Regulation of Insulin-stimulated Glucose Transport in the Isolated Rat Adipocyte

CAMP-INDEPENDENT EFFECTS OF LIPOLYTIC AND ANTI-LIPOLYTIC AGENTS*

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This paper examines the modulation of insulin-stimulated glucose transport activity in rat adipose cells by ligands for receptors (R) that mediate stimulation (Rs; lipolytic) or inhibition (Ri; anti-lipolytic) of adenylate cyclase. The changes in glucose transport activity and cAMP, as assessed by 3-O-methylglucose uptake and (-+/-) cAMP-dependent protein kinase (A-kinase) activity ratios, respectively, were monitored under conditions that maintain steady-state A-kinase activity ratios (Honnor, R. C., Dhillon, G. S., and Londos, C. (1985) J. Biol. Chem. 260, 15122-15129). Removal of endogenous adenosine with adenosine deaminase decreased insulin-stimulated glucose transport activity by ~30%, which was prevented or restored with Rs agonists such as phenylisopropyladenosine, nicotinic acid, and prostaglandin E, . These changes in transport activity were not accompanied by changes in A-kinase activity ratios, indicating that Ri-mediated effects on transport are independent of cAMP changes.

Addition of an Ri ligand, isoproterenol, in the presence of adenosine increased kinase activity but did not change glucose transport activity. Conversely, upon removal of adenosine, addition of Rs ligands such as isoproterenol, adrenocorticotropic hormone, or glucagon strongly inhibited transport (≥50%) and stimulated kinase activity. However, subsequent addition of phenylisopropyladenosine nearly restored transport activity without alteration of A-kinase activity. These data and additional kinetic experiments suggest that Rs-mediated glucose transport modulations are also independent of cAMP. The interchangeability of ligands for both Rs and Ri receptors in modulating transport activity suggests that these cAMP-independent effects are mediated by the stimulatory (N) and inhibitory (N) guanyl nucleotide-binding regulatory proteins of adenylate cyclase.

All Rs- and Ri-induced changes in transport activity occurred without a change in glucose transporter distribution, as assessed by d-glucose-inhibitable cytochalasin B binding, suggesting that Rs and Ri ligands modulate the intrinsic activity of the glucose trans-

porter present in the plasma membrane.

Studies from this (1-3) and Kono’s (4-6) laboratories have led to the formulation of the translocation hypothesis to explain insulin stimulation of glucose transport in rat adipose cells. Essentially, it is proposed that insulin induces the translocation of glucose transporters from a large intracellular pool to the plasma membrane by a rapid, reversible, and energy-dependent process. Accordingly, increased glucose transporter concentration in the plasma membrane, rather than a change in transporter intrinsic activity, is thought to be the basis of the increased \( V_{\text{max}} \) for glucose transport activity in response to insulin. This concept has been extended to explain certain pathophysiological conditions in which insulin resistance or a diminished response to insulin is associated with a diminished intracellular pool of glucose transporters in the basal state and, consequently, fewer transporters available for translocation to the plasma membrane in response to the hormone (7-9).

Lipolytic agents which stimulate adenylate cyclase, such as catecholamines, ACTH, and glucagon, in the absence of endogenous adenosine, acutely inhibit the \( V_{\text{max}} \) for both basal and insulin-stimulated glucose transport activity. This inhibition may be reversed by antilipolytic agents which act via receptors to inhibit adenylate cyclase, such as adenosine, nicotinic acid, and prostaglandins (10-16). It has been suggested, by implication, that these modulations in glucose transport activity result from changes in cellular cAMP concentration and the resultant changes in cAMP-dependent protein kinase activity. Hereafter, we shall refer to cAMP-dependent protein kinase as A-kinase and use this activity to reflect intracellular cAMP concentrations (17-19).

To assess the putative relationship between changes in cAMP concentration, glucose transport activity, and glucose transporter subcellular distribution, we have applied the stringently defined protocol for adipose cell incubations reported by Honnor et al. (20, 21). By eliminating the transient “peaking” phenomenon in kinase activity in response to lipolytic agents, this procedure permits the establishment and maintenance of relatively invariant A-kinase activity levels for up

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1 The abbreviations used are: ACTH, adrenocorticotropic hormone; PGE1, prostaglandin E1; A-kinase, cAMP-dependent protein kinase; Bt,cAMP, dibutyryl cAMP; PIA, \( N^\text{3}-[R-(-)-1-methyl-2-phenethyl] \) adenosine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Ro-20-1724, dl-4-(3-butoxy-4-methoxybenzyl)-2-imidazolidi-none; R- and Rs receptors that mediate stimulation or inhibition, respectively of adenylate cyclase; N and N, the stimulatory and inhibitory guanyl nucleotide-binding proteins, respectively, of adenylate cyclase.

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to 30 min following application of a lipolytic stimulus. Thus, the relationship between steady-state A-kinase activity and glucose transport activity can be assessed, allowing one to determine whether or not changes in transport by adenylate cyclase stimulators and inhibitors result from changes in cellular cAMP concentration. For increased sensitivity, we have examined these effects in the insulin-stimulated state although a previous report describes comparable findings in the basal state (16). The outcome of such studies reported here strongly suggests that 1) the alterations in the V_{max} for both lipolytic and anti-lipolytic hormones occur predominantly through a cAMP-independent process, and 2) the changes in glucose transport activity observed in the intact cell are the result of alteration in the intrinsic activity of the glucose transporters residing in the plasma membranes and not in the translocation of glucose transporter.

**EXPERIMENTAL PROCEDURES**

**Materials**—(-)-Isoproterenol, Bt_{4}-cAMP, PGE_{1}, Hepes, adenosine deaminase, and the A-kinase inhibitor were purchased from Sigma. PI-4 kinase from bovine brain, nicotinic acid from Eastman Kodak, ACTH_{1-24} from Behring Diagnostics, and glucagon from Lilly.

Sources for all other reagents were as reported previously for the glucose transport (3, 22) and A-kinase (20) procedures. Rats of the CD strain were supplied by the Charles River Breeding Laboratories. Insulin was a generous gift of Dr. Ronald E. Chance, Lilly. The cyclic nucleotide chelator and Ro-20-1724 were, respectively, 10 mM and 500 

**Cell Preparation and Measurement of Glucose Transport Activity**—Adipose cells were isolated from the epididymal fat pads of 180-200-g rats fed ad libitum with standard National Institutes of Health chow. The fat pads were digested with collagenase according to the method of Rodbell (23) as modified by Cushman et al. (24). All incubations were carried out in Krebs-Ringer buffer, pH 7.4, supplemented with 30 mM Hepes, 10 mM HCO_{3}, 5% (w/v) bovine serum albumin, and 2.5 mM glucose. Following their isolation, the cells were homogenized and A-kinase activity ratios determined. The data are presented as the -cAMP/+cAMP activity ratios, with corrections made for non-A-kinase activities (20).

**Subcellular Membrane Fractions and Determination of Glucose Transporters**—Isolated cells prepared from 12 g of adipose tissue (12-15 rats) were diluted to a final volume of 72 ml with the medium described by Cushman et al. (24). The medium described by Cushman et al. (24) and its composition were as follows: 500 ml of a saline solution containing 17 mM NaCl, 1.2 mM KCl, 1.2 mM CaCl_{2}, 1.2 mM MgCl_{2}, 12.5 mM glucose, and 175 mM NaHC0_{3} at pH 7.4 at 37 °C. The saline solution was pre-cooled to 4 °C and was used to make the saline solution from 500 ml of polypropylene containers at 37 °C with moderate shaking (70 strokes/min) in the above described shaking water bath. Following incubation, the cells were washed twice at 17 °C in a solution of 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 255 mM sucrose, and immediately homogenized. Three subcellular membrane fractions were then prepared by differential ultracentrifugation as previously described (22): a plasma membrane fraction, a high-density and a low-density microsomal membrane fraction. Incubation of cells with either lipolytic or anti-lipolytic hormones and agents had no effect on the subcellular fractionation of membranes, as assessed by the distribution of marker enzyme activities (22).

**RESULTS**

**Effects of Cell Mixing Speed**—Under the protocol established by Honnor et al. (20), rapid agitation of lipolytically stimulated adipocytes to ensure removal of released fatty acids by the medium albumin is essential for eliminating the transient peaking of A-kinase activity ratios. Fig. 1 presents a comparison of the time courses of kinase activity ratios in response to isoproterenol under both rapid and slow mixing conditions. With the slower, conventional shaking speed (40 strokes/min), kinase activities reached a peak within a few minutes and declined dramatically over 30 min, whereas with the higher mixing speed (110 strokes/min), elevated A-kinase activities were maintained throughout the incubation period.

Table I depicts representative experiments comparing the effects of cell mixing speeds on both glucose transport activities and the A-kinase activity ratios. Comparable inhibitions of insulin-stimulated glucose transport activities were observed irrespective of either the rate of cell mixing or the kinase activity ratios. Thus, despite considerable decreases in A-kinase activity at the slower shaking speed, the inhibition of transport activity persisted, suggesting that the maintenance of elevated kinase activity is not necessary for transport inhibition.

**Effects of Removal of Endogenous Adenosine**—Adenosine, a potent inhibitor of both adenylate cyclase and lipolysis, is inevitably present in isolated adipose cell suspensions (26, 27). The above experiments were performed in the presence of adenosine deaminase in order to convert endogenous adenosine to inosine, which is not an agonist of adenosine receptors. Fig. 2 shows the effects of adenosine removal with adenosine deaminase on both insulin-stimulated glucose transport activity in isolated adipose cells.
were taken for A-kinase activity determinations. Transport activities, glucose transport activities were measured and parallel samples were taken for A-kinase activity determinations. Transport activities represent the mean values of triplicate samples and A-kinase activity ratios are the means of triplicate determinations of duplicate samples. For Experiments B and C, data are shown for 30 min, a time at which the A-kinase activity ratios had declined from the high values seen at 5 min (see Fig. 1). The Table shows only one example of results at the higher shaking speed, Experiment D, since all other experiments presented in this paper were performed at this speed (see Figs. 4–8).

| Incubation condition  | Shaking speed | Glucose transport activity | A-kinase activity ratio |
|-----------------------|---------------|----------------------------|-------------------------|
|                       | Control       | 5 min                      | 0.28                    |
|                       | ISO* + ADA*   | 30 min                     | 0.80                    |
|                       | Control       | 5 min                      | 1.55                    |
|                       | ISO + ADA     | 30 min                     | 1.15                    |
|                       | Control       | 5 min                      | 3.05                    |
|                       | ISO + ADA     | 30 min                     | 1.10                    |
|                       | Control       | 15 min                     | 2.60                    |
|                       | ISO + ADA     | 30 min                     | 0.88                    |

*ISO, isoproterenol.
*ADA, adenosine deaminase.

transport activity and the A-kinase activity ratio. Adenosine removal led to a 30% reduction in the maximum rate of 3-O-methylglucose transport irrespective of whether deaminase was added before, together with, or after the addition of insulin. Adenosine deaminase addition either prior to or simultaneous with insulin did not alter the course of insulin stimulation of transport activity (Panels A and C), and transport activity declined with a t½ of 60–90 s when adenosine was removed subsequent to establishment of the transport response with insulin (Panel B). In all cases, the lower transport rate resulting from adenosine depletion occurred without a change in the A-kinase activity ratio. Kinase activity did rise slightly upon addition of deaminase prior to insulin, but rapidly returned to control levels after insulin addition (Panel C). All of the actions of adenosine deaminase seen in Fig. 2 were prevented or reversed by 100 nM PIA, a potent adenosine receptor agonist that is not metabolized by deaminase. Taking the adenosine-depleted condition as the reference point, Table II and Fig. 2 show that adenosine or PIA increased insulin-stimulated glucose transport by 40%. Similarly, in an adenosine-free medium, other antilipolytic agents that inhibit adenylate cyclase, such as PGE, and nicotinic acid, prevented the decrease in glucose transport activity upon adenosine removal with adenosine deaminase (Table II). Moreover, the enhancement of transport activity by these agents was not accompanied by a change in the A-kinase activity ratio (data not shown). Thus, both the reduction in insulin-stimulated glucose transport activity by metabolism of endogenous adenosine with adenosine deaminase and the enhancement of transport by ligands for adenylate cyclase inhibitory receptors occur without changes in A-kinase activity ratios. In all subsequent discussions we will refer to that condition in which endogenous adenosine has been removed by adenosine deaminase as the "ligand-free" state.

Effects of Isoproterenol—The effects of isoproterenol in the presence of endogenous adenosine on both glucose transport activity and A-kinase activity are illustrated in Fig. 3. Applying a protocol similar to that seen in Fig. 2B, cells were
obtained from triplicate samples. The results of A-kinase ratio are the means of triplicate determinations of duplicate samples. Results of transport rates are the means of triplicate samples. Results of transport rates are expressed as percentages of the value obtained from triplicate samples.

The effects of 200 nM isoproterenol in a ligand-free environment are shown in Fig. 4. Two minutes after the simultaneous addition of the β-adrenergic agonist and adenosine deaminase, the A-kinase activity ratio reached the maximum value, 1.0, which was maintained for the remainder of the incubation. Isoproterenol also produced a time-dependent (t½ ~ 2 min) ~70% inhibition of glucose transport activity (Fig. 4). The isoproterenol concentration dependency for both the inhibition of glucose transport and the stimulation of A-kinase is illustrated in Fig. 5. Note that the control value for transport activity depicted in Fig. 5 represents the insulin stimulated level of the ligand-free state. Under these conditions, the isoproterenol concentration-dependent increase in A-kinase activity was essentially paralleled by the decrease in glucose transport activity. Half-maximally effective concentrations for modulation of both responses were approximately 30-40 nM, suggesting that both systems are mediated through the same β-adrenergic receptor. However, the data in Figs. 4 and 5 are to be contrasted with those in Fig. 3, in which isoproterenol, in the presence of adenosine, leads to an increase in kinase activity without any decrease in transport activity.

Effects of Combinations of Isoproterenol and PIA—The experiments depicted in Figs. 6-8, involving manipulations with combinations of ligands for the adenosine and β-adrenergic receptors, demonstrate a dissociation between the effects of these ligands on glucose transport activity and A-kinase activity ratios. Fig. 6 shows the effect of PIA addition to cells that had been challenged first by isoproterenol in a ligand-free environment. In the initial phase, isoproterenol inhibited transport activity by ~50% and elevated the A-kinase activity ratio to 1.0. Subsequent addition of PIA rapidly (t½ = 2 min) restored the glucose transport to ~90% of the initial insulin-stimulated rate but, surprisingly, had little effect on A-kinase activity. The data in Fig. 7 show that the temporal sequence of ligand addition determined whether or not PIA lowered isoproterenol-stimulated A-kinase activity. PIA, when added together with isoproterenol, prevented the rise in kinase activity induced by a moderately high concentration of the β-adrenergic agonist. However, addition of PIA after exposure to isoproterenol failed to reduce kinase. Despite the temporal dependency for achieving PIA effects on A-kinase, the adenosine receptor agonist consistently maintained or restored glucose transport activity under all conditions tested.

Fig. 8 shows the effects of varying concentrations of both isoproterenol and PIA on glucose transport activity and A-kinase activity ratios. Two features of the data are to be
noted. First, the PIA concentration requirement for reversing isoproterenol-induced transport inhibition was independent of the isoproterenol concentration (see “Discussion”). Second, as in Fig. 7B, all changes in transport activity elicited by PIA occurred without a change in the kinase activity ratio. Fig. 8, top, also shows that the final level to which PIA restored transport activity was dependent upon the concentration of isoproterenol in the incubation, but seemingly independent of the kinase activity, which was maximal for all isoproterenol concentrations tested.

Other Lipolytic and Antilipolytic Agents—In Table III the actions of isoproterenol and PIA are compared with two other lipolytic hormones, ACTH and glucagon, and two other antilipolytic agents, PGE, and nicotinic acid. As with isoproterenol (Fig. 3), ACTH and glucagon did not inhibit glucose transport activity in the presence of adenosine (Table III). However, in a ligand-free environment, all three lipolytic hormones at maximally effective concentrations induced a 50–60% inhibition of transport compared to the 63% inhibition observed in the absence of adenosine. In the ligand-free state, Bt2cAMP induced only a 20% inhibition of transport activity, which had been inhibited by isoproterenol. These results are consistent with the notion that all the hormones, both lipolytic and antilipolytic, appear to exert their respective actions on insulin-stimulated glucose transport activity interchangeably, suggesting mediation by common mechanisms distal to the initial hormone receptor interactions.

Effects of Bt2cAMP—The data in Table IV compare the effects of Bt2cAMP and isoproterenol on glucose transport activity. In the ligand-free state, Bt2cAMP induced only a 20% inhibition of transport compared to the 63% inhibition observed in the presence of adenosine.
Adipocytes were incubated for 15 min in the presence of 7 nM insulin, and then for an additional 15 min in the further presence of the ligands indicated. Values represent the means ± S.E. of triplicate determinations, expressed as a percentage of the transport rate of control, which is the activity measured in the presence of adenosine deaminase. As previously reported, insulin-stimulated glucose transport activity in isolated adipocytes was followed by a further 15-min incubation in the presence of 1 unit/ml adenosine deaminase and the additions as indicated in the Table. Transport activities are expressed as the percent of control, insulin-stimulated cells measured in the presence of adenosine deaminase plus insulin. For this experiment the control glucose transport rate was 3.2 ± 0.21 fmol/cell/min. Values shown represent the means ± S.E. of triplicate determinations.

### Table III: Regulation of Adipocyte Glucose Transport Activity

| Incubation condition | Glucose transport activity | % of control |
|----------------------|---------------------------|--------------|
| Adenosine deaminase  | Isoproterenol             | Other additions |
| 1 unit/ml            | 200 nM                    | % of control |
| +                    | None                      | 100          |
| +                    | None                      | 143 ± 6      |
| +                    | Isoproterenol, 1000 nM    | 138 ± 6      |
| +                    | ACTH, 100 nM              | 133 ± 9      |
| +                    | Glucagon, 1000 nM         | 139 ± 3      |
| +                    | Isoproterenol, 15 nM      | 79 ± 9       |
| +                    | Isoproterenol, 1000 nM    | 44 ± 6       |
| +                    | ACTH, 1.5 nM              | 76 ± 7       |
| +                    | ACTH, 100 nM              | 39 ± 3       |
| +                    | Glucagon, 75 nM           | 73 ± 6       |
| +                    | Glucagon, 1000 nM         | 51 ± 6       |
| +                    | Isoproterenol, 15 nM      | 47 ± 7       |
| +                    | Isoproterenol, 1000 nM    | 56 ± 6       |
| +                    | glucagon, 75 nM           | 56 ± 6       |
| +                    | Isoproterenol, 1000 nM    | 36 ± 3       |
| +                    | ACTH, 1000 nM             | 100          |
| Experiment B         |                           |              |
| +                    | None                      | 100          |
| +                    | None                      | 143 ± 7      |
| +                    | None                      | 48 ± 7       |
| +                    | PIA, 1000 nM              | 124 ± 9      |
| +                    | PIA, 1 nM                 | 84 ± 11      |
| +                    | NA, 1000 nM               | 124 ± 10     |
| +                    | NA, 100 nM                | 80 ± 9       |
| +                    | PGE2, 10 nM               | 94 ± 7       |
| +                    | PGE2, 0.5 nM              | 57 ± 3       |
| +                    | PIA, 1 nM + NA, 100 nM    | 102 ± 4      |
| +                    | PIA, 1 nM + PGE2, 0.5 nM  | 93 ± 14      |
| +                    | PIA, 1000 nM              | 131 ± 4      |
| +                    | NA, 1000 nM               | 100          |

* NA, nicotinic acid.

### Table IV: Comparison of the effects of isoproterenol and Bt-cAMP on insulin-stimulated glucose transport activity in isolated rat adipose cells

| Incubation condition | Glucose transport activity | % of control |
|----------------------|---------------------------|--------------|
| Adenosine deaminase  | Isoproterenol             | Other additions |
| Control              | -                         | 154 ± 7      |
| +                    | Isoproterenol, 1 nM       | 130 ± 4      |
| +                    | Bt-cAMP, 1 nM             | 82 ± 7       |

Bt-cAMP inhibited transport activity by 47% whereas isoproterenol induced only a 16% inhibition. Thus, the effects of Bt-cAMP and isoproterenol on glucose transport activity are modulated differently by adenosine.

**Subcellular Distribution of Glucose Transporters**—A previous report from this laboratory suggested that isoproterenol in combination with adenosine deaminase induced both a reduction in the intrinsic activity of the glucose transporters in the plasma membrane and an impairment of the insulin-induced translocation of glucose transporters from the intracellular pool to the plasma membrane (16). Applying the new incubation protocols as described in Fig. 5, Fig. 9 shows that these conclusions require re-evaluation. As previously reported, insulin's stimulatory effect on glucose transport activity in the presence of adenosine was accompanied by an approximately 2.5-fold increase in the concentration of glucose transporters in the plasma membranes, as assessed by cytochalasin B binding. However, incubation with insulin in a ligand-free environment did not alter the plasma membrane glucose transporter concentration despite a 30% decrease in glucose transport activity observed in the intact cell. Moreover, in contrast to previous observations, the addition of isoproterenol in the absence of adenosine did not induce a significant decrease in the number of transporters in the plasma membranes, despite eliciting a 70% reduction in insulin-stimulated 3-O-methylglucose transport activity in intact cells. Finally, addition of PIA subsequent to isoproterenol stimulation restored transport activity to 50% of the initial activity seen with insulin plus adenosine, again without changing the concentration of glucose transporters in the plasma membranes. Correspondingly, the insulin-induced 60% reduction in transporters residing in the low density microsomal fraction was not modified by either adenosine or isoproterenol. Also, there were no significant changes in the low concentrations of glucose transporters found in the high-density microsomal fraction in the presence or absence of any of the above agents (data not shown).

**DISCUSSION**

Rat adipocyte adenylate cyclase is regulated by two opposing circuits, each containing a complement of stimulatory (R) and inhibitory (Ri) receptors and their associated GTP-binding regulatory proteins, Nt and N, respectively (28–30). The R, and Ri receptors have been shown to regulate the cAMP-independent antilipolytic insulin effect in adipose cells, leading to the proposal that the R-N circuits impinge on processes other than adenylate cyclase (31). In this report we have attempted to answer two questions. First, with the use of A kinase activity ratios to monitor cellular cAMP concentrations, can one attribute R, and Ri effects on glucose transport activity to changes in cAMP? Second, we asked whether these R, and Ri effects resulted from changes in the subcellular distribution of the glucose transporters or from changes in intrinsic activity of the transporter. In both cases, we employed maximally insulin-stimulated cells to optimize our ability to measure small changes in transport activity. However, in our previous study (16), qualitatively similar effects of R, and Ri ligands were observed in both basal and insulin-stimulated cells. The evidence appears to suggest that R, and Ri modulate the intrinsic activity of the plasma membrane glucose transporter through a cAMP-independent mechanism.
Consider first the effects of adenosine on glucose transport rates. Insulin-stimulated transport activity is significantly decreased upon removal of the adenosine that inevitably appears as a contaminant of isolated adipocyte suspensions, especially at the relatively high cell concentrations used routinely in glucose transport studies (Figs. 2, 7, and 8; Table II) (26). When added to cells in the ligand-free state (adenosine removed), the adenosine receptor agonist PIA enhances glucose transport activity by 40%. Despite these large fluctuations in glucose transport activity, A-kinase activity ratios remain low (≤0.05) and unchanged, which is explained by the presence of supramaximal insulin concentrations (See Fig. 2C). Another apparent A-kinase-independent effect of the adenosine receptor agonist is observed upon fixing kinase ratios at varying levels by adding an R, ligand (isoproterenol) to cells in an otherwise ligand-free environment (Figs. 6–8). That is, if one first establishes inhibited transport rates and high kinase levels with isoproterenol, subsequent addition of PIA nearly restores transport activity but the kinase activity remains elevated and unchanged (see below). Finally, with lipolysis, an R, and R,-mediated process linked tightly to cAMP metabolism, increasing concentrations of R, ligands are required to reverse the actions of increasing concentrations of R, ligands (21). However, the PIA concentration required to reverse transport inhibition by increasing isoproterenol concentrations does not vary (Fig. 8). Therefore, insofar as A-kinase activity ratios reflect cellular cAMP concentrations, adenosine receptor-mediated changes in glucose transport in insulin-stimulated adipocytes are not mediated by changes in cAMP.

Ligands for other adipocyte R, receptors, such as nicotinic acid and prostaglandinshave been shown to mimic the effects of adenosine and PIA on transport activity both in the absence (Table II) or presence of isoproterenol (Table III). Furthermore, the effects of these agents are all additive at submaximal concentrations (Table II). Thus, it is likely that the cAMP-independent effects of the R, receptors are mediated by factors separate from but common to all of the R, receptors, presumably the N, regulatory proteins.

Although not as clear-cut as the data with the R, effectors, the data presented herein argue against cAMP involvement in glucose transport inhibition by R, ligands, such as isoproterenol, the limitation being the inevitable increase in cAMP and A-kinase activity upon application of an R, stimulant. Nevertheless, the following disparities may be noted. First, stimulation of kinase activity ratios to nearly 0.5 by isoproterenol in an adenosine-replete medium does not inhibit transport activity (Fig. 3). However, with increasing isoproterenol concentrations in an adenosine-free medium, significant inhibition of glucose transport is seen as the kinase ratio approaches 0.5 (Fig. 5). Similarly, the inhibition of transport by isoproterenol in an adenosine-free medium persists when, under slow cell mixing conditions, the A-kinase activity ratio declines to 0.19–0.41 (Table I). Thus, one may activate A-kinase activity ratios to 0.5 with isoproterenol and, depending on the incubation conditions, see either a marked inhibition or no effect on glucose transport activity.

Second, dependent on the incubation condition (compare Figs. 5 and 8), progressive glucose transport inhibition may be seen with increasing isoproterenol concentrations beyond those required to achieve kinase ratios of 1.0. Finally, the observation that R, reversal of the effects of isoproterenol apparently occurs through a cAMP-independent process tends to raise doubts about the role of cAMP in the mediation of transport inhibition by isoproterenol in the first instance. Taken together, these results strongly suggest a cAMP-independent inhibition of glucose transport by β-adrenergic effectors. Analogous with the R, system discussed above, the finding that other R, ligands, such as corticotropin and glucagon, mimic isoproterenol and exhibit additive effects on glucose transport at submaximal concentrations (Table III) suggests that the R, receptors modify transport activity via their GTP regulatory protein, N.
in intracellular pool to the plasma membrane (16). By contrast, in this paper we show that glucose transport regulation by isoproterenol and adenosine occurs without a change in the subcellular distribution of the transporter, indicating that R, and R, receptors modulate intrinsic transporter activity within the plasma membrane. The present study employed lower cell concentrations, shorter incubation times, and more vigorous cell mixing conditions than earlier studies. Another important difference is the sequence of addition of the various hormones and agents. In this paper, we report data on cells that were stimulated first by insulin, following which R, and R, ligands were tested, whereas previously the R, ligands and insulin were added simultaneously. Whether the different methodologies account for the different results remains to be determined.

The hypothesis proposed here that R, and R, ligands alter the intrinsic activity or turnover number of those glucose transporters residing in the plasma membrane is in marked contrast with previous studies of insulin resistance, such as seen in the high-fat fed rat (8) and the diabetic rat (9), where the observed 40-50% inhibition of insulin-stimulated glucose transport activity directly correlates with the concentration of plasma membrane glucose transporters. The converse has also been observed in the young Zucker rat (33) and the hyperinsulinemic rat (34) in which insulin hyper-responsive- ness is associated with an increase in the concentration of transporters in the plasma membranes. The ability to readily detect these changes in transporter concentration using the cytochalasin B binding assay clearly demonstrates that the assay is of sufficient sensitivity to have detected changes in transporter distribution induced by R, and R, ligands had they occurred.

More recently, Joost et al. (35, 36) have demonstrated that the inhibition of insulin-stimulated glucose transport activity in the intact adipose cell induced by isoproterenol in the absence of adenosine can be retained in preparations of isolated plasma membranes by homogenizing the cells in the absence of adenosine can be retained in preparations of isolated plasma membranes by homogenizing the cells in the presence of KCN. Moreover, the preservation of this inhibited glucose transport activity occurs without alteration of the concentration of glucose transporters in the plasma membrane, which remains unchanged with isoproterenol treatment. These results appear therefore to directly demonstrate the inhibitory action of isoproterenol on the intrinsic activity of the plasma membrane glucose transporter.

Coupled with the realization that recent technical improvements in manipulating isolated adipocytes permit establishment and maintenance of steady-state A-kinase activity ratios (20), an impetus for re-examining the actions of R, and R, effectors on insulin-stimulated glucose transport activity was the finding that these ligands modify inhibition of lipolysis by insulin (31). Lipolytic activity in a ligand-free environment is significantly less sensitive to insulin than lipolytic activity seen in the presence of both R, and R, ligands (31), and such regulation applies to both cAMP-related and cAMP-independent insulin effects on lipolysis. Similarly, in studies previously reported (12, 32), we and others have found that both isoproterenol and adenosine modulate the sensitivity to insulin in initiating the glucose transport response, which might reflect an action of these ligands on the insulin signaling process and subsequent transporter translocation. It should be noted that to eliminate such effects in this study, a saturating concentration of insulin was employed in all experiments.

An enigma presented by the results is the inability of PIA to reduce A-kinase activity ratios in cells previously exposed first to insulin and then to isoproterenol. Note that this sequence of ligand addition is critical for eliciting resistance to the adenosine receptor agonist. This phenomenon did not result from an apparent overshoot of cAMP to concentrations beyond those required to activate kinase, since resistance to PIA was observed at kinase activity ratios well below 1.0 (Fig. 7). Tests on adenylate cyclase activities in purified plasma membranes from such cells revealed that they were normally responsive to inhibition by GTP, PIA, and other inhibitory ligands. Whatever the mechanism, resistance to R, ligands is elicited only in insulin-stimulated cells, since in many experiments we found that cells first exposed to isoproterenol in the absence of insulin were highly susceptible to subsequent inhibition of kinase with PIA (21). It is of interest that this peculiar lack of response to PIA occurs despite an adenosine receptor-mediated effect on the restoration of glucose transport activity, indicating that cells may differentially regulate different responses to R, or R, N, signals. A potentially related anomaly is the relationship between the inability of PIA to fully restore isoproterenol-inhibited glucose transport and the initial degree of inhibition induced by the increasing isoproterenol concentrations (Fig. 8). Only the extent of restoration of glucose transport activity and not the sensitivity to PIA is correlated to the degree of inhibition. This absolute loss of glucose transport activity may well be related to the, albeit unphysiological, effect of BtzcAMP (Table IV). This agent also induces an absolute decrease in insulin-stimulated glucose transport which is insensitive to restoration by the antilipolytic hormones. These effects potentially reflect a temporal response of the adipose cells to elevated cAMP/A-kinase activity leading to a general secondary, slowly reversible desensitization of the initial mechanism regulating glucose transport.

In summary, our data indicate that R, and probably R, ligands modify glucose transport activity in isolated adipose cells by a cAMP-independent mechanism(s) involving regulation of the intrinsic activity of the glucose transporter in the plasma membrane. Of perhaps broader significance than transport regulation is the demonstration of involvement of adipocyte receptor complexes known primarily for their link- age to adenylate cyclase in events apparently unrelated to cAMP, a finding reminiscent of the modulation by these receptors of the cAMP-independent inhibition of lipolysis by insulin (31). Based on arguments presented above and previously (31), we speculate that in exerting their cAMP-inde- pendent actions, R, and R, receptors act through their respective GTP regulatory complexes, N, and N. Thus, regulation of the glucose transporter would join the family of other R,- and R,-mediated, cAMP-independent processes, such as β-adrenergic receptor-mediated inhibition of MgATP (37) and N-formyl peptide receptor-mediated arachidonic acid release (38). Examples in which other insulin responsive systems appear to be modulated by R-N complexes include phosphodiesterase activation in adipose (39) and liver (40) cells and phosphatidylinositol turnover (41). Thus, there is a growing body of evidence suggesting that the concerted regulation of lipogenesis and lipolysis in rat adipocytes involves the interaction of R-N complexes with insulin at many levels.

Acknowledgments—We wish to thank Mary Jane Zarnowski, Dana R. Yer, and Douglas L. Johnson for expert technical assistance. We also thank Drs. Soraya Naghshineh and Hans Joost for making data available prior to publication.

S. Naghshineh, unpublished observations.

R. C. Hosanor and C. Londos, unpublished observations.
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