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Detection of GLUT4 release to the PM by immunofluorescence

Upon insulin stimulation the cells became immunostained with anti-HA antibodies known to enter the intact PM (Dawson et al., 2001). The HA-tagged exofacial domain of GLUT4 originally exposes to the GLUT4 vesicle lumen and only becomes accessible to the antibodies in the extracellular media if the GLUT4 vesicles fuse with the PM. Accordingly, and consistent with the earlier observations (Malide et al., 1997; Dawson et al., 2001), when the cells were incubated with insulin for 5–15 min at 37°C degree before immunostaining with anti-HA immunofluorescence (Fig. S2 A, red) became highly colocalized with GFP-fluorescence of GLUT4 (Fig. S2 A). In contrast, when the cells were first pretreated for 10 min with 100 nM wortmannin, which is known to inhibit insulin signaling, GFP fluorescence showed a punctate pattern with no colocalization with anti-HA immunofluorescence (Fig. S2 B).

FRAP measurements of GLUT-GFP lateral mobility in the PM

Bleaching of GLUT-GFP confined in the TIRF area was performed by turning laser power to the maximum (70 mW). Radius of the circular area of the cell subjected to TIRF depended on the level of squeezing of each particular cell between the coverslips, and varied from 10 to 30 μm. More than 80% of initial fluorescence was bleached out in 3 s. After that, laser power was set to the minimum of level (6 mW) and, with the shutter opening every 15 s, snapshots of TIRF area were acquired for 5–10 min. Stacks of images were further transferred to ImageJ and processed as described below. Mean gray value of the circular ROI representing bleached TIRF area of the cell was calculated for each image and plotted versus time. The experimental data was fitted with a theoretical curve (Axelrod et al., 1976; Soumpasis, 1983; Klein et al., 2003) using the least square algorithm in Maple software (Maplesoft): $I(t) = I_0 + (F_0 - F_{\infty})e^{-k_1t} + (I_2t) + I_1(2\pi t)$, where $t$ is the time, $F_0$ is the fitting parameter, $F_\infty$ is the fluorescence intensity of the bleached area immediately after bleaching, $F_0$ is the fluorescence intensity of the bleached area after recovery to the constant level, $I_1$ and $I_2$ are modified Bessel functions. Characteristic time $\tau$ obtained from fitting procedure was used to calculate lateral diffusion coefficient for GLUT-GFP from the formula: $D = R^2/4\tau$, where $R$ is the radius of the bleached area.

Kinetic analysis of GLUT4 recycling in primary adipose cells

In adipose cells, GLUT4 cycles between the following main locations (Holman et al., 1994; Lee et al., 1999; Bryant et al., 2002): (a) GLUT4 vesicles (constituting the GLUT4-releasable pool); (b) the rest of subcellular GLUT4 that is not directly delivered to the PM upon insulin stimulation (mainly “endosomal” GLUT4); and (c) GLUT4 associated with the PM, i.e., GLUT4 exposed to the extracellular medium and thus accessible for antibody labeling. The basal distribution of GLUT4 among these three general pools is highly dependent on the experimental system under consideration. In freshly prepared isolated rat adipose cells, most of the GLUT4 is found in the GLUT4 vesicles and little in either the “endosomal” pool or PM; in 3T3-L1 adipocytes, intracellular GLUT4 is roughly divided between the GLUT4 vesicles and the “endosomal” pool, with little in PM; and as reported here and previously (Malide et al., 1997), in primary rat adipose cells cultured for increasing periods, the distribution shifts with culture duration from that observed in the freshly prepared cells to one quite similar to that observed in the 3T3-L1 adipocytes. As noted in the text, we have performed the present TIRFM studies under conditions such that the basal GLUT4 distribution approximates that in freshly prepared isolated rat adipose cells cultured for increasing periods, the distribution shifts with culture duration from that observed in the freshly prepared cells to one quite similar to that observed in the 3T3-L1 adipocytes. Accordingly, and consistent with the earlier observations (Malide et al., 1997; Dawson et al., 2001), when the cells were incubated with insulin for 5–15 min at 37°C before immunostaining with anti-HA immunofluorescence (Fig. S2 A, red) became highly colocalized with GFP-fluorescence of GLUT4 (Fig. S2 A). In contrast, when the cells were first pretreated for 10 min with 100 nM wortmannin, which is known to inhibit insulin signaling, GFP fluorescence showed a punctate pattern with no colocalization with anti-HA immunofluorescence (Fig. S2 B).
A 3-pool model is the minimal one describing the behavior of GLUT4 in rat adipose cells (Holman et al., 1994); moreover, the steady-state distribution of GLUT4 between the three pools described above is consistent with morphological observations (Malide et al., 1997). We extend this model to account for GLUT4 mobility; thus, subdividing GLUT4 vesicles into moving and stationary pools.

The vesicle is considered moving if it makes a detectable path (see Fig. 1, A and C). The scheme of this 4-pool model is presented in Fig. S4 A. The following GLUT4 pools are considered: (1) pool 1, GLUT4 in vesicles moving in the TIRF zone; (2) pool 2, GLUT4 in vesicles that remain static in the TIRF zone; (3) pool 3, GLUT4 in the PM or in vesicles fixed with the PM so that the GLUT4 is fully exposed to the extracellular medium and accessible to extracellular antibodies; (4) pool 4, the rest of subcellular GLUT4.

As GLUT4 vesicles are uniformly distributed near the PM (see Fig. 1), we assume that the number of GLUT4 vesicles (both moving and stationary) in the TIRF zone is directly proportional to the total number of GLUT4 vesicles in a cell. The same holds for the PM GLUT4. Thus, the dynamics of the fraction of the GLUT4 visible in the TIRF zone correctly represents the overall dynamics of the GLUT4 in the whole adipocyte. Accordingly, we consider the behavior of a fraction of GLUT4, C, corresponding to a single TIRF zone. This fraction is distributed between the four pools described above so that C1 + C2 + C3 + C4 = C and C remains constant. For simplicity, we assume that C = 1. The system of equations describing GLUT4 dynamics is as follows (System 1):

\[
\begin{align*}
\dot{C}_1(t) &= -k_1 C_1 + k_2 C_2 + 5C \\
\dot{C}_2(t) &= -(k_2 + k_3)C_2 + k_1 C_1 \\
\dot{C}_3(t) &= -k_4 C_3 + 3C \\
\dot{C}_4(t) &= -k_4 C_4 + 4C
\end{align*}
\]

From the literature, we know that ~5% of GLUT4 is in the PM (i.e., C3 = 0.05), and that the endocytosis rate constant k4 = 0.06 min⁻¹ (Holman et al., 1994; Malide et al., 2000); from our data, we obtain C1/C2, k1, and k2 (see Table S1). The 3 remaining unknowns, k3, k5, and C1, are defined by solving the System 2 equations.

The system of equations is a system of three algebraic equations (System 2):

\[
\begin{align*}
\dot{C}_1(t) + \dot{C}_2(t) + \dot{C}_3(t) - \dot{C}_4(t) &= \Delta S \\
(k_2 + k_3)C_2 + k_1 C_1 &= \Delta S \\
-k_4 C_3 + k_4 C_4 &= \Delta S
\end{align*}
\]

### Basal steady-state distribution

From the literature, we know that ~5% of GLUT4 is in the PM (i.e., C3 = 0.05), and that the endocytosis rate constant k4 = 0.06 min⁻¹ (Holman et al., 1994; Malide et al., 2000); from our data, we obtain C1/C2, k1, and k2 (see Table S1). The 3 remaining unknowns, k3, k5, and C1, are defined by solving the System 2 equations.

### Table S1. The parameters of the kinetic model and steady-state distribution of GLUT4

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Basal</th>
<th>Stimulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>k1</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>k2</td>
<td>1/150</td>
<td>1/150</td>
</tr>
<tr>
<td>k3</td>
<td>1/1000</td>
<td>1/600</td>
</tr>
<tr>
<td>k4</td>
<td>10⁻¹</td>
<td>10⁻³</td>
</tr>
<tr>
<td>k5</td>
<td>10⁻³</td>
<td>10⁻³</td>
</tr>
<tr>
<td>C1 (GLUT4 moving)</td>
<td>0.08</td>
<td>0.01</td>
</tr>
<tr>
<td>C2 (GLUT4 stationary)</td>
<td>0.85</td>
<td>0.34</td>
</tr>
<tr>
<td>C3 (PM)</td>
<td>0.05</td>
<td>0.58</td>
</tr>
<tr>
<td>C4 (subcellular red)</td>
<td>0.02</td>
<td>0.07</td>
</tr>
</tbody>
</table>

k1 is estimated from the mean distance d the vesicle passes before getting tethered for the first time: k1 = d/V, where V is the mean vesicle velocity. The means were obtained from Fig. S3, and V = 13 (5 ± 4, μm in the basal state and 10 ± 2, μm in the stimulated state, respectively). k4 is the number of GLUT4 vesicles detected in a ROI of 100 μm² randomly selected in a snapshot of the TIRF zone and measured in the number of moving vesicles in a ROI of the same area (the numbers were averaged over 20 different cells). We note that while at each moment many vesicles are stationary, in the basal state the attachment to the PM is mostly reversible; vesicles will continue moving until they reach the fusion zone. Cells with or without insulin at the same conditions. Note low immunofluorescence staining and punctate pattern of GLUT4-GFP fluorescence resembling the basal state distribution (as in Fig. 1 C).
Insulin steady state

From the literature, we know that ~50% of GLUT4 is translocated to the PM (C3) and ~30% remain in GLUT4 vesicles (C1 + C2) and that \( k_4 \) remains equal to 0.06 min\(^{-1}\) (Holman et al., 1994, Malide et al., 2000); from our data, we measure changes in \( k_1 \) (see Table S1). So the unknowns are \( k_2, k_3, k_5, \) and \( C_1 \) (or \( C_2 \)). They cannot all be determined from the System 2 equations, so we assume that \( k_5 \) remains unchanged and find approximate steady-state values for the remaining unknowns. To verify the assumption, we fit the kinetics of the increase of PM GLUT4 (and the respective reduction of vesicular GLUT4, see Fig. S4, B and C) upon insulin stimulation (solving the System 1 equations using the matrix exponent method) and find that the kinetics are indeed well described assuming that only \( k_1 \) and \( k_3 \) are changed by insulin. If \( k_3 \) is assumed to remain unchanged, the model does not describe either the kinetics of the insulin response or the steady-state distribution of GLUT4 in the insulin-stimulated state.

Conclusion

We conclude that insulin primarily increases the probability of tethering and also stimulates priming of vesicles tethered to specific fusion sites on the PM. This conclusion is well supported by biochemical kinetic data obtained using rat adipose cells.
References


Klein, C., T. Pillet, J. Chambaz, and R. Drouet. 2003. Determination of plasma membrane fluidity with a fluorescent analogue of sphingomyelin by FRAP measure-


Malide, D., G. Ramos, S.W. Cushman, and J.W. Slot. 2001. Immunocytochemical evidence that GLUT4 translocation explains the stimulation of glucose