Communication

The Existence of an Optimal Range of Cytosolic Free Calcium for Insulin-stimulated Glucose Transport in Rat Adipocytes*

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We have examined the effects of extracellular and intracellular Ca$^{2+}$ concentrations upon basal and insulin-stimulated 2-deoxyglucose uptake in isolated rat adipocytes. In the absence of extracellular Ca$^{2+}$, both basal and insulin-stimulated glucose uptake were significantly reduced. Insulin-stimulated glucose transport was optimal at 1 and 2 mM Ca$^{2+}$. Further increases in extracellular Ca$^{2+}$ concentration (3 mM) significantly diminished insulin-stimulated glucose uptake. When intracellular Ca$^{2+}$ concentrations were augmented by ionomycin (1 μM), insulin-stimulated glucose uptake was significantly reduced at extracellular Ca$^{2+}$ concentrations of 2 and 3 mM. The levels of intracellular free Ca$^{2+}$ concentrations were then measured with Ca$^{2+}$ indicator fura-2. The correlation between the levels of intracellular free Ca$^{2+}$ and the magnitude of insulin-stimulated glucose uptake revealed that the optimal effect of insulin is observed at Ca$^{2+}$ levels between 140 and 370 nM. At both extremes outside of this window, both low and high levels of intracellular Ca$^{2+}$ result in diminished cellular responsiveness to insulin.

These data suggest that intracellular calcium concentrations may exert a dual role in the regulation of cellular sensitivity to insulin. First, there must exist a minimal concentration of intracellular calcium to promote insulin action. Second, increased levels of intracellular calcium may provide a critical signal for diminution of insulin action.

The role of Ca$^{2+}$ in mediating multiple cellular functions has been well established (1–7). We have recently demonstrated that both insulin and glyburide increased cytosolic free calcium concentrations, [Ca$^{2+}$], in isolated rat and human adipocytes (8, 9). These observations, as well as an existing controversy relative to the role of Ca$^{2+}$ in the mechanism of insulin action (10, 11), prompted us to investigate in greater detail the relationship between extracellular and intracellular Ca$^{2+}$ concentrations and insulin-stimulated glucose transport. Furthermore, our findings of increased [Ca$^{2+}$], in adipocytes isolated from obese and hyperinsulinemic subjects (9) indicated that intracellular Ca$^{2+}$ may be a critical factor in postreceptor step modulation of cellular responsiveness to insulin. In this study, we assessed the effects of various concentrations of extracellular and intracellular Ca$^{2+}$ upon basal and insulin-stimulated glucose transport in isolated rat adipocytes.

EXPERIMENTAL PROCEDURES

Material—Porcine insulin was a gift from Lilly. Fura-2, fura-2AM, and ionomycin were purchased from Behring Diagnostics, collagenase from Worthington, verapamil from Sigma, [3H]-2-deoxyglucose from Amersham Corp., and [3H]-glucose from Du Pont-New England Nuclear.

Preparation of Isolated Adipocytes—Male Sprague-Dawley rats weighing 200–250 g were used in these studies. Isolated adipocytes were prepared from epididymal fat pads according to the method of Rodbell (12). Animals were allowed food and water ad libitum before they were killed.

2-Deoxyglucose Transport—Adipocytes (2 x 10$^6$ cells) were incubated in the absence and in the presence of increasing concentrations of insulin for 60 min at 37 °C. Concentrations of extracellular Ca$^{2+}$ ranged between 0 and 3 mM as indicated below. Glucose uptake was initiated by addition of [3H]-2-deoxyglucose (0.2 μCi). After 3 min of incubation, the reaction was terminated by transferring 200-μl aliquots of incubation mixture to the microfuge tubes (containing 100 μl of silicone oil) and centrifuging the tubes in a Beckman Microfuge. The cell pellets were counted for radioactivity present in a Beckman liquid scintillation counter. The results were corrected for diffusion by subtracting the uptake of L-glucose in the absence of insulin.

Measurements of Intracellular Calcium—The fluorescence of control and fura-2 loaded cells was measured using a Turner Model 340 spectrofluorometer as previously described (8). During Ca$^{2+}$ measurements, the cells were incubated in 2.4 ml of Krebs-Hepes’ buffer containing 118.4 mM NaCl, 4.89 mM KCl, 1.2 mM MgCl$_2$, 1.18 mM KH$_2$PO$_4$, 1.25 mM NaHCO$_3$, 20 mM Hepes, 5 mg/ml bovine serum albumin, and 30 mg/dl glucose at pH 7.4. Extracellular Ca$^{2+}$ concentration ranged from 0 to 3 mM as indicated below. The final cell calcium concentration was approximately 2 x 10$^6$ cells/ml. Tissue or buffer fluorescence in the absence of fura-2 did not change in response to either insulin, verapamil, or ionomycin. Similarly, neither addition of fat emulsion nor rat fat droplets to the fura-2-Ca$^{2+}$-complex changed the fluorescent signal. The measurements were also performed by the dual wavelength technique (8, 13) at the excitation wave lengths 347 and 387 nm. Although the relative changes in [Ca$^{2+}$], were the same, the absolute values were approximately 2.5 times lower, probably as a result of tissue viscosity interference with the measurements (14, 15). Statistics—The results of these studies are presented as mean ± S.E. of three to five experiments and compared using either paired or unpaired t test as indicated.

RESULTS

In initial experiments, we have assessed the influence of extracellular concentrations of Ca$^{2+}$ on basal and insulin-stimulated 2-deoxyglucose uptake in isolated adipocytes (Fig. 1). In these experiments, the cells were incubated without insulin (termed here, basal transport), with two submaximally effective concentrations of insulin (0.3 and 1 ng/ml) and with a maximally effective insulin concentration (25 ng/ml). The studies were performed at varying levels of Ca$^{2+}$ in the extracellular fluid (0, 0.01, 0.1, 1, 2, and 3 mM). The basal rate of glucose transport was reduced in the absence of extracellular Ca$^{2+}$ (p < 0.05 versus 0.01 mM, and <0.01 versus other Ca$^{2+}$)

* The abbreviation used is: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
FIG. 1. The effect of extracellular Ca\(^{2+}\) concentrations on basal and insulin-stimulated 2-deoxyglucose uptake in adipocytes. The results are presented as nmol of 2-deoxyglucose taken up by 250,000 cells in 3 min (A) or as a percent above the basal rate (B). Each point represents the mean value of three or four independent experiments. The S.E. bars are omitted for the clarity. Concentrations of extracellular Ca\(^{2+}\) are shown on the right.

FIG. 2. The effect of intracellular Ca\(^{2+}\) concentrations on basal and insulin-stimulated 2-deoxyglucose uptake. The cells were exposed to 1 \(\mu\)M ionomycin for 10 min before the measurements of glucose uptake were initiated. The results represent the mean values of three or four separate experiments and are expressed as in Fig. 1.

regardless of the Ca\(^{2+}\) concentration in the extracellular fluid (when it was present), the basal rate of glucose transport was practically identical.

The response to insulin stimulation was minimal in the absence of extracellular Ca\(^{2+}\) and optimal at 1 and 2 mM Ca\(^{2+}\). However, an increase of the extracellular Ca\(^{2+}\) concentration to 3 mM resulted in a significant decrease in insulin-stimulated glucose transport \((p < 0.01 \text{ versus } 1 \text{ and } 2 \text{ mM at all insulin concentrations})\). This is particularly evident when the results of the experiments are expressed as the percent of stimulation above the basal rate of glucose transport (Fig. 1B). As can be seen, insulin-stimulated glucose transport was diminished under two conditions: 0 and 3 mM extracellular Ca\(^{2+}\).

To examine the effect of intracellular Ca\(^{2+}\) concentrations upon basal and insulin-stimulated glucose transport, we have performed similar experiments in the presence of the calcium ionophore, ionomycin. Ionomycin (1 \(\mu\)M) was added to the incubation mixture 10 min prior to initiation of glucose uptake measurements. In the absence of extracellular Ca\(^{2+}\), both basal and insulin-stimulated 2-deoxyglucose uptake were lower than in the presence of extracellular Ca\(^{2+}\) \((p < 0.01 \text{ at all points})\) and not significantly different from values observed in the absence of ionomycin (Fig. 2).

In the presence of extracellular Ca\(^{2+}\), ionomycin did not alter the rate of basal glucose transport, which was identical to that seen without ionomycin. Similarly, the rate of basal glucose transport was not significantly affected by various concentrations of extracellular Ca\(^{2+}\). In these experiments, the optimal response of glucose transport to insulin was seen at a Ca\(^{2+}\) concentration of 1 mM \((p < 0.01 \text{ versus } 0.01, 2, \text{ and } 3 \text{ mM Ca}^{2+} \text{ at all insulin concentrations})\). In the presence of ionomycin and either 2 or 3 mM extracellular Ca\(^{2+}\), insulin-stimulated glucose transport was significantly diminished as compared with lower Ca\(^{2+}\) concentrations. Again, when the results were expressed as a percent above the basal rate, the inhibitory influence of high Ca\(^{2+}\) concentrations in the presence of ionomycin was particularly apparent (Fig. 2B). Interestingly, in the presence of ionomycin, 2 mM extracellular Ca\(^{2+}\) now exerted an inhibitory effect on glucose transport \((p\)
The role of Ca\(^{2+}\) in mediating insulin action remains controversial. Clausen et al. (1), Kissebah et al. (2), and others (3-7) have emphasized the role of intracellular Ca\(^{2+}\) as a mediator of insulin action. Nevertheless, evidence regarding this hypothesis has also been presented (10, 11). Introduction of Ca\(^{2+}\) indicators quin-2 and fura-2 has provided the means for direct determinations of [Ca\(^{2+}\)], in insulin target cells. In muscle cells, these measurements failed to demonstrate an effect of insulin on [Ca\(^{2+}\)], despite continuous stimulation of glucose transport (11, 16). In adipocytes, however, we were able to observe a dose-dependent effect of insulin and glyburide upon [Ca\(^{2+}\)] (8, 9). The effect of insulin was rapid, seen within the first 2-4 min of exposure and reaching a maximum at 10-14 min.

Although insulin enhances both glucose transport and [Ca\(^{2+}\)], the direct relationship between these two variables has not been established. The effect of insulin upon [Ca\(^{2+}\)] seems somewhat delayed when compared with the insulin effect on glucose transport. However, glucose transport meas-

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**Table I**

| Insulin (ng/ml) | Control | Ionomycin (1 µM) | Verapamil (30 µM) |
|----------------|---------|-----------------|------------------|
| 0.0 pg/ml      | 0.18 ± 0.02 | 0.20 ± 0.02 | 0.18 ± 0.01 |
| 0.3 ng/ml      | 0.32 ± 0.03 | 0.35 ± 0.02 | 0.25 ± 0.03* |
| 1.0 ng/ml      | 0.50 ± 0.02 | 0.55 ± 0.03 | 0.41 ± 0.02* |
| 25.0 ng/ml     | 0.64 ± 0.03 | 0.70 ± 0.04 | 0.50* ± 0.02* |
| None           | 0.16 ± 0.01 | 0.15 ± 0.02 | 0.18 ± 0.02 |
| 0.3 µg/ml      | 0.27 ± 0.01 | 0.28 ± 0.03 | 0.22 ± 0.02 |
| 1.0 µg/ml      | 0.40 ± 0.03 | 0.52 ± 0.03* | 0.33 ± 0.03* |
| 25.0 µg/ml     | 0.54 ± 0.03 | 0.65 ± 0.02* | 0.43 ± 0.03* |

*p < 0.05 versus control.
urements are usually performed after 30–60 min of cell exposure to insulin. It is conceivable that optimal levels of intracellular Ca\(^{2+}\) are achieved during this preincubation period, which then results in adequate glucose transport: when the glucose tracer is added to the cells. As is evident from this and previous (17–19) communications, insulin-stimulated glucose transport is diminished in the absence of extracellular Ca\(^{2+}\), strongly supporting the possibility that there exists a tight relationship between insulin-stimulated increases in Ca\(^{2+}\) influx and glucose transport. On the other hand, we have also demonstrated (8) that ambient glucose concentrations modulate the effect of insulin on [Ca\(^{2+}\)].

Regardless of whether or not intracellular Ca\(^{2+}\) plays a role in initiating and/or mediating insulin action, our recent (9) and present observations strongly suggest that high [Ca\(^{2+}\)], may be a mechanism for deactivation or possibly termination of insulin action. In vivo insulin and glucose infusion (euglycemic clamp) in normal volunteers resulted in higher levels of [Ca\(^{2+}\)]. In isolated adipocytes and in the loss of cellular responsiveness to insulin or glyburide. However, adipocytes isolated from obese subjects demonstrated high levels of intracellular Ca\(^{2+}\) and were also unresponsive to subsequent stimulation with insulin or glyburide (9).

Similar induction of insulin resistance was demonstrated by Garvey et al. (20) and Mandarino et al. (21). Garvey and his colleagues (20) have produced insulin resistance by incubating rat adipocytes with high concentrations of insulin and glucose for 24 h. Neither glucose nor insulin alone were active in this regard. Mandarino et al. (21) observed a significant reduction in insulin-stimulated glucose transport in adipocytes isolated from normal subjects at the 6th hour of insulin and glucose infusion (euglycemic clamp). In the light of our observations, it is possible that in both cases, exposure of adipocytes (either in vivo or in vitro) to high concentrations of insulin and glucose might have resulted in increased [Ca\(^{2+}\)], which, in turn, diminished cellular responsiveness to insulin.

The mechanism whereby elevated [Ca\(^{2+}\)], induced cellular insensitivity to insulin is unknown. It is intriguing to postulate that elevated levels of intracellular Ca\(^{2+}\) provide a feedback signal for termination of insulin action. It is conceivable that Ca\(^{2+}\), either itself or via activation of protein kinase C, may inhibit various processes involved in mediating insulin action. Another possibility is that intact Ca\(^{2+}\) fluxes, in and out of cells, are required to maintain an optimal rate of glucose transport. In recent studies with chromaffin cells, Artalejo et al. (22) demonstrated that intracellular concentrations of Ca\(^{2+}\) modulate the rate of Ca\(^{2+}\) influx. In their studies, increased concentrations of intracellular Ca\(^{2+}\) deactivated Ca\(^{2+}\) channels and reduced the rate of Ca\(^{2+}\) influx. Furthermore, chelation of intracellular Ca\(^{2+}\) with quin-2 prevented deactivation of Ca\(^{2+}\) entry, whereas enhancement of the [Ca\(^{2+}\)], with the ionophore A23187 rapidly increased the rate of deactivation of Ca\(^{2+}\) influx. If the maintenance of Ca\(^{2+}\) influx is indeed required to optimize glucose transport, then deactivation of this process by high and/or sustained intracellular concentrations of Ca\(^{2+}\) may result in diminution of insulin-stimulated hexose transport.

In conclusion, there appears to be an optimal level of [Ca\(^{2+}\)], for insulin-stimulated glucose transport. One must realize, however, that our method measures total intracellular Ca\(^{2+}\) concentration and does not take into account the possible localized effects of Ca\(^{2+}\) or changes in Ca\(^{2+}\) concentration within discrete cellular compartments. Nevertheless, our data suggest that high and/or sustained levels of intracellular Ca\(^{2+}\) may function as a postreceptor feedback sensor to diminish cellular responsiveness to insulin. In obesity or in normal subjects receiving glucose/insulin infusions, increasing intracellular Ca\(^{2+}\) may result in overt insulin resistance (9). Further investigations are necessary to elucidate the role of Ca\(^{2+}\) as a physiological terminator of insulin action.

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