7% to 40% of the total ATPase activity [23,30]. Since detergent treatment renders membranes leaky to Ca^{2+} [31], untreated vesicles were used for the Ca^{2+} transport studies. The kinetics, energy of activation, and ionic requirements of microsomal Ca^{2+} transport were quite similar to those found for (Ca^{2+} + Mg^{2+})-ATPase activity in deoxycholate-treated microsomes [23,27,30]. These similarities are summarized in Table I. In addition, both processes were maximal at 1 mM Mg^{2+}, but inhibited by higher Mg^{2+} concentrations. At 5 mM Mg^{2+}, Ca^{2+} transport and (Ca^{2+} + Mg^{2+})-ATPase activity were reduced to 87 and 82%, respectively, of maximal values.

To further compare the ionic requirements of Ca^{2+} transport and (Ca^{2+} +

TABLE I

COMPARISON OF THE KINETICS OF CALCIUM TRANSPORT AND (Ca^{2+} + Mg^{2+})-ATPase ACTIVITY

Calcium transport was measured in untreated microsomes as described in Materials and Methods. Data for (Ca^{2+} + Mg^{2+})-ATPase activity in deoxycholate-treated microsomes were derived from Ref. 26, 27 and 30.

	Calcium transport (μM)	(Ca^{2+} + Mg^{2+})-ATPase activity (μM)
K_m for ATP (low concentration)	7	36
K_m for ATP (high concentration)	100	118
K_m for Ca^{2+}	0.4	0.6
K_m for Mg^{2+}	132	125
Energy of activation (in kcal/mol)	23.4	21.5

Mg^{2+})-ATPase, the monovalent cation specificity of the system was examined. Omission of K^+ lowered Ca^{2+} transport and (Ca^{2+} + Mg^{2+})-ATPase activity to 30.3 and 21.9%, respectively, of the values obtained in the presence of 100 mM K^+ (Table II). This effect was not the result of reduced ionic strength, since substitution of 100 mM Li^+ for K^+ gave similar low values. Other monovalent cations could partially substitute for K^+ (Table II). The order of effectiveness in supporting Ca^{2+} transport was $K^+ > Na^+ = NH_4^+ > Ca^{2+} > Li^+$. The same order of effectiveness was found for (Ca^{2+} + Mg^{2+})-ATPase activity, with the exception of Cs^+ which could not be tested since it interfered with the ^{32}P extraction step of the assay.

The effectiveness of potential inhibitors on Ca^{2+} transport and (Ca^{2+} + Mg^{2+})-ATPase activity is shown in Table III. Sodium azide had little effect on either process, whereas 50 μM *p*-chloromercuribenzene sulfonate completely inhibited both activities. A similar correlation between Ca^{2+} transport and (Ca^{2+} + Mg^{2+})-ATPase activity was revealed by the effect of two local anesthetics. Dibucaine at a concentration of 3 mM completely inhibited both processes, whereas 6 mM procaine lowered Ca^{2+} transport and (Ca^{2+} + Mg^{2+})-ATPase activity by only 10—20%.

TABLE II

EFFECT OF MONOVALENT CATIONS ON CALCIUM TRANPORT AND (Ca^{2+} + Mg^{2+})-ATPase ACTIVITY

Calcium transport was measured in untreated microsomes and (Ca^{2+} + Mg^{2+})-ATPase activity in deoxycholate-treated microsomes using the standard incubation medium minus potassium, and containing the Tris salts of ATP and oxalate. Monovalent cations at a concentration of 100 mM were added as indicated. Results are expressed as percents of the activity present with 100 mM KCl. Each value represents the mean (± S.E.) of three microsomal preparations.

Monovalent cation added to incubation medium	Ca^{2+} transport (%)	(Ca^{2+} + Mg^{2+})-ATPase activity (%)
KCl	100	100
NaCl	82.1 ± 3.7	76.9 ± 6.6
NH_4Cl	89.9 ± 4.7	72.2 ± 4.4
CsCl	63.4 ± 4.7	—
LiCl	37.5 ± 2.0	23.6 ± 2.3
None	30.3 ± 3.9	21.9 ± 3.5

TABLE III

EFFECT OF INHIBITORS ON CALCIUM TRANSPORT AND (Ca^{2+} + Mg^{2+})-ATPase ACTIVITY

Calcium transport was measured in untreated microsomes and (Ca^{2+} + Mg^{2+})-ATPase activity in deoxycholate-treated microsomes using the standard incubation medium. Results are expressed as percent of the activity present in the standard incubation medium without inhibitors. Each value represents the mean (± S.E.) of three microsomal preparations.

Inhibitor added to standard incubation medium	Ca^{2+} transport (%)	(Ca^{2+} + Mg^{2+})-ATPase activity (%)
None	100	100
NaN_3 (10 mM)	93.5 ± 1.8	105 ± 1.9
p-Chloromercuribenzene sulfonate (50 μM)	0.8 ± 0.5	0
Procaine (6 mM)	91.7 ± 5.5	80.5 ± 1.8
Dibucaine (3 mM)	0	0.4 ± 0.4

Discussion

The regulation of intracellular Ca^{2+} in noncontractile cells by endoplasmic reticulum represents a potentially critical factor in cellular metabolism. These studies have further characterized the previously described Ca^{2+} transport system of adipocyte endoplasmic reticulum [9] by utilizing a rapidly obtained microsomal preparation. The rapid method for preparing microsomes (endoplasmic reticulum) was necessary to study both Ca^{2+} transport and (Ca^{2+} + Mg^{2+})-ATPase activity. The rapidly prepared microsomes exhibited a 7—8-fold increase in Ca^{2+} transport rate compared to the standard microsomal preparation. Preliminary data (unpublished observations) indicate that the (Ca^{2+} + Mg^{2+})-ATPase activity is similarly enhanced in the rapidly prepared preparation. These data suggest that the lability of (Ca^{2+} + Mg^{2+})-ATPase activity is responsible for the loss of Ca^{2+} transport in the routine preparation; however, other factors such as increased Ca^{2+} permeability of the membrane may also play a role. Therefore, employing this preparation, we were able to investigate both Ca^{2+} transport and (Ca^{2+} + Mg^{2+})-ATPase activity at 37°C under conditions which reflect initial reaction rates.

The finding that Ca^{2+} accumulation by these microsomes was nonlinear in the presence of 5 mM oxalate was somewhat surprising since oxalate linearizes transport in many systems through precipitation of Ca^{2+} within the vesicles [32,33]. Several explanations for such nonlinearity might be proposed. One possibility, that the rate of Ca^{2+} transport into the vesicles decreased during the incubation period, can be rule out since uptake of $^{45}Ca^{2+}$ was at least 85% as rapid after 3 min of incubation as at the beginning of the assay. An alternate explanation, that the vesicles were leaky for Ca^{2+}, is unlikely because Ca^{2+} accumulation could be linearized by lowering the incubation temperature to 15 or 20°C. It seems most likely that the exponential time course of Ca^{2+} accumulation was due to increased passive diffusion of Ca^{2+} out of the vesicles as internal Ca^{2+} concentration increased. The steady-state approached after 3—4 min would thus reflect equal rates of Ca^{2+} uptake and efflux, similar to the rapid Ca^{2+} exchange at steady-state which occurs in sarcoplasmic reticulum vesicles [34,35]. The apparent failure of 5 mM oxalate to precipitate significant

amounts of accumulated Ca^{2+} could be explained if the rates of oxalate entry into vesicles and/or Ca^{2+} oxalate precipitation were slower at 37°C than the rate of Ca^{2+} accumulation. Consistent with this explanation, high oxalate concentration as well as low temperature linearized Ca^{2+}-accumulation by microsomes.

These studies of the Ca^{2+} transport system have revealed a striking similarity in the characteristics of Ca^{2+} uptake and $(Ca^{2+} + Mg^{2+})$-ATPase activity (Table I). Ca^{2+} transport in untreated microsomal vesicles resembled the $(Ca^{2+} + Mg^{2+})$-ATPase activity of deoxycholate-treated vesicles regarding optimum concentrations and K_m values for ATP, Mg^{2+}, and Ca^{2+}; energy of activation; dependence on monovalent cations; and degree of inhibition by *p*-chloromercuribenzene sulfonate, procaine, dibucaine, and 5 mM Mg^{2+}. The only apparent exception to the close relationship between transport and enzyme activity was the difference in K_m values at low ATP concentrations which suggests that the processes may become uncoupled at substrate concentration below 100 μM. The similarity of kinetic properties and ionic requirements for Ca^{2+} transport and $(Ca^{2+} + Mg^{2+})$-ATPase activity resembles that found in sarcoplasmic reticulum where the correlation is taken as evidence for a $(Ca^{2+} + Mg^{2+})$-ATPase mediated mechanism of Ca^{2+} uptake [34,36]. Therefore, Ca^{2+} transport in adipocyte endoplasmic reticulum appears to be linked to Ca^{2+}-stimulated ATP hydrolysis, analogous to the mechanism of transport in sarcoplasmic reticulum.

The Ca^{2+} transport systems of endoplasmic reticulum and sarcoplasmic reticulum may be further compared. Consideration of the stoichiometry of the system using initial rates of Ca^{2+} transport and the value for the initial rate of $(Ca^{2+} + Mg^{2+})$-ATPase activity of 13.7 nmol ATP/mg per min obtained under similar conditions with untreated microsomes [23,27] indicates that 1.4 Ca^{2+} molecules are transported per ATP hydrolyzed. Similar Ca^{2+}/ATP ratios of 1 : 1 or 2 : 1 are characteristic of Ca^{2+} transport in cardiac and skeletal sarcoplasmic reticulum [1,34,37]. The order of effectiveness of monovalent cations in supporting Ca^{2+} transport by adipocyte endoplasmic reticulum, as well as the inhibition by low concentrations of a sulfhydryl inhibitor (*p*-chloromercuribenzene sulfonate) is identical to that found in sarcoplasmic reticulum [32,38—40], providing further evidence for similar mechanisms of transport.

The major difference between sarcoplasmic reticulum and adipocyte endoplasmic reticulum is the 10—100-fold faster rate of Ca^{2+} transport by sarcoplasmic reticulum which probably reflects a higher concentration of $(Ca^{2+} + Mg^{2+})$-ATPase proteins in these membranes [41]. A second difference is the amount of Ca^{2+} which can be accumulated by the two types of vesicles. Calculations of the Ca^{2+} concentration within the endoplasmic reticulum vesicles indicate that adipocyte endoplasmic reticulum can accumulate a maximum of 0.7 mM Ca^{2+} (intravesicular concentration) in the absence of oxalate. In contrast, sarcoplasmic reticulum vesicles can accumulate Ca^{2+} to a concentration of 3—10 mM [42,43]. This difference might suggest that endoplasmic reticulum membranes are more permeable to internal Ca^{2+}. However, the rate of Ca^{2+} efflux at steady-state, presumably equivalent to the initial rate of Ca^{2+} uptake of 18.5 nmol/mg per min, is no higher than the rates of passive Ca^{2+} efflux reported for sarcoplasmic reticulum of 10—20 nmol/mg per min at 24°C

[44] and 30 nmol/mg per min at 37°C [45]. The greater capacity of sarcoplasmic reticulum for retention of Ca^{2+} might be related instead to a higher concentration of Ca^{2+}-binding proteins. Adipocyte endoplasmic reticulum has Ca^{2+} binding sites with an affinity similar to that of calsequestrin, the major Ca^{2+}-binding protein in sarcoplasmic reticulum [1,46], but the capacity of this binding site is almost 100× higher in sarcoplasmic reticulum. This suggests that a larger percent of the Ca^{2+} accumulated by sarcoplasmic reticulum is bound within the vesicles thus reducing the amount lost by passive diffusion.

The Ca^{2+} transport system of adipocyte endoplasmic reticulum appears to have potential physiological significance for regulation of intracellular Ca^{2+} distribution. The concentrations of ATP, Mg^{2+}, and K^+ required for maximum rate of Ca^{2+} accumulation are consistent with typical cellular levels [47,48], suggesting that these factors would not be rate limiting in vivo. The low K_m for Ca^{2+} (0.36—0.67 μM) and the concentration of Ca^{2+} eliciting maximum uptake rates (1—3 μM) are within the estimated range for cytoplasmic free Ca^{2+} of 0.1—1.0 μM [5]. Both the rate and capacity of the Ca^{2+} transport system seem adequate to allow the endoplasmic reticulum to rapidly alter cytoplasmic Ca^{2+} levels or to act as a buffer system in maintaining a constant cytoplasmic Ca^{2+} concentration.

The Ca^{2+} transport systems of both liver [49,50] and adipocyte endoplasmic reticulum, recently have been shown to be hormone sensitive. In the adipocyte system pretreatment of intact cells by insulin elicits a 30% stimulation of Ca^{2+} transport in subsequently obtained endoplasmic reticulum vesicles [29], whereas pretreatment with epinephrine produces a small, but significant, inhibition of transport [51]. Further study of the effect of hormones and potential regulatory agents on Ca^{2+} transport and (Ca^{2+} + Mg^{2+})-ATPase activity in adipocyte endoplasmic reticulum should provide insight into the mechanism of hormone action on this transport system, as well as the role of Ca^{2+} in hormone-mediated cellular events.

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