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INSULIN STIMULATION OF ADIPOCYTE MEMBRANE GLUCOSE TRANSPORT

A GRADED BIOLOGIC RESPONSE INSENSITIVE TO BILAYER LIPID DISORDERING

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Abstract—Aspects of the mechanism by which insulin stimulates the membrane glucose transport system were examined by (1) assessing the influence of the bilayer lipid structure on transport stimulation characteristics, and (2) considering the form of the insulin dose–response curve. We tested the effects of membrane lipid perturbation on the insulin stimulation process. Benzyl alcohol, at concentrations (25 mM) that grossly fluidize lipids forming the adipocyte membrane bilayer matrix, caused 50% inhibition of intrinsic transporter activity. However, this membrane perturbation had no significant effect on either the insulin dose–response curve (conducted at 37°) or the time–course of the insulin stimulation of hexose transport (conducted at 32°). These data are difficult to rationalize in terms of a model in which transport stimulation involves interaction of transporters and hormone-bound receptors that is limited by lateral diffusion of these proteins in the fluid lipid bilayer. Curve-fitting experimental insulin dose–response data for stimulation of 2-deoxy-D-glucose and D-glucose uptake provided an estimate of an insulin "association constant" for transport regulation that may be compared with recent insulin receptor binding data. Similar magnitude constants were obtained whether estimated directly from plots of transport velocity versus arithmetic hormone dose, or by extrapolation from linear segments of sigmoidal velocity versus log dose plots, or from inverse (Lineweaver–Burk-type) plots of the insulin dose–response data. Insulin apparently regulates transport by associating with a binding site, having an apparent dissociation constant which is determinable through kinetic measurements of hexose uptake ($K_{D_{app}}$ approx. 17–40 pM). This is in good agreement with the dissociation constant, K_D, determined from Scatchard plots of recent binding data to adipocytes, for a class of receptors representing the "high affinity" binding sites for insulin. Insulin dose–response curve simulations also indicated that the stimulation process may be classified in pharmacologic terms as a typical graded biologic response and may involve insulin association with a site that regulates transport rates in a manner kinetically analogous to allosteric modulation of a V-series enzyme by a noncompetitive ligand. From the results we suggest that (1) a relatively close association occurs between transport and receptor proteins in the membrane, where the relative activation of transport depends on the fractional occupancy of functional high affinity receptors by insulin, and (2) the insulin stimulation of transport involves regions of the membrane that are not influenced significantly by disordering the membrane lipid matrix.

Insulin stimulates the glucose transport system with a time [1], temperature [2, 3], oxygen [4], and perhaps ATP [5, 6] dependent process requiring the presence of an intact cell. Insulin binds to a specific, cell surface receptor which may subsequently assume a distinct conformation [7, 8]. The hormone alters gross structural properties of the surface membrane, reflected by phosphorylation of a wide variety of membrane proteins, alterations in the binding of cytochalasin B, and activation of numerous other membrane functional properties [9–11].

It is possible that insulin influences cell membrane glucose transport and other functions by a relatively indirect, complex mechanism in the membrane. Indeed, the hydrophobic glucose transporter and the insulin binding tetrameric receptor are physically distinct proteins [11–14]. Some data have been interpreted as indicating that transport activation is due to insertion of additional transporters into the membrane from intracellular vesicle stores [9], although direct evidence for this is yet necessary [10, 15–17].

However, other evidence suggests that the initial insulin binding event and transporter stimulation may be relatively closely coupled and limited to the membrane locus [10]. Also, exposure of adipocytes with agents that perturb structures associated with the membrane (including the Golgi apparatus-disintegrating agents monensin and leupeptin and the cytoskeleton-perturbing agents cytochalasin B and D) does not affect the ability of insulin to stimulate the uptake of glucose [18–20].

Detailed structural features/components of the surface membrane that are necessary to optimize the insulin stimulation mechanism are at present not

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well-defined. Removal of adipocyte membrane surface sialic acid residues with neuraminidase can abolish completely the insulin stimulation process [21]. The alcohol series from methanol to octanol, at doses that fluidize essentially the entire membrane lipid bilayer matrix, thereby inhibiting transporter function, surprisingly do not diminish the subsequent normal fold increase above basal transport caused by saturating insulin [22, 23]. We here examine further the influence of the membrane lipid bilayer fluidity/structure on the time–course of the insulin stimulation process and the insulin dose–response curve.

The glucose transport system in the adipocyte and other cell membranes follows Michaelis–Menten expressions for enzyme catalysis in both the absence and presence of saturating doses of insulin [24]. It is possible that the enzymatic character of the transport protein in the membrane provides clues to help explain the mechanism of action of insulin. In this study we examine whether the activation of transport by various doses of insulin in adipocytes is describable in pharmacologic terms as a graded biologic response [25], exhibiting features in common with activation of allosteric enzymes by non-substrate-like ligands.

MATERIALS AND METHODS

Adipocyte isolation and incubation conditions. Adipocytes were isolated as described in earlier studies [10, 26] from male Sprague–Dawley rats (Simonsen Laboratories, Gilroy, CA). The epididymal adipose tissue was minced and digested in buffer containing NaCl (110 mM), KCl (5 mM), $CaCl_2$ (1 mM), $MgSO_4$ (2.5 mM), KH_2PO_4 (1 mM), morpholinopropane sulfonic acid (MOPS) (25 mM), 20 mM NaOH, pH 7.4 (physiologic MOPS), with 4% (w/v) albumin (fatty acid free) (all from the Sigma Chemical Co., St. Louis, MO) and 1 mg of collagenase (lot CLS 43 N131, Worthington, Freehold, NJ) per ml. Cells were filtered and washed in MOPS buffer containing 1% albumin (approx. 305 mOsM) and then diluted to approximately 10^6 cells/ml buffer. Cell counts were determined by direct microscopic examination of small samples of cell suspension diluted 10-fold.

Hexose transport measurements. The initial rate of uptake of 2-deoxy-D-[^{3}H]glucose was measured essentially as described by Gliemann *et al.* [27] and Olefsky [24]. Cells were incubated at a fixed temperature (37°) either with or without insulin (Sigma) for 10 min before addition of labeled substrate (0.1 mM, final specific radioactivity 4 mCi/mmol). Uptake was terminated after 10 min by centrifuging 200-μl portions of cell suspension through dinonyl phthalate oil in 400-μl centrifuge tubes in an Eppendorf microfuge (Brinkmann) for 3 sec. The tubes were sliced at the cell/oil interface and counted for ^{3}H radioactivity in liquid scintillation fluid (United Technologies Packard) in a Searle liquid scintillation counter. 2-Deoxy-D-glucose passes through the membrane and is phosphorylated by the same mechanisms as for D-glucose but is not further metabolized inside the cell [28]. Uptake of this glucose analog under the conditions used directly measures the membrane transport process in both the absence and the presence of insulin. Trapped and noncarrier-mediated uptake of hexose was estimated by the inclusion of 50 μM cytochalasin B in the assay medium.

The time-dependence of the insulin stimulation of transport at 32° was conducted by measuring uptake for 5 min with high specific activity substrate (8 mCi/mmol), where substrate was added at various times after the addition of insulin. Data are expressed as a function of total cell incubation time with insulin.

The effects of alcohol were assessed on the time–course and the dose–response curve as indicated earlier [26] by adding the alcohol (with adjustment for constant ionic strength) prior to insulin and substrate. In all cases, uptake was corrected for the amount of label present in cytochalasin B (50 μM) treated cell suspensions that represented trapping and nonspecific diffusion of substrate.

Insulin dose–response curves at 37° were generated by diluting stock insulin (initially made in pH 3 buffer at 1 mg/ml) into physiologic MOPS 4% albumin buffer (pH 7.4) so that 10-μl aliquots of each were added to obtain the listed final insulin concentrations. Cells were here pretreated with each insulin dose for at least 5 min prior to addition of substrate.

Dose–response curve fitting. Insulin treatment of intact adipocytes leads to activation of membrane glucose [29] and 2-deoxyglucose [24] transport rate (V_{max}) without change in K_m for the system [24, 29]. One possibility is that the net results of transport activation may be compared or modeled by allosteric activation of a V-series enzyme [30], where binding of an allosteric ligand to a site distinct from the substrate binding site causes activation of V_{max} without change in K_m for the enzyme, [31]. In this case:

$$V_I = V_B + (\sigma V_B - V_B)/(1 + K_D/I)$$

where V_B is the basal velocity of the transport of substrate (hexose) from outside to inside the cell, V_I is the transport rate in the presence of insulin (at various doses), K_D is the apparent insulin dissociation constant, I is the insulin concentration, and σ is the fold increase above basal transport rate due to maximal concentrations of insulin.

This expression may be used to generate dose–response curves for an allosteric-type model and to determine any dependence of the curve on variations in K_D and σ. Theoretic dose–response curves may be compared with experimentally-determined data to test whether this model is inappropriate for the insulin stimulation of transport (under conditions in which the initial substrate concentration is constant for the basal and insulin-stimulated states).

The above analysis is fundamentally similar to the application of the law of mass action to the dose–response relationship describing biologic responses to drugs [32]. These processes reflect the combination of ligand molecules with a single class of receptors, where the magnitude of the response is directly related to fractional receptor occupancy, with a maximal response corresponding to occupancy of all functional receptors. Here, one ligand molecule binds with one receptor site, and a negligible fraction of total ligand present in a sample actually combines with cell receptors.

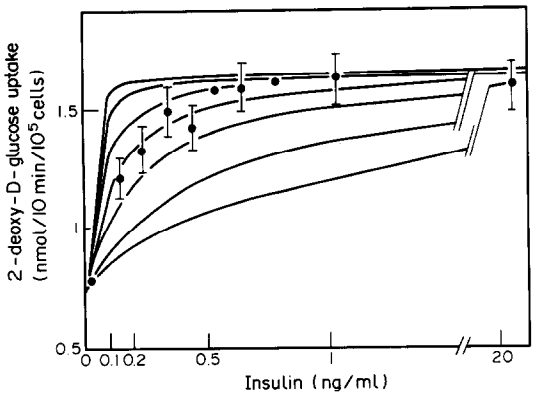

Fig. 1. Simulated insulin dose–response curves for stimulation of hexose transport system by insulin. Points were computed assuming that the basal rate of uptake (V_B) is 0.58 nmol hexose/10 min per 10^5 cells. The K_D for insulin (e.g. the apparent insulin "dissociation constant") was varied from 0.01 (top curve) to 0.02, 0.05, 0.10, 0.20, 0.50 and 1.0 (bottom curve) ng/ml, respectively, and the value of σ was 2.7. Points represent theoretic transport rates in the presence of various levels of added insulin (ng/ml). The actual experimental insulin dose–response curve for stimulation of 2-deoxy-D-glucose uptake in adipocytes is also indicated on the figure (●—●). Adipocytes were prepared and treated with or without insulin at indicated doses for 5 min prior to measurement of 2-deoxy-D-glucose uptake as indicated previously in Ref. 26. Points are average transport rates in the presence of various levels of added insulin. The experimental data are effectively bounded by simulated curves having a K_D of 0.05 and 0.2 ng/ml.

RESULTS

Dose–response curve properties. Insulin dose–response curves for stimulation of hexose transport system activity were theoretically generated by first assuming a fixed basal rate of transport of 0.58 nmol hexose/10 min per 10^5 cells [26]. Predicted insulin dose–response curves are indicated in Fig. 1, where various levels of insulin were used to compute predicted transport velocities V_I over the range 0.1 to 20 ng/ml hormone. The effect of varying values of σ on curve shape is not shown, since σ is typically 2–3 under our experimental conditions [10].

An experimentally-generated insulin dose–response curve is also shown for a hormone dose range employed for the above-generated curves in Fig. 1. These data were obtained from an earlier study [26] conducted as indicated in Materials and Methods, in which cells were pretreated with insulin for 5 min prior to a subsequent assay of the uptake of 2-deoxy-D-glucose. The theoretically-generated dose–response curve that represents the best fit or estimate of the actual curve would have a K_D value of approximately 0.1 ng/ml (17 pM) since the experimental points (see also control dose–response curve in Fig. 3) are bounded by curves with K_D of 0.05 and 0.2 ng/ml hormone.

The deoxy-glucose data were also analyzed by plotting in the form of log dose–response curves and as Lineweaver–Burk-type plots, where the inverse velocities are plotted as a function of inverse insulin (e.g. agonist) dose. Log dose–response plots convert hyperbolic curves into a sigmoidal shape. The central linear portion was employed to extrapolate a K_D (as in Ref. 32) and yielded a value of 0.18 ng/ml (31 pM). For the inverse velocity (e.g. inverse percent stimulation of transport) versus inverse dose plot, an apparent $-1/K_D$ at the intersection of the abscissa yielded a dissociation constant of 0.14 ng/ml (24 pM) insulin (Fig. 2). Included on the plot are data from Fig. 3 and from other studies analyzed in this manner for comparison purposes (see Fig. 2).

Simulated insulin dose–response curves were also compared with the stimulation of natural D-glucose uptake by insulin over a dose range employed for the above dose–response curves. The data were obtained from an earlier study [26] where D-glucose uptake

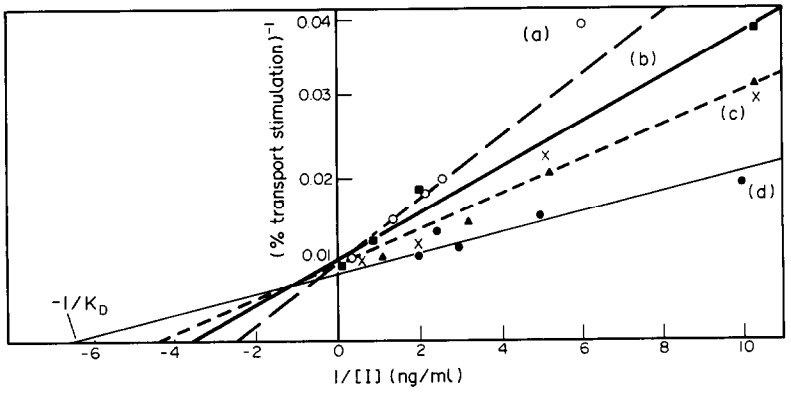

Fig. 2. Inverse (or double-reciprocal, Lineweaver–Burk-type) plot of the percent stimulation by insulin of transport above basal at various insulin doses. Data are from insulin dose–response curves generated in this study (see Fig. 3) and from various published dose–response data for 2-deoxy-D-glucose transport in adipocytes. Least squares lines were drawn through each set of data points, and all had high correlation coefficients (not shown). The X-intercept ($-1/K_D$) values range from approximately 0.14 to 0.5 ng/ml (24–80 pM) hormone. Lines drawn through individual dose–response data sets are indicated as follows: (A) data from Garvey *et al.* (○—○) [33]; (B) from Olefsky [34] (■); (C) data from Olefsky [24] (▲--▲) and from the present study (see Fig. 3) (×--×) are indicated by one line, due to close positioning of the points; (D) from Sauerheber *et al.* [26] (●).

(corrected for passive diffusion through the use of cytochalasin B) was measured by accurately determining the concentration of glucose in the medium both before and after a 1- to 2-hr incubation. Uptake in this case reflects the membrane transport rate of sugar since the transport step rate limits overall uptake in the absence of hormone at this glucose level (1 mM) and up to saturating levels of hormone [26]. The basal rate of uptake is in good quantitative ageement (within a factor of 2) with the transport rate obtained from the deoxy-glucose measurements. A theoretical curve that best estimated the curvature of the actual data had a K_D value of 0.1 ng/ml insulin (not shown).

Alcohol effects on time–course/dose–response of insulin action. To determine whether structural properties of the lipid bilayer of the membrane play a role in mediating the transfer of information from insulin binding sites to the glucose transporter, the bilayer lipid disordering agent benzyl alcohol was tested for effects on the insulin dose–response curve and the time-dependence of the insulin stimulation of hexose transport. Benzyl alcohol (25 mM) caused no significant effect on the dose–response curve (Fig. 3) within experimental error. Here, data are reported as the percent activation above basal caused by insulin; this normalizes the effects of alcohol on the intrinsic transport rate. Lower alcohol doses did not affect the dose–response curve detectably, even though transport rates were inhibited (not shown). Although higher alcohol doses likely shift the dose–response curve significantly, we did not investigate concentrations above 25 mM since the marked inhibition of the basal transport rate would require significant procedural modification to yield accurate dose–response curves.

The time-dependence of the insulin stimulation process was studied at 32°, where the stimulation time is prolonged conveniently to approximately 10 min. In agreement with earlier findings, the alcohol caused a 50% inhibition of basal and maximally insulin-stimulated transport rates, without affecting the maximal fold increase due to saturating insulin (Fig. 4). Unexpectedly, the time–course for the stimulation of transport was unaffected by alcohols over the range 5–25 mM. In each case, full stimulation was achieved within approximately 10–15 min insulin exposure time. Higher doses of alcohol cause more dramatic inhibition of transport and were not employed here for detailed time–course measurements.

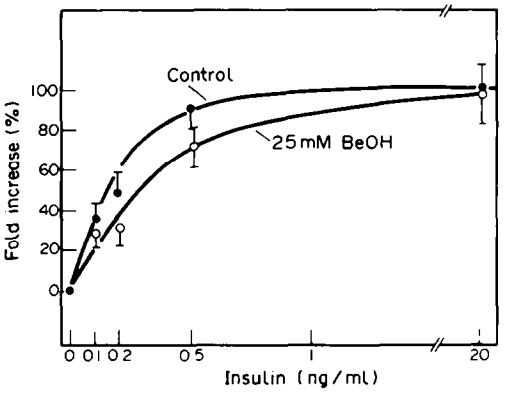

Fig. 3. Effects of benzyl alcohol on the insulin dose–response curve of hexose transport stimulation. The dose–response curves were generated as indicated in Materials and Methods for 2-deoxy-D-[^{3}H]glucose uptake, in the presence (○) and absence (●) of 25 mM benzyl alcohol. Uptake (percent of maximum at saturating insulin levels) is reported as a function of insulin concentration. Assays were conducted for 10 min, as indicated in the text. Data are from one of three experiments with freshly-prepared batches of cells.

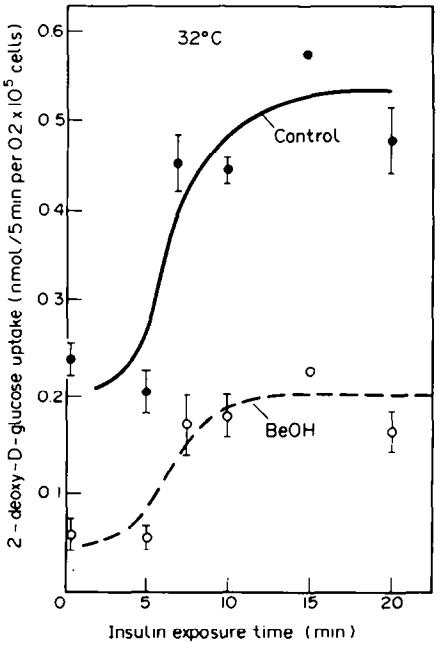

Fig. 4. Dependence of the time–course of the insulin stimulation of hexose uptake on alcohol. The time-dependence of 2-deoxy[^{3}H]glucose uptake stimulation by insulin (40 ng/ml) was assessed in the absence (●) and presence of (○) of 25 mM benzyl alcohol at 32°, as indicated in Materials and Methods. Uptake of hexose (conducted for 5 min throughout) is reported as a function of total insulin exposure time, which was varied from 0 to 20 min. The data are representative of three similar experiments with separately-prepared cell batches.

DISCUSSION

The time-dependence of the insulin stimulation of hexose transport in adipocytes, and also the insulin dose–response curve, were unaffected by treatment of cells with 25 mM benzyl alcohol. This alcohol dose caused an increase in lipid fluidity in adipocyte (and other cell) membranes, generally throughout the bilayer matrix, that may also be induced by a 5° increase in the membrane temperature [22, 23, 35, 36]. Transport responsiveness (fold increase) and sensitivity (half-maximal stimulation dose) to insulin, and insulin receptor–transporter coupling kinetics are, therefore, unaffected by this degree of lipid perturbation. Perhaps the hormone signaling mechanism is not described by diffusionally-limited con-

tact between receptor and transporter in the plane of the membrane [37]. The structural features of the lipid bilayer required to support the insulin stimulation process thus remain to be defined.

The actual insulin dose–response curves for 2-deoxy-glucose (Fig. 1) and D-glucose uptake stimulation were effectively mimicked by the theoretical curve generated (from Michaelis–Menten assumptions) for a dissociation constant (for the interaction of insulin with a site responsible for transport stimulation) of approximately 0.1 ng/ml (17 pM). Similar values (17 pM) were obtained from experimental data for insulin stimulation of transport, whether plotted as arithmetic dose–response, log dose–response (not shown), or as inverse (e.g. Lineweaver–Burk-type) plots. It is also of interest that Lineweaver–Burk-type plots, (Fig. 2), of the inverse of the percent insulin stimulation of 2-deoxy-glucose transport versus the inverse of the hormone concentration, for numerous earlier published insulin dose–response curves, as well as the data presented in Fig. 3, yielded apparent dissociation constants ranging from approximately 24–80 pM.

Although specific insulin receptors occur in adipocytes, where the affinity of the receptor is usually estimated at approximately 1–3 nM [47], recent data of binding to both rat [2] and human adipocytes [39] indicate a K_D for insulin at lower concentrations than emphasized in earlier studies [38]. For example, Taylor *et al.* [40] found that mono[^{125}I]insulin labeled at tyrosine 14 on the A chain binds to adipocytes at 37° and indicates an apparent specific, saturable, high-affinity insulin receptor where total binding is half-maximal at approximately 120 pM insulin.

Plotting the data of Taylor *et al.* according to Scatchard yields a typical curvilinear plot, where a high affinity binding site having a binding constant estimated at approximately 60 pM, may be a reasonable representation of a fraction of the binding data observed (Fig. 5). Here, the graphic method of Rosenthal [41, 42] was employed to correct for simultaneous binding of hormone to both sites at all insulin doses tested to obtain a better estimate of binding for each putative site separately. In a separate study [43] using similarly-labeled insulin, binding data with human adipocytes also revealed a "high affinity site", [44, 45] where extrapolation of a 5-point linear segment of the Scatchard plot yields a K_D of approximately 80 pM [43].

The agreement between indirectly-measured, apparent K_D in functional transport studies (17–80 pM), and dissociation constants directly-determined through recent insulin binding measurements (in the physiologic range of hormone concentrations), is thus reasonable, considering the assumptions that must be made to analyze the data. One advantage of using functional measurements to assess the binding constants is that nonspecific effects [46], and potentially incomplete reversability of binding that typically complicate physical binding measurements are eliminated. Finally, in an early study [38, 47], a good correlation between binding and functional measurements was obtained for insulin when the physiological range was considered.

Many receptor-mediated biological responses to drugs and other agonists are "graded" [25], such as catecholamine, ACTH and glucagon-stimulated adenylate cyclase [48], and display hyperbolic dose–response curves when reported on an arithmetic axis, sigmoidal curves when plotted as a function of log dose, and yield straight lines when plotted as inverse response versus inverse concentration (according to Lineweaver–Burk) [32]. Other agonist-modulated phenomena, such as glucose-induced insulin release and other stimulus–secretion processes, and also those involving multiple binding sites and cooperative interactions, usually follow sigmoidal dose–response curves on an arithmetic plot, and are thus comparable to the pharmacologic "quantal" or "all-or-none" response [25].

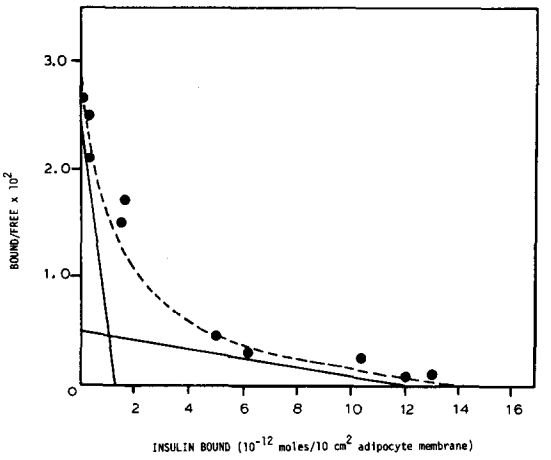

Fig. 5. Scatchard plot of data presented by Taylor *et al.* [40] (with kind permission) for insulin binding to adipocytes at 37°. Binding was performed with ^{125}I labelled insulin at tyr A14, as indicated in ref. 40. Estimates of binding parameters were made with the graphic method of Rosenthal [41, 42] where linear lines representing estimates of binding to low and high affinity sites were fitted by trial and error. Lines drawn from the origin through the field of the graph at regular intervals assisted in generating the predicted Scatchard plot, by the addition of linear segments from the origin to the high affinity site line and from the origin to the low affinity site line (41) (not shown). The experimental points (●) lie consistently close to the predicted curve drawn through the computed points (----).

In all cases examined in this study, the actual insulin dose–response curves were hyperbolic when plotted as a function of the arithmetic insulin dose (Figs. 1 and 3) [and were sigmoidal on a log scale (see Refs. 11 and 49)], typical of a "graded" response, where a single insulin molecule interacts with a single class of receptors, and the relative stimulation of transport is directly related to the fractional occupancy of functional receptors.

Since grossly perturbing the lipid phase was without measurable effects on the time-dependence of hexose transport stimulation, it is possible that select membrane lipid domains contain functional proteins that are sensitive to the occupied insulin receptor, such as the glucose transporter, cAMP phosphodiesterase, and any proteins involved in the anti-lipolytic effect of insulin. The receptor and functional protein molecules might form macromolecular complexes [37] or may be simply bounded/retained within structurally-distinct membrane lipid domains.

Recent X-ray crystallographic studies of proteins indicate that small rotational motions of packed helices, due to conformation changes in amino acid side chains, mediate the overall changes in 3-dimensional structure of a variety of allosteric enzymes, [50] which in some cases can exhibit a time-dependence not unlike insulin stimulation of transport [30].

If the above allosteric model is indeed applicable to the stimulation of glucose transport by insulin, then the hormone may cause the conversion of membrane transporters to a more active conformation, as suggested earlier [51]. This could perhaps be achieved [20] through direct interaction of the occupied high affinity receptor with the transport system, or via less direct coupling involving other proteins associated with the membrane. Indeed, insulin binding to plasma membranes and cells causes widespread alterations in the physical and organizational properties of the membrane [52, 53] over time–courses similar to that observed for the activation of hexose transport.

We conclude from the presented data that the kinetically simplest model should be considered for the mechanism of activation of hexose transport by the occupied insulin receptor.

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