Ability of insulin to increase calcium binding by adipocyte plasma membranes

(atomic absorption/magnesium/equilibrium analysis)

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ABSTRACT Calcium specifically binds to adipocyte plasma membranes, demonstrating two classes of binding sites having affinity constants of 4.5 × 10⁴ M⁻¹ and 2.0 × 10⁵ M⁻¹. Insulin (100 micromolars/ml) added directly to the isolated plasma membranes caused no alteration in calcium binding, whereas incubation of the adipocytes with 100 micromolars/ml of insulin resulted in a 25.0 ± 1.6% increase in calcium binding to the subsequently isolated plasma membranes. The increase in calcium binding produced by insulin resulted from an increase in the maximum binding capacities of both classes of binding sites without alteration in their affinity constants. Additionally, a second pool of calcium in adipocyte plasma membranes has been identified by atomic absorption analysis; it was more than two times larger than the maximum binding capacity of the calcium binding system. This pool of calcium was stable, did not participate in the ⁴⁵Ca²⁺ exchange, and was unaltered by insulin treatment. A similar stable pool of magnesium exists in plasma membranes and was also unaffected by insulin treatment. The increased capacity of the isolated plasma membranes to bind calcium after insulin treatment of the cells may represent an important bioregulating mechanism and supports the concept that calcium may play an important role in the effector system for insulin.

The metabolic effects of insulin are thought to be produced by the interaction of insulin with its receptors on the plasma membranes (1, 2). This concept necessitates the involvement of a second messenger or an effector system to explain the mechanism of the complex cellular pleiotropic response to insulin. Considerable evidence suggests that adenine 3′,5′-cyclic monophosphate (cyclic AMP) does not fulfill the criteria of a second messenger for insulin in adipocytes (3, 4) and other cells (5, 6). Alternatively, the divalent cations, calcium and magnesium, have been proposed as possible second messengers for insulin (7–10), with a large body of indirect evidence to support this theory. Krah has reported that insulin causes an increased intracellular magnesium concentration in rat hemiuteri (11) and adipose tissue (12) and that calcium and magnesium were necessary for maximum insulin stimulation of protein synthesis (12). Agents which promote an increased concentration of intracellular calcium, such as ouabain, procaine, lanthanum, and calcium ionophores, have been reported to mimic the antilipolytic action of insulin (9, 13, 14). Insulin was found to alter efflux of ⁴⁵Ca²⁺ from preloaded fat pads and adipocytes (8, 10). Numerous intracellular enzymes have been reported to be both insulin and calcium sensitive (15–18). Changes in calcium concentrations have been shown to affect pyruvate dehydrogenase [pyruvate-lipoate oxidoreductase (decarboxylating and acceptor-acetylating), EC 1.2.4.1] (16), triglyceride lipase (triacylglycerol acyl-hydrolase, EC 3.1.1.3) (17, 19), adenylate cyclase [ATP pyrophosphate-lyase (cycling), EC 4.6.1.1] (20), 3′,5′-cyclic AMP phosphodiesterase (3′,5′-cyclic AMP 5′-nucleotidohydrolase, EC 3.1.4.17) (21), and glycogen synthase (UDP glucose:glycogen 4-α-glucosyltransferase, EC 2.4.1.11) (18) isolated from adipocytes. Thus, small changes of calcium concentration at critical intracellular loci could have significant metabolic impact.

The evaluation of cellular calcium distribution and regulation is difficult because intracellular calcium is highly compartmentalized with estimated concentrations ranging from 0.1 to 10 μM in the cytosol to 1–20 mM in the mitochondria (22) and microsomes (23). Plasma membranes contain an additional, potentially important calcium pool and demonstrate a high transmembrane calcium gradient (22). Since direct cytosol calcium measurements in hormone-sensitive cells are not feasible with present technology, isolated subcellular fractions commonly have been used to evaluate the control of subcellular calcium distribution. Such studies have yielded considerable insight into the mechanism of intracellular calcium homeostasis.

The adipocyte provides an ideal cell for analysis of the influence hormones may have upon the control of intracellular calcium. The isolated adipocyte demonstrates numerous well-characterized, measurable, hormone-sensitive processes (for review see ref. 24), can be obtained in a homogeneous cell suspension (25), and can be fractionated into highly purified and characterized subcellular fractions (26). Calcium binding to purified adipocyte plasma membranes has been characterized previously (27, *). The present study analyzes the effect of insulin upon calcium binding to adipocyte plasma membranes, showing that incubation of adipocytes with insulin significantly increases calcium binding to isolated plasma membranes. In addition, a nonexchangeable or stable pool of calcium was identified by atomic absorption and was unaltered by insulin.

EXPERIMENTAL PROCEDURES

Materials. Male Wistar rats (125 g) were obtained from National Laboratory Animal Co., O’Fallon, Mo. Chemicals were obtained from the following sources: ⁴⁵CaCl₂ (approximately 1.0 mCi/μmol), New England Nuclear, Boston, Mass.; bovine-serum albumin (fraction V) and collagenase (type I from Clostridium histolyticum), Sigma Chemical Co., St. Louis, Mo.; and EDTA and lanthanum oxide, J. T. Baker Chemical Co., Phillipsburg, N.J. The bovine-serum albumin contained no insulin-like activity as determined by the lack of stimulation of glucose oxidation in adipocytes. Porcine insulin (lot PJ5682) was a gift from Dr. M. Root, Eli Lilly. All other reagents were obtained from standard sources. A multiple chambered filtering manifold (no. 3025) and 0.45 μm (HAWP) filters were purchased from Millipore Corp., Bedford, Mass. All reagents were prepared in deionized and filtered water as outlined previously*. Toluene–Omnifluor (New England Nuclear, Boston, * J. M. McDonald, D. E. Bruns, and L. Jarett, submitted for publication.

Abbreviations: Bₘₐₓ, maximum binding capacity; Kₐ, affinity constant.
Table 1. Insulin increases calcium binding

| Calcium, μM | Control | Increase after insulin treatment | % Increase after insulin treatment | P <  
|------------|---------|----------------------------------|-----------------------------------|--------
| 1          | 0.052 ± 0.009 | 0.011 ± 0.002 | 21.2 ± 3.8 | 0.001 |
| 26         | 0.56 ± 0.05 | 0.15 ± 0.04 | 26.7 ± 7.3 | 0.05  |
| 252        | 2.58 ± 0.25 | 0.74 ± 0.22 | 28.7 ± 8.6 | 0.025 |
| 502        | 5.17 ± 0.53 | 1.1 ± 0.5  | 21.3 ± 9.7 | 0.05  |
| 1002       | 6.30 ± 0.71 | 1.7 ± 0.44 | 27.0 ± 7.0 | 0.005 |
| Total      | 25.0 ± 1.6  |                       | 25.0 ± 1.6  | 0.001 |

Plasma membranes were prepared in parallel from insulin-treated and control cells as outlined in Experimental Procedures. The calcium binding assays were performed by Millipore filtration in incubation media containing 25 mM Tris-HCl, pH 7.0, 0.1 M KCl, and CaCl₂ concentrations ranging from 1 to 1002 μM (0.3-0.8 μCi of ⁴⁵CaCl₂). The incubation temperature was 24°C and the standard incubation time was 10 min. Each calcium binding assay was performed in triplicate. A total of 10 paired preparations was analyzed. The P values were obtained using the paired t test. The calcium binding in controls and the absolute increase and percent increase in calcium binding after insulin treatment are indicated ± SEM.

Mass.) was the scintillant used. Atomic absorption analysis was performed on a Perkin Elmer model 303 atomic absorption spectrophotometer.

Preparation of Plasma Membranes. Isolated adipocytes were prepared by the method of Rodbell (25) with use of 0.5 mg of collagenase per ml of modified Krebs–Ringer phosphate buffer, at 37°C, pH 7.4, containing 1.1 mM D-glucose, 3 mg of bovine-serum albumin/ml, and 1.3 mM calcium. The cells were washed three times, resuspended, and preincubated at 37°C in two equal aliquots for 10 min in modified Krebs–Ringer bicarbonate buffer, pH 7.4, containing 1.1 mM D-glucose, 3 mg of bovine-serum albumin per ml, and 1.3 mM calcium equilibrated with 95% O₂. The stock porcine insulin was dissolved in 0.1 M HCl and diluted with 0.1% bovine-serum albumin to the desired concentration.

After preincubation, 1 aliquot of cells received 100 micromolars of insulin per ml diluted in 0.1% bovine-serum albumin (insulin cells) and the other (control cells) received an equivalent amount of 0.1% bovine-serum albumin. These were incubated for 10 min at 37°C. Both aliquots were then washed one time and homogenized at 4°C in 0.25 M sucrose buffered with 10 mM Tris-HCl, pH 7.4, again adding 100 micromolars of insulin per ml or 0.1% bovine-serum albumin to the appropriate cells at both steps. The plasma membranes were isolated from both sets of cells in a parallel fashion by a modification (28) of the method of McKeel and Jarett (26), except that EDTA was omitted throughout the procedure. The omission of EDTA did not alter protein distribution or purity of the isolated fractions*. Proteins were determined by the method of Lowry et al. (29).

Calcium Binding Assay. The equilibrium calcium binding assays were performed by Millipore filtration, utilizing 0.45 μm filters as outlined previously*. The calcium content of concentrated samples of all reagents was measured by atomic absorption spectrophotometry, revealing a consistent 0.3 μM calcium contamination in the buffer, and all calculations included this calcium. The standard assay medium contained 25 mM Tris-HCl at pH 7.0, 0.1 M KCl, and 1.0 mM calcium with 0.3-0.8 μCi of ⁴⁵CaCl₂ in a total volume of 0.3 ml. The standard incubation was 10 min at 24°C. Assays were initiated by the addition of 20-35 μg of plasma membrane protein and terminated by applying 0.25 ml aliquots to the filters and immediately washing three times with 5 ml aliquots of 0.25 M sucrose.

Calcium and Magnesium Determinations by Atomic Absorption. Aliquots (1.0 ml) of isolated plasma membranes (1.0-1.75 mg of protein per ml) suspended in 0.25 M sucrose buffered with 10 mM Tris-HCl at pH 7.4 were solubilized with 0.1 ml 10 M NaOH and heated to 75°C in a water bath for approximately 5 min. Following this, 1 ml of a solution containing 0.01 M lanthanum oxide, 0.025 M HCl, 0.02 M EDTA, and 0.08 M NaOH was added. Samples were again heated to 75°C for approximately 5 min and then analyzed by atomic absorption spectrophotometry. Calcium and magnesium standards were prepared from CaCO₃ (National Bureau of Standards standard reference material no. 915) and a reference magnesium acetate standard (Ultrex, J. T. Baker Chemical Co.). The validity of this technique was established (unpublished).

RESULTS

Characteristics of calcium binding to adipocyte plasma membrane

The kinetic and equilibrium characteristics of calcium binding to isolated plasma membranes from adipocytes have been described (27, *). Calcium binding was specific, saturable, and totally displaceable by excess unlabeled calcium, and demonstrated two distinct classes of binding sites. Fig. 1 illustrates a Scatchard analysis of a typical calcium binding experiment assayed using the standard conditions of the present study. Affinity constants and maximum binding capacities derived by fitting the experimental data to computer-generated curves for two independent classes of binding sites agreed within 5-25% of those obtained directly from the Scatchard plots. Results were reproducible with affinity constants and maximum binding capacities (± SEM; n = 4 preparations) of 4.5 ± 0.4 × 10⁴ M⁻¹ and 1.83 ± 0.25 nmol of calcium per mg of protein for the high affinity site and 2.0 ± 0.2 × 10³ M⁻¹ and 13.7 ± 0.9 nmol/mg of protein for the low affinity site.

Effect of insulin upon calcium binding

Insulin concentrations from 65 micromolars/ml to 3 millimolars/ml added directly to the system using control plasma membranes (isolated from cells not incubated with insulin) had no significant effect upon calcium binding in 35 paired experiments assayed at calcium concentrations from 1 μM to 1 mM (not shown). Insulin is known to bind to the plasma membranes under these conditions (30). In contrast, plasma membranes isolated from cells which had been incubated with 100 micromolars of insulin per ml demonstrated a 25.0 ± 1.6% increase of calcium binding at all calcium concentrations tested compared to parallel controls (Table 1). The mean percent increase of binding after insulin treatment of the cells was similar at each
of the five standard calcium doses analyzed, which ranged from 1 μM to 1 mM, and each was statistically significant. The mean absolute change in calcium binding obviously increased with progressively higher doses of calcium as the amount of calcium bound increased. The calcium concentrations tested were such as to induce binding to both the high and the low affinity binding sites.

Analysis of a representative preparation by double reciprocal plots is illustrated in Fig. 2. The Michaelis constants and thus the apparent affinity constants (K_a) for both high (3.0 × 10^4 M^{-1}) and low (2.8 × 10^3 M^{-1}) affinity calcium binding sites were unaltered by insulin treatment, whereas the maximum binding capacities (B_{max}) for both classes of sites were increased approximately 25% by insulin treatment. The calculated values for the B_{max} from a series of experiments are seen in Table 2, and the increases caused by insulin were highly significant (P < 0.01).

**DISCUSSION**

Two distinct pools of calcium have been identified in rat adipocyte plasma membranes. An exchangeable pool was reflected by the ^45Ca^{2+} binding studies, and a larger nonexchangeable or stable pool was reflected by atomic absorption analysis. The existence of two such calcium pools in the plasma membranes is consistent with observations on subcellular fractions from

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**Table 2. Insulin increases maximum binding capacity of high and low affinity calcium binding sites**

<table>
<thead>
<tr>
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<th>Control</th>
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<th>% Increase after insulin treatment</th>
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<tr>
<td><strong>High affinity site</strong></td>
<td>1.26 ± 0.15</td>
<td>1.61 ± 0.16</td>
<td>27.8 ± 3.0</td>
</tr>
<tr>
<td><strong>Low affinity site</strong></td>
<td>13.0 ± 1.1</td>
<td>17.1 ± 0.7</td>
<td>31.5 ± 8.6</td>
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Plasma membrane isolation from insulin-treated and control cells and the calcium binding assays were performed as outlined in Table 1. The maximum binding capacities (B_{max}) were determined using double reciprocal plots of binding data from four paired preparations in which calcium binding was determined at more than seven different calcium concentrations. Calcium binding at each concentration was assayed in triplicate. The values shown represent the mean ± SEM from four paired preparations. The differences between control and insulin-treated cells were significant using the Student paired t test (P < 0.01).

**Effect of insulin on calcium and magnesium content of isolated plasma membranes**

Atomic absorption analysis of the calcium content of isolated plasma membranes revealed a pool of calcium which was more than two times larger than the maximum binding capacity of the ^45Ca^{2+} binding system. Previous characterization of the exchangeable calcium binding system indicated that, following isolation of the plasma membranes, the exchangeable sites were essentially unoccupied. Therefore, the atomic absorption measurements did not reflect any significant amount of this exchangeable calcium compartment. Table 3 shows that insulin treatment did not significantly alter the pool of calcium that is measured by atomic absorption. In six of the 10 experiments represented, equilibrium calcium binding assays were performed (data included in Table 1) and the plasma membranes from the insulin-treated cells demonstrated increased calcium binding capacity. The magnesium content also was measured and no significant difference between the control and insulin-treated preparations was found.

**Table 3. Effect of insulin upon stable pools of calcium and magnesium**

<table>
<thead>
<tr>
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<th>Pool content, nmol/mg of protein</th>
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<tr>
<td><strong>Calcium</strong></td>
<td></td>
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<tr>
<td>(n = 10)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>32.8 ± 1.1</td>
</tr>
<tr>
<td>Insulin</td>
<td>33.2 ± 1.8</td>
</tr>
<tr>
<td><strong>Magnesium</strong></td>
<td></td>
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<tr>
<td>(n = 5)</td>
<td></td>
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<tr>
<td>Control</td>
<td>11.0 ± 0.6</td>
</tr>
<tr>
<td>Insulin</td>
<td>12.0 ± 0.2</td>
</tr>
</tbody>
</table>

Plasma membranes were isolated from control and insulin-treated cells and calcium and magnesium content were determined by atomic absorption as outlined in Experimental Procedures. The number of paired preparations used is indicated by n. Each value represents the mean ± SEM.
other cells (22). Adipocyte microsomes and mitochondria have been found also to contain two such distinct pools of calcium (unpublished).

Insulin treatment of adipocytes produced an increase in the ability of the isolated plasma membranes to bind calcium. This was associated with an increase in the maximum binding capacity of both the high and low affinity calcium binding sites. The affinity constants for both of the classes of sites were unaffected by the insulin treatment of the adipocytes. This phenomenon was not reproduced by the addition of the hormone directly to the isolated plasma membranes. The necessity for insulin to interact with the intact cell in order to demonstrate an insulin effect on cellular metabolism is not unique. Such a requirement must be met in order to demonstrate insulin stimulation of glucose uptake into isolated adipocyte plasma membrane vesicles (31) and stimulation of 3':5'-cyclic AMP phosphodiesterase in adipocytes (32).

The maximum calcium capacity of the adipocyte plasma membranes (nonexchangeable pool plus the maximum binding capacity of the exchangeable pool) contain 46.0 nmol of calcium per mg of protein in the control preparations and 50.1 nmol/mg of protein in the insulin-treated preparations. This represents approximately a 9% increase in the total calcium capacity of the plasma membranes which has resulted entirely from the 25% increase in binding capacity of the exchangeable pool. This change, although small in percentage, represents a change of 0.25 fmol of calcium per cell, based on the plasma membrane content per cell. This quantity of calcium could profoundly alter the adipocyte calcium distribution and thereby have significant impact on metabolic processes. For example, this amount of calcium could alter the cytosol calcium by approximately 12 μM, based on cytosol volume per adipocyte. This change in cytosol calcium concentration would be more than an order of magnitude, based on the cytosol calcium levels of other mammalian cells (22). Hales et al. (33), using ultrastructural x-ray microprobe analysis of adipocytes, found the plasma membranes to contain a significant quantity of calcium, but they could not detect a change induced by insulin. The 9% change in plasma membrane total calcium capacity caused by insulin would not be expected to be detectable by the ultrastructural x-ray studies. Yet this change could clearly be responsible for metabolic responses.

Several alternatives exist to explain how insulin might result in the appearance of additional calcium binding sites. First, insulin could cause a change in conformation or charge of the plasma membrane, exposing new calcium binding sites. This could be a direct effect or secondary to formation of a second messenger that interacts with the membrane. Second, the increased calcium binding might relate to the increased phosphorylation of specific adipocyte plasma membrane proteins induced by insulin (34). Studies using liver and myocardial plasma membranes have shown that calcium binding was increased following membrane phosphorylation (35). A third alternative involves a possible critical interaction between calcium and magnesium at the plasma membrane. Calcium binding has been shown to be sensitive to changes in the concentration of ionized magnesium*. The finding that the stable pool of magnesium (atomic absorption measurements) appeared to be unaltered by insulin does not exclude this possibility, as a pool of free (noncomplexed) magnesium ions may exist which might be altered by insulin treatment and such an alteration might not be reflected by the atomic absorption measurements. Although it cannot be excluded, it seems highly unlikely that insulin treatment of the adipocyte resulted in enrichment of the plasma membrane fraction with a cellular component with additional calcium binding sites, especially since both classes of binding sites are affected. The insulin-induced increase in calcium binding cannot be accounted for by a calcium or other ion pump, since no energy source was included in the isolation procedure or in the calcium binding incubation system, such that no gradient could be created, let alone maintained, in excess of control.

The present study has shown that insulin treatment of adipocytes resulted in an increased capacity for the plasma membrane to bind calcium, using physiological concentrations of insulin and a highly purified plasma membrane preparation. Others have found insulin to decrease calcium binding, but used either pharmacological concentrations of insulin (2.5–250 milliunits/ml) added directly to liver plasma membranes (36); a heterogeneous membrane system such as fat cell ghosts (10), which contain nuclei, mitochondria, endoplasmic reticulum, and cytosol inside plasma membrane sacs; or artificial lipid monolayers that contained no insulin receptors (37).

It has been suggested that an increased cytosol concentration of calcium is responsible for the pleiotropic response of insulin (8–10). The key component of this hypothesis has been the necessity for insulin to decrease calcium binding to plasma membranes, resulting in an increased cytosol calcium level (8, 9). The present data do not support this mechanism for raising cytosol calcium, since the actual binding capacity for calcium by the plasma membrane has been found to be increased. However, this increase in calcium binding could still result in an increase in cytosol calcium if the plasma membrane is in equilibrium with both the extracellular and intracellular calcium with a gradient toward the cytosol. The increased calcium binding capacity of the plasma membrane could cause more extracellular calcium to bind to the membrane and in turn a new, higher equilibrium state between the plasma membrane and cytosol would be reached. This could relate to either a passive or active transport system. Another alternative is that the changes in calcium binding to the plasma membranes produced by insulin are unrelated to changes in the cytosol concentration of calcium but relate directly to alterations in plasma membrane processes controlled by insulin, such as the transport of glucose, amino acids, and ions. Furthermore, increasing the cytosol calcium concentrations, if it is necessary for insulin action on intracellular metabolic processes, could result from the ability of insulin to alter the calcium content of intracellular organelles such as mitochondria and endoplasmic reticulum (38) without directly altering calcium influx into or efflux from the cell. Regardless of which of the above interpretations is correct, it is clear that insulin can affect cellular calcium distribution and that this may play a central role in the mechanism of insulin action.

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