Properties of Passive Binding of Calcium to Endoplasmic Reticulum from Adipocytes*

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DAVID E. BRUNS,‡ JAY M. MCDONALD,§ AND LEONARD JARETT

From the Division of Laboratory Medicine, Departments of Pathology and Medicine, Washington University School of Medicine and Barnes Hospital, St. Louis, Missouri 63110

Calcium binding to isolated adipocyte microsomes enriched in endoplasmic reticulum has been characterized. Binding was concentration-dependent, saturable, and totally dissociable. Steady state was reached within 20 min at all calcium concentrations tested. Three apparent classes of binding sites were identified in kinetic and steady state studies using calcium concentrations from 1 μM to 10 mM. The affinity constants (and maximum binding capacities) as determined by computer analysis for the three classes were 2.1 × 10^4 M^-1 (0.25 nmol of calcium/mg of protein), 1.3 × 10^6 M^-1 (1.1 nmol/mg), and 1.3 × 10^9 M^-1 (35 nmol/mg). The dissociation rate constants for the high and intermediate affinity classes of sites were 1.6 × 10^-3 s^-1 and 2.0 × 10^-7 s^-1, respectively, and the association rate constant for the high affinity sites was 8 × 10^9 M^-1 s^-1. The affinity constant calculated from the rate constants was 5.0 × 10^6 M^-1 for the high affinity sites in agreement with the value obtained in studies at steady state.

The three classes of binding sites were specific for calcium. Magnesium was a noncompetitive inhibitor of calcium binding to all three classes of sites with a K_i of 9 to 12 mM. Calcium binding at 1 μM calcium was 50% inhibited by 18 μM La^3+, 600 μM Sr^2+, or 2.7 mM Ba^2+.

These data represent the first analysis of passive calcium binding to endoplasmic reticulum from nonmuscular cells and the first report of corresponding rate constants for either endoplasmic or sarcoplasmic reticulum. The characteristics of the binding are consistent with the properties of calcium transport by endoplasmic reticulum of adipocytes. The characteristics and specificity of the calcium binding constitute further evidence that endoplasmic reticulum plays an important role in cellular calcium homeostasis.

Calcium appears to play an important role in the control of metabolic processes (1) and has been suggested as a second messenger or effector for certain hormones including insulin (2, 3). Evaluation of these roles of calcium requires detailed investigation of the cellular homeostasis of the ion. Such studies are made difficult by the high degree of subcellular compartmentalization of calcium (4) which complicates the interpretation of studies with intact cells. Recent studies (5-8) in our laboratory have concentrated on the use of subcellular fractions of adipocytes to characterize cellular calcium metabolism in this hormonally responsive cell. Insulin has been found to increase calcium binding to plasma membranes (5), to increase the total calcium content of microsomes, and to increase the exchangeable calcium pool in mitochondria (6), indicating that insulin affects calcium metabolism in at least these subcellular organelles. It was concluded from these studies that calcium is not the direct second messenger for insulin, but probably plays an important role in the final effector system in the mechanism of insulin action.

While it is accepted that events at both the plasma membrane and mitochondria must be considered in studies of calcium metabolism (9), the possible role of the endoplasmic reticulum in calcium homeostasis of noncontractile cells has been largely ignored. Only recently have reports identified active calcium transport systems in endoplasmic reticulum vesicles of liver (10), kidney (11), and adipocytes (8). The properties of these transport systems have strongly suggested that the endoplasmic reticulum in these cells, like the sarcoplasmic reticulum in muscle, plays an important role in controlling cytosol calcium concentrations. In further support of this concept in adipocytes is the recent report of dense deposits of calcium in the endoplasmic reticulum of this cell demonstrated by x-ray microprobe analysis (12).

The present report is the first characterization of passive calcium binding to endoplasmic reticulum isolated from nonmuscular cells. Three distinct classes of binding sites were identified which had characteristics similar to those of calcium binding sites in sarcoplasmic reticulum. The properties of calcium binding to the highest affinity sites of the endoplasmic reticulum were consistent with this binding being an initial step in the active transport of calcium by this subcellular organelle (8). The data reported contribute to an understanding of the mechanism of active calcium transport by endoplasmic reticulum (13) and re-emphasize the importance of this organelle in cellular calcium homeostasis.

**Experimental Procedures**

*Materials*—Male Wistar rats, weighing 120 g, were purchased from National Laboratory Animal Co., O'Fallon, Mo. Collagenase (type I) from Clostridium histolyticum and bovine serum albumin...
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(Fraction V) were purchased from Sigma Chemical Co., St. Louis, Mo. Crude microsomal material used was free of insulin-like activity in bioassay (stimulation of glucose oxidation in fat cells) and immunossay. Omnifluor and "CaCl₂ (approximately 1 mCi/μmol) were purchased from New England Nuclear, Boston, Mass. All other materials were of reagent grade quality and obtained from standard sources. All reagents were prepared in water deionized by a mixed bed ion exchange resin (Culligan, Inc., Northbrook, Ill.) and filtered through a 0.25-μm nominal pore size filter (Ultipor, FTM Corp., Cortland, N.Y.). The calcium content of concentrated water specimens measured by atomic absorption was below the limits of detection (approximately 0.5 μM). Calcium determinations on concentrated biological samples revealed nonmeasurable levels in all except sucrose, which at the final concentrations used in the binding assay introduced approximately 0.3 μM calcium and these concentrations were incorporated into calculations of the data.

Multiple sample filtration manifolds and 0.45-μm pore size filters (type HAWP) were purchased from Millipore Corp., Bedford, Mass. Prior to use, filters were presoaked for 60 min in 0.25 M KCl and washed on the manifold with 10 ml of 0.25 M KCl.

Adipocyte Microsomal Fraction—Isolated fat cells were prepared by a modification of the method of Rodbell (14) using 0.5 mg of collagenase/ml of modified (15) Krebs-Ringer bicarbonate or phosphate buffer containing 11 mM Na-hexanoate, 13 mM calcium, and 30 mM of bovine serum albumin/ml. The microsomal fraction, highly enriched in endoplasmic reticulum, was prepared by a modification (15) of the method of McKee and Jarett (16). Isolated fat cells were homogenized in 0.25 M sucrose buffered to pH 7.4 with 10 mM Tris/HCl (Tris/sucrose). The homogenate was centrifuged at 20,000 × g for 15 min and the resulting supernatant centrifuged at 160,000 × g for 60 min to obtain the microosomal pellet which was resuspended in Tris/sucrose. EDTA was omitted from the originally described buffers (16) which did not affect the protein distribution or the purity of the fraction as assessed by measurements of succinic dehydrogenase, 5'-nucleotidase, and cytochrome c reductase using the enzyme assays described previously (7, 16). Microsomes employed in the calcium binding studies were used as soon as resuspended or were quickly frozen in small aliquots at −70°C and thawed immediately prior to use. Storage in this fashion did not affect the binding properties of the fraction.

Calcium Binding Assay—Calcium binding was assayed using a Millipore filtration technique previously validated for calcium binding to adipocyte plasma membranes by comparison to equilibrium dialysis (7). All studies were performed in the presence of 0.1 M KC1 to minimize nonspecific cation binding and Donnan effects and the pH was buffered to 7.0 at 24°C (except as indicated) using 25 mM Tris/HCl. The standard incubations were performed in polystyrene tubes in a total volume of 0.3 ml containing 0.3 to 0.8 μCi of "CaCl₂ and total calcium concentrations of 1 μM to 10 mM. The assay was initiated by the addition of 20 to 50 μg of microsomal protein in a volume of 15 to 20 μl; the tubes were incubated in a 24°C water bath with gentle shaking for 20 min. Bound calcium was separated from free calcium by filtration of 250-μl aliquots through 0.45-μm membrane filters. The filters were washed immediately with three 5-ml aliquots of 0.25 M sucrose with a total washing time of 15 s. The filters were dried in scintillation vials and the bound calcium determined by liquid scintillation counting using toluene/Omnifluor as the scintillant. Aliquots from matched tubes without protein were filtered, washed, dried, and counted to allow correction for nonspecific binding of calcium to the filters. Aliquots of buffer were dried on filter discs and counted under the same conditions to determine the specific activity of calcium.

Bound calcium demonstrated a linear dependence on added microsomal protein over a range of 0 to 50 μg of protein with 1, 26, or 250 μM calcium in the buffer. Preliminary experiments with Ca/EGTA buffers over a range of calculated (17) free calcium from 1 to 10 μM resulted in a mean apparent affinity constant for the high affinity sites of 5.3 (±1.7) × 10⁵ M⁻¹ (n = 4) which was similar to that obtained in the standard assay (2.1 × 10⁷ M⁻¹) over the same range of calcium concentrations. Previous studies of calcium transport by adipocyte endoplasmic reticulum showed a similar agreement between calcium transported and equilibrated calcium (Culligan, Inc., Northbrook, Ill.) whereas, in this case, EDTA buffers (8) and EGTA buffers were not employed in further studies because of difficulties with the use of the buffer under the conditions of varying pH, temperature, Mg²⁺, Sr²⁺, Ba²⁺, and La³⁺ concentrations investigated in this study.

Protein Determination—Protein was determined by the method of Lowry et al. (18) using bovine serum albumin as standard.

Analysis of Data—Linear regressions were performed by computer least squares fit of the data. First order dissociation rate constants (k) were obtained using the SAAM program (19) with sequential approximation (Culligan, Inc., Bedford, Mass.) and least squares fitting on an IBM 360 computer. The time curves of dissociation exhibited an extremely rapid phase which could not be adequately evaluated and two slower phases. The slower phases fitted with a biexponential function of the form

\[ R(t) = D_1 e^{-k_1 t} + D_2 e^{-k_2 t} \]

where \( B_1 \) = amount of calcium bound at time t, \( D_1 \) and \( D_2 \) = total amount of calcium dissociated during the first and second measurable phases, and \( k_1 \) and \( k_2 \) the corresponding dissociation rate constants. The SAAM program was used to determine values for the constants in Equation 1 from experimental values of \( B \) and \( t \) at \( t > 0 \). The amount of calcium dissociated during the extremely rapid phase was calculated as \( B_1 - (D_1 + D_2) \). The association rate constant \( (k_a) \) was derived for the high affinity sites with the aid of a programmable calculator using iterative nonlinear least squares analysis (20) based on the asymptotic inverse exponential equation,

\[ B(t) = B_e \cdot e^{-k_e t} + D_e \cdot e^{-k_d t} \]

where \( B_e \) = amount of calcium bound at time t, \( D_e \) and \( D_d \) = total amount of calcium added, and \( k_e \) and \( k_d \) the corresponding dissociation rate constants. The SAAM program was used to determine values for the constants in Equation 1 from experimental values of \( B \) and \( t \) at \( t > 0 \). The amount of calcium dissociated during the extremely rapid phase was calculated as \( B_e - (D_e + D_d) \). The association rate constant \( (k_a) \) was derived for the high affinity sites with the aid of a programmable calculator using iterative nonlinear least squares analysis (20) based on the asymptotic inverse exponential equation,

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RESULTS

Time Course of Calcium Binding—At 1 μM "Ca²⁺ (the lowest concentration used in these studies) an approach to steady state was observed over 20 min (Fig. 1). At higher calcium concentrations (2, 25, and 500 μM), binding was progressively more rapid, reaching apparent equilibrium within 15 s at 500 μM, and remained constant for 1 h (not shown). A 20-min incubation time was employed for standard assays.

Determination of Binding Constants—Calcium binding to adipocyte microsomes was a concentration-dependent saturating...
tuble process reaching a plateau at 5 to 7 mM calcium (inset Fig. 2). The Hill coefficient was 0.67 for the data shown. Three apparent orders of binding sites were present when steady state data were analyzed by Scatchard (22) analysis (Fig. 2). The points illustrated on the high, intermediate, and low affinity portions of the Scatchard plot in Fig. 2 correspond, respectively, to calcium concentrations of 1 to 11 μM, 16 to 100 μM, and 250 to 1000 μM. Estimated affinity constants (K_a) and maximum binding capacities (B_max) obtained directly from slopes and extrapolated intercepts of Scatchard plots were quite precise showing standard errors of 10, 20, and 25% for high, intermediate, and low affinity classes of sites, respectively, in four experiments with three microsomal preparations. Binding constants obtained in this manner are not accurate when multiple classes of sites are present (23). Consequently, constants were estimated using the computer-aided techniques (Table I). Three classes of sites were necessary to obtain good fit of the data. The bound calcium calculated from the best fit curve agreed with the experimentally determined values within the experimental error of the assays at all calcium concentrations. In contrast, the computer-generated best fit curve assuming only two classes of sites showed errors of over 50% in the estimated bound calcium.

Dissociation of Bound Calcium - The binding of "Ca" at 26 μM was allowed to reach steady state and dissociation was studied by the addition of either excess calcium (1.7 to 250 mM) or EGTA (250 μM). The resulting time curves of dissociation were identical in both cases, indicating that the curvilinear Scatchard plot was not attributable to negative cooperativity. The dissociation was triphasic (Fig. 3) consistent with dissociation from the three classes of binding sites.

The rapid, intermediate, and slow phases of dissociation appeared to reflect dissociation of calcium from the low, intermediate, and high affinity classes of sites, respectively. When binding was carried out at an initial calcium concentration of 500 μM, 60 to 62% of the bound calcium was dissociated during the rapid phase. This was consistent with the amount of calcium bound to the low affinity sites at 500 μM calcium as determined from binding constants obtained at steady state (63%). This would indicate that the low affinity sites account for the rapid phase of dissociation. Similarly, when the initial Ca^2+ concentration was 1.2 μM, with approximately 70% of the binding to the high affinity sites, 70% of the dissociation was accounted for by the slow phase of dissociation. In experiments at 1.2, 26, and 500 μM calcium, the amounts of calcium association were fitted with a model for three noninteracting classes of binding sites to determine the affinity constant (K_a) and maximum binding capacity (B_max) of each site as described under "Experimental Procedures." The free energy change (ΔF) was derived from the relation ΔF = RT ln K_a. Association (k_on) and dissociation (k_off) rate constants were determined as described under "Experimental Procedures" using data from kinetic studies at 24°C performed as described in the legends of Figs. 1 and 3, respectively. The affinity constant from kinetic studies, K_a, was derived from the relation K_a = k_on/k_off. Reliable estimates of the kinetic parameters indicated by N.D. could not be obtained by the present analytical techniques because of the rapidity of the processes.

<table>
<thead>
<tr>
<th>Class</th>
<th>K_a (M^-1)</th>
<th>B_max (nmol/mg)</th>
<th>k_on (s^-1)</th>
<th>k_off (s^-1)</th>
<th>K_a (M^-1)</th>
<th>ΔF (kcal/mol)</th>
</tr>
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<tr>
<td>High affinity</td>
<td>3.1 x 10^4</td>
<td>0.28</td>
<td>800</td>
<td>1.6 x 10^5</td>
<td>5.0 x 10^4</td>
<td>7.3</td>
</tr>
<tr>
<td>Intermediate</td>
<td>1.3 x 10^4</td>
<td>1.1</td>
<td>N.D.</td>
<td>0.02</td>
<td>5.0 x 10^5</td>
<td>5.7</td>
</tr>
<tr>
<td>Low affinity</td>
<td>1.3 x 10^3</td>
<td>35</td>
<td>N.D.</td>
<td>N.D.</td>
<td>2.9</td>
<td></td>
</tr>
</tbody>
</table>

FIG. 2. Scatchard plot of calcium binding to adipocyte microsomes. The incubation medium contained 0.1 mM KCl, 0.3 to 0.9 μCi of "CaCl", and 25 mM Tris/HCl, pH 7.0. Total calcium concentration was varied between 1 μM and 1 mM. Incubation was performed at 24°C for 20 min. Each point represents the mean of triplicate determinations. Inset, dependence of calcium bound on calcium concentrations. Conditions were the same as above and calcium concentrations up to 10 mM are shown.

FIG. 3. Dissociation of "Ca" bound at 26 μM calcium. Calcium binding was carried out using the standard assay medium containing 26 μM calcium. Time zero represents the time of addition of 20 mM CaCl. At each time point, bound "Ca" was determined by filtration of 250-μl aliquots of the total assay medium. Each point represents the mean of two separate determinations using a single preparation of microsomes. The interrupted lines with the rate constants (k_on and k_off) shown represent the two exponential terms in the equation of the best fit curve.
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Kinetics of Binding and Dissociation. Data from the time curves of binding and of dissociation were analyzed with the aid of a computer modeling program (19) as described under "Experimental Procedures." Dissociation rate constants (Table I) were obtained for both the high and intermediate affinity sites using data obtained at 26 μM initial calcium (Fig. 3); the lack of obtainable data points in the rapid phase of dissociation prevented determination of a rate constant for the low affinity sites. The dissociation rate constant for the slowest phase (high affinity sites) was also estimated from studies at 1.2 μM initial calcium. The value obtained was 1.6 ± 0.2 × 10⁻³ s⁻¹ (n = 3) which was the same as the value found at 26 μM initial calcium, indicating that the dissociation rate constant was not a function of the initial occupancy of the binding sites.

The time curves of calcium binding at 1.0 and 1.2 μM calcium (Fig. 1) could be modeled as a single exponential approach to steady state plus an "instantaneous" phase. The major portion of binding occurred during the slow exponential phase, consistent with the amount of binding to the high affinity sites at this concentration of calcium. The association rate constant for this phase was 8 × 10⁶ M⁻¹ s⁻¹ (Table I). Using this association rate constant and the dissociation rate constant for the high affinity sites gave a Kₐ of 5 × 10⁻⁸ M⁻¹ which was in close agreement with the value of 2.1 × 10⁻⁸ M⁻¹ found for the high affinity sites in steady state studies.

pH Dependence of Calcium Binding. The pH dependence of calcium binding was studied at 1 and 26 μM calcium (Fig. 4). A relative peak of binding was seen at pH 7.0 with 1 μM calcium. This peak is similar to the pH 7.2 optimum reported by Cohen and Selinger (24) for calcium binding by muscle microsomes. At 26 μM calcium, with more than 60% of the bound calcium associated with the intermediate and low affinity sites, the binding increased progressively with increase of pH and the relative peak at pH 7.0 was obscured.

Temperature Dependence of Calcium Binding. Calcium binding at temperatures from 4-45°C using 26 μM calcium indicated 30% less binding at 4°C than at the other temperatures tested. The amounts of calcium bound at 24, 30, 37, and 45°C were all similar to each other with overlap of the standard errors of the means. A suggestion of a small peak in binding at 30°C was investigated by a calcium binding study at 30°C using 16 μM calcium concentrations and the same preparation of microsomes used in Fig. 2. The Scatchard plot of the binding data obtained at 30°C revealed a curve essentially identical with Fig. 2 obtained at 24°C with negligible changes in the affinity constants and maximum binding capacities.

Effects of Other Cations upon Calcium Binding. All three classes of calcium binding sites demonstrated a high relative specificity for calcium. Magnesium was a noncompetitive inhibitor of calcium binding to all three classes of sites (illustrated in Fig. 5 for the high and intermediate affinity sites) with a Kᵢ of 9 to 12 mM for all three classes of sites as determined by Dixon plots (25). Barium and strontium concentrations of 2.7 and 0.6 mM, respectively, were required for 50% inhibition of the binding of 1 μM calcium, reflecting binding primarily to the high affinity sites. The inhibition of calcium binding by Sr²⁺ was similar to that found for sarcoplasmic reticulum by Meissner (26) who reported 60% inhibition at 0.8 mM Sr²⁺ (using cholate-extracted vesicles). At calcium concentrations reflecting binding to the intermediate and low affinity sites, equimolar concentrations of Ba²⁺ or Sr²⁺ produced only a 2 to 19% inhibition of calcium binding.

Lanthanum was a noncompetitive inhibitor of calcium binding. A La³⁺ concentration of 18 μM produced 50% inhibition of binding of 1 μM Ca²⁺. This is essentially identical with the inhibition of calcium-specific binding at 1 μM calcium reported for sarcoplasmic reticulum (27) and adipocyte plasma membranes (7). The similarity in inhibition of calcium binding by lanthanides in different binding systems suggests that the effect of these ions on the calcium binding is nonspecific and related to their high charge density (27).

Effects of Other Agents on Calcium Binding. Ruthenium red had no effect on calcium binding at a concentration of 5 μM which was found in this laboratory to totally block calcium binding at 1 μM calcium and reflected the binding associated with the high affinity sites. At 26 μM calcium, with more than 60% of the bound calcium associated with the intermediate and low affinity sites, the binding increased progressively with increase of pH and the relative peak at pH 7.0 was obscured.

Fig. 4. Dependence of calcium binding on pH. Assays were performed using the standard conditions except that the pH was varied as shown. A, at 1 μM calcium. Each point represents the mean of two experiments, each performed in triplicate. B, at 25 μM calcium. Each point represents the mean of three experiments, each performed in triplicate.
transport (8) and high affinity binding by mitochondria isolated from adipocytes. This indicates that the calcium binding cannot be attributed to mitochondrial contamination of the microsomal fraction. Procaine/HCl at 10 mM inhibited calcium binding by 18 to 19% at calcium concentrations reflecting binding to all three classes of sites. Procaine/HCl has been found to produce a similar 10 to 20% inhibition of the calcium transport by adipocyte endoplasmic reticulum at this concentration of the anesthetic.

**DISCUSSION**

This report characterizes calcium binding to adipocyte microsomes. Both equilibrium and kinetic studies demonstrated three classes of binding sites. Binding was rapid, totally dissociable, and specific for calcium. The K_s for calcium binding to the high affinity sites was calculated to be 2 to 5*10^-8 M which corresponds to the estimated level of calcium in the cytosol of a variety of cells (4).

The three apparent orders of calcium binding sites observed by Scatchard analysis appear to represent independent sites and not negatively cooperative binding. This is supported by several findings. The dissociation of bound calcium demonstrated three kinetic components and the dissociation rate constant found for the high affinity sites was independent of the initial occupancy of the binding sites. The amount of calcium associated with each of the three phases of dissociation could be predicted from the steady state binding constants which were derived assuming no cooperativity. The high affinity constant derived from kinetic analysis assuming independent classes of sites agreed closely with the value found in steady state studies. Most importantly, dissociation was identical when studied by addition of either excess EGTA or calcium. Finally, the high affinity sites appear to exhibit a pH optimum which is not shared by the other sites.

Characterizations of microsomal calcium binding have been performed previously only in skeletal muscle and the binding constants derived have been based solely on studies at steady state. The high affinity constants reported for muscle microsomes have ranged from 2 to 10*10^-8 M^-1 (26-28). These values are comparable to the value of 2 to 5*10^-8 M^-1 found in the present study with or without EGTA buffers from both steady state and kinetic analyses. Another class of calcium-specific binding sites has been reported for muscle microsomes (27), with an affinity constant of 3*10^-9 M^-1 (24) and the affinity constant of 1.3*10^-9 M^-1 found for the intermediate affinity sites of adipocyte microsomes. These comparative data reflect a striking qualitative similarity between the calcium binding properties of sarcoplasmic reticulum and adipocyte endoplasmic reticulum. In contrast to these similarities of the affinity constants for the high and intermediate sites, the maximum binding capacities of each of these classes are 100-fold higher in muscle microsomes (24, 26-28) indicating a marked quantitative difference between the two systems.

In contrast to the findings for the higher affinity sites the number of low affinity sites in adipocyte endoplasmic reticulum may be comparable to the number in sarcoplasmic reticulum. Our previous studies of calcium binding to plasma membranes compared the Millipore technique to equilibrium dialysis (7). For binding with K_s = 10^4 M^-1, the two techniques were essentially identical, whereas for binding with K_s = 10^9 M^-1 the B_max was 2.5 times greater by dialysis than by the filtration technique. Both methods yielded the same K_s. Thus the maximum binding capacity of the low affinity class of sites is underestimated by the Millipore technique and is probably >90 nmol/mg of protein rather than the value of 35 nmol/mg derived using the filtration technique. This is relatively close to the amount of low affinity calcium binding to sarcoplasmic reticulum which is on the order of 100 nmol/mg (13). The similarity of low affinity binding to sarcoplasmic reticulum and adipocyte endoplasmic reticulum suggests that this binding is not directly related to the rate of calcium transport since the transport rates are markedly different in the two systems.

The properties of the high affinity calcium binding are consistent with this binding being an early step in calcium transport by the adipocyte endoplasmic reticulum. The dissociation constants for the high affinity sites measured with and without Ca/EGTA buffers were 2 and 5 *10^-7 M, respectively, in excellent agreement with the values of 2 and 4 *10^-7 M for the K_s of the transport system measured under these same two conditions. Both the passive binding to the high affinity sites and the velocity of calcium transport exhibited a pH optimum of 7.0. Finally, procaine/HCl produced a similar inhibition of both calcium binding and transport in the adipocyte endoplasmic reticulum. These similarities strongly suggest that high affinity binding represents binding of calcium to the transport sites.

The maximum binding capacity of the high affinity class of sites would appear to reflect the number of calcium transport sites in the adipocyte endoplasmic reticulum. In sarcoplasmic reticulum the amount of high affinity calcium binding is approximately 10 to 14 nmol of calcium/mg of membrane protein (26-28). This can be entirely accounted for by the binding of 2 mol of calcium/mol of the calcium pump protein (26) which constitutes between 5 and 7 nmol/mg of membrane protein (13). By comparison, high affinity calcium binding to the adipocyte endoplasmic reticulum was only 0.28 nmol of calcium/mg of protein, which suggests that the molar concentration of pump protein in this system would be almost 2 orders of magnitude less than in sarcoplasmic reticulum if the same conversion factor is valid. Similarly, the maximum velocity of calcium transport by the adipocyte endoplasmic reticulum was two orders of magnitude less than that reported for sarcoplasmic reticulum (8). This implies that the calcium transport proteins in muscle and adipocyte reticulum operate at similar rates, at least in vitro. This interesting conclusion will require further investigation.

The small effect of procaine/HCl on calcium binding is of considerable interest since procaine has been used to study the hypothesis that calcium is a second messenger for insulin (3). Procaine has been reported to mimic several of insulin's actions on the intact adipocyte (29-31) without alteration of cAMP concentration. The insulin-like properties of the local anesthetics have been attributed to their ability to raise cytosol calcium concentrations (29), supposedly by decreasing calcium binding to plasma membranes (32, 33). Klascbahl et al. (3) have characterized calcium binding to fat cell ghosts (which are plasma membrane sacs containing mitochondria, endoplasmic reticulum, nuclei, and cytosol), reporting two classes of binding sites with affinity constants an order of magnitude higher than those of the high and intermediate affinity classes in the present report and 2 orders of magnitude greater than those of isolated adipocyte plasma membranes (7). In the fat cell ghost system, procaine (0.1 mM), like insulin (100 micromolar/ml), decreased the binding of 5 *10^-7 M calcium by approximately 30%. This was attributed to decreased calcium binding to the plasma membranes or endoplasmic reticulum, or both.
In contrast, studies with highly enriched subcellular fractions of adipocytes have revealed that even 10 mM procaine decreased calcium binding to endoplasmic reticulum by less than 20% when added directly to the membranes and had no effect on calcium binding to the isolated plasma membranes (7). Furthermore, the maximum binding capacities reported for the ghosts were at least 3 to 7 times those found for plasma membranes (7) and 40 to 50 times those for the endoplasmic reticulum. Thus, the findings with isolated subcellular fractions do not support the suggestion of Kissebah et al. that the effects of insulin and procaine on calcium binding to fat cell ghosts are the result of a decrease in passive binding to the plasma membranes and endoplasmic reticulum.

This report re-emphasizes the importance of studying the calcium metabolism and interactions with isolated subcellular organelles in order to understand the complex mechanisms of cellular calcium homeostasis. The present study on calcium binding coupled with the data on the calcium transport by the adipocyte endoplasmic reticulum (9) makes it important to consider the role of this organelle as well as mitochondria in the maintenance of calcium homeostasis.

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