

Phosphorylation state of the GLUT4 isoform of the glucose transporter in subfractions of the rat adipose cell: Effects of insulin, adenosine, and isoproterenol

(lipolytic hormones/antilipolytic hormones/cyclic AMP-dependent protein kinase)

H. NISHIMURA*, J. SALTIS*†, A. D. HABBERFIELD*‡, N. B. GARTY§¶, A. S. GREENBERG§, S. W. CUSHMAN*, C. LONDOS§, AND I. A. SIMPSON*||

*Experimental Diabetes, Metabolism and Nutrition Section, Diabetes Branch, and §Membrane Regulation Section, Laboratory of Cellular and Developmental Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892

Communicated by Rachmiel Levine, September 24, 1991 (received for review July 8, 1991)

ABSTRACT The acute effects of insulin, adenosine, and isoproterenol on the activity, subcellular distribution, and phosphorylation state of the GLUT4 glucose transporter isoform were investigated in rat adipocytes under conditions carefully controlled to monitor changes in cAMP-dependent protein kinase (A-kinase) activity. In contrast to GLUT1, which has not been shown to be phosphorylated even when cells are exposed to any of the above agents, GLUT4 was partially phosphorylated (0.1–0.2 mol/mol) when the activity of the A-kinase was suppressed, and remained unchanged in response to insulin. Isoproterenol elicited a 64% inhibition of insulin-stimulated glucose transport activity in the absence, but not the presence, of adenosine receptor agonists. However, in either the presence or the absence of agonists, A-kinase was activated as assessed by examining the phosphorylation of the major adipocyte A-kinase substrate, perilipin. Similarly, under either condition, phosphorylation of GLUT4 was enhanced 1.4-fold in the intracellular membranes, but no significant change was observed in the plasma membrane. In the absence of adenosine receptor agonists, isoproterenol exerted a small (14%) but significant inhibition of the insulin-induced translocation of GLUT4 but had no effect on the translocation of GLUT1. Thus, changes in the phosphorylation state and/or subcellular distribution of GLUT4 cannot account for the inhibition of insulin-stimulated glucose activity induced by isoproterenol.

Insulin stimulates glucose transport activity in rat adipose cells primarily by inducing the translocation of the transporter isoforms GLUT1 and GLUT4 from an intracellular location to the plasma membrane (1–4). GLUT1 is widely distributed whereas GLUT4, the predominant form in adipose cells (4–6), is found only in tissues where insulin regulates glucose transport activity (i.e., white and brown adipose tissue, heart, and skeletal muscle; for review see refs. 7 and 8). Hereafter, *insulin-stimulated glucose transport activity* will be abbreviated as transport activity.

A second level of transport regulation is exerted by agents that modulate adenylyl cyclase and lipolysis (9–11). Lipolytic agents (isoproterenol, glucagon, and corticotropin) inhibit transport activity, but only in the absence of antilipolytic agents (adenosine, nicotinic acid, and prostaglandin E₁). Further, Smith *et al.* (12) and Kuroda *et al.* (13) demonstrated that both the transport inhibition by lipolytic agents and the augmentation by antilipolytic agents did not result from changes in transporter location.

An unresolved issue is whether the changes in transport activity mediated by these various agents are related to the changes in cAMP. Currently, two distinct mechanisms have

been proposed. Kuroda *et al.* (13) demonstrated an apparent dissociation between isoproterenol-mediated stimulation of cAMP-dependent protein kinase (A-kinase) and inhibition of transport activity, suggesting that changes in A-kinase-mediated phosphorylation are not responsible for transport inhibition. This was supported by Joost *et al.* (14), who showed that the GLUT1 isoform of the glucose transporter was not phosphorylated in response to insulin, adenosine, or isoproterenol. More recently, however, with the advent of anti-GLUT4 antibodies, James *et al.* (15) and Lawrence *et al.* (16) have demonstrated that (i) GLUT4 is phosphorylated “in intact cells,” (ii) GLUT4 is a “cell-free” target of A-kinase, and (iii) “cell-free” phosphorylation is augmented by lipolytic agents. Hence, they proposed that transport activity in the adipose cells is regulated by an A-kinase-dependent phosphorylation of GLUT4.

We have compared transport activity and the phosphorylation state of GLUT4 under conditions carefully controlled to monitor simultaneous changes in transport activity and A-kinase activity. We confirmed that GLUT4 is phosphorylated and that isoproterenol mediates a small increase in GLUT4 phosphorylation. This enhanced phosphorylation is confined to intracellular membranes and occurs both in the presence and in the absence of adenosine receptor activation, but isoproterenol inhibits transport activity only in the absence of adenosine receptor agonists, suggesting that phosphorylation does not explain the inhibition of transport activity.

METHODS

Animals and Cell Preparation. Adipose cells were isolated from 50–56 male rats (170–200 g, CD strain, Charles River Breeding Laboratories) as described (17). The cells (0.8–1.2 × 10⁶ per ml) were preincubated for 90 min at 37°C in the presence or absence of ³²P₁ [0.1 mCi (3.7 MBq)/ml] in a Krebs–Ringer medium, pH 7.4, containing 2.5 mM glucose, 10 mM sodium bicarbonate, 0.1 mM sodium phosphate, 30 mM Hepes, and 5% (wt/vol) albumin. To suppress A-kinase

Abbreviations: A-kinase, cAMP-dependent protein kinase; PIA, (R)-N⁶-(1-methyl-2-phenylethyl)adenosine; 8DPCPX, 8-cyclopentyl-1,3-dipropylxanthine.

†Present address: Baker Medical Research Institute, P.O. Box 348, Prahran, Victoria 3181, Australia.

‡Present address: Amgen Incorporated, Amgen Center, Thousand Oaks, CA 91320.

¶Present address: The Weizmann Institute of Science, Rehovot, Israel.

||To whom reprint requests should be addressed at: Experimental Diabetes, Metabolism and Nutrition Section, Diabetes Branch, National Institute of Diabetes and Digestive and Kidney Diseases, Building 10, Room 5N102, National Institutes of Health, Bethesda, MD 20892.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.

activity, 200 nM adenosine and 10 nM (*R*)-*N*⁶-(1-methyl-2-phenylethyl)adenosine (PIA) were included in the incubation medium. After centrifugation through dinonylphthalate and resuspension in an equal volume of the above medium without ³²P_i, the cells were subjected to the protocols outlined below.

Cell Incubation and Processing. Both control and radiolabeled cells were incubated in parallel and subjected to the protocols outlined in Fig. 1. Specific methods are described below. Following removal of 200- μ l aliquots for transport activity determinations (18), the remaining cells were fractionated.

Preparation of Subcellular Membrane Fractions. Cells from each incubation were centrifuged through dinonylphthalate (5 ml) and resuspended in 20 ml of homogenization buffer (20 mM Tris/1 mM EDTA/255 mM sucrose, pH 7.4) containing phosphatase inhibitors (0.2 mM vanadate, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate) at 17°C. The cells were homogenized and plasma membranes and intracellular membranes were prepared (17). For the reasons noted by Shibata *et al.* (19), the intracellular membranes correspond directly to the membranes erroneously named "low-density microsomes" (20). Membrane protein was assayed using bicinchoninic acid by a modification of the method of Smith *et al.* (21) according to the manufacturer's instructions (Sigma). Cytochalasin B binding was determined in the subcellular fractions from unlabeled cells (17). To confirm reproducible fractionation, membranes were analyzed for GLUT1 and GLUT4 by Western blotting.

Immunoprecipitation of Glucose Transporters. Immunoprecipitation of GLUT4 from radiolabeled cells was as described (14), with 1 mg of solubilized membrane protein and either a mouse monoclonal antibody (1F8, a gift from Paul Pilch, Boston University) or a rabbit antibody raised against the 15-amino acid C-terminal sequence of GLUT4 (a gift from Hoffman LaRoche). The bound complex was washed sequentially with SDS- and NaCl-containing buffers (22). The immune complex was eluted with sample buffer and 80% of the sample subjected to SDS/PAGE (23). The gels were dried and autoradiographed at -70°C for 12 hr. ³²P-labeled glucose transporter was quantitated by cutting out the band for Cerenkov counting. To determine the distribution of GLUT4 the remaining 20% was Western blotted using 1F8 and

¹²⁵I-labeled sheep anti-mouse IgG. Transporters were quantitated by γ counting of excised bands.

Assessment of A-Kinase Activity. A-kinase activity was assessed by viewing the behavior of the major adipocyte A-kinase substrate, perilipin, in SDS/PAGE. This protein is phosphorylated in unstimulated cells (quiescent A-kinase) and migrates in SDS/PAGE as a 62-kDa protein, but it gains an additional 5 mol of phosphate per mol as the \pm cAMP A-kinase activity ratio rises to 0.35-0.5, at which point it migrates as a 65/67-kDa doublet (24, 25). Thus, the migration patterns of the various phosphorylation species of perilipin provide a sensitive indicator of A-kinase activity (25).

Determination of ATP-Specific Radioactivity. Two hundred microliters of the cell suspension was added to 22 μ l of 25% perchloric acid, mixed vigorously, and placed on ice. The precipitated samples were centrifuged for 10 min at 10,000 \times g at 4°C, and 100 μ l of supernatant was added to 100 μ l of solution consisting of 4.2 M KOH, 1 M HEPES (pH 7.6), and water (1:2:7). ATP was determined in 10- μ l aliquots of a 1:10 dilution of the neutralized supernatants by luminometry in a Packard Picolite luminometer with a firefly luciferin/luciferase kit (Analytical Luminescence Laboratories, San Diego, CA). Aliquots of the neutralized supernatant were assayed also for ATP labeled in the γ position by quantitatively transferring the terminal phosphate to the A-kinase peptide substrate Kemptide, with an excess of A-kinase [purified from rat adipocytes (26)]. The A-kinase reaction mixture contained 100 μ M Kemptide, 20 mM Mops (pH 7.0), 16 mM magnesium acetate, 4 mM dithiothreitol, 10 μ M cAMP, and usually 20 μ l of the neutralized supernatant. The reaction was initiated with A-kinase and conducted for 1 hr at 30°C to ensure complete transfer. The reactions were terminated and ³²P-labeled peptide was purified (27). The specific activity of ATP was determined to be \approx 500 cpm/pmol, which is consistent with previous observations (28, 29).

Calculations. Statistical significance was tested with one-way analysis of variance followed by Duncan's multiple range test and a paired Student's *t* test, as appropriate, and differences were accepted as significant at the *P* < 0.05 level.

RESULTS

Since assessments of cellular phosphorylation require \approx 90 min of incubation with ³²P_i at a reduced P_i concentration (28), we tested the consequences of these incubation conditions on glucose transport activity (Table 1). Prolonged incubation enhanced both basal and insulin-stimulated transport activity while blunting the inhibitory response to isoproterenol from 46.0 \pm 1.4% to 28.0 \pm 0.6 (*P* < 0.01). These effects were reversed by washing the cells through oil and resuspending them in fresh low-P_i medium, the method employed to ensure a maximal response to isoproterenol.

The role of phosphorylation in the regulation of transport activity was assessed by four protocols (Fig. 1). In each case

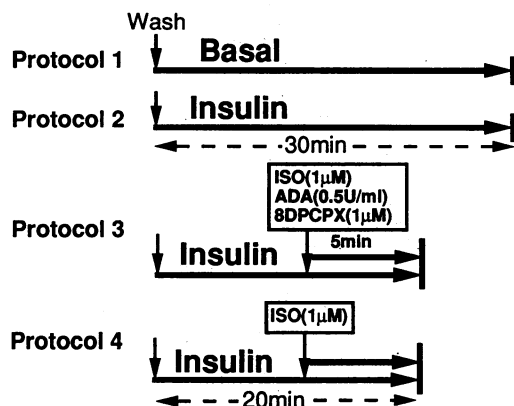


FIG. 1. Incubation protocols. Rat adipose cells were preincubated in the presence of low P_i (0.1 mM) with or without ³²P (0.1 mCi/ml) for 90 min, washed through dinonylphthalate, and resuspended in fresh Krebs-Ringer buffer, and treated with one of the following protocols: 1, no further additions for 30 min [BAS (basal)/ADO (adenosine)]; 2, addition of insulin (0.66 μ M) for 30 min (INS/ADO); 3, addition of insulin (0.66 μ M) for 15 min followed by addition of isoproterenol (ISO, 1 μ M), 8-cyclopentyl-1,3-dipropylxanthine (8DPCPX, 1 μ M), and adenosine deaminase (ADA, 2.5 μ g/ml, 0.5 unit/ml) for 5 min (INS/ISO); 4, addition of insulin (0.66 μ M) for 15 min followed by isoproterenol (1 μ M) for 5 min (INS/ADO/ISO).

Table 1. Effect of P_i equilibration on isoproterenol-mediated inhibition of insulin-stimulated glucose transport activity

	Transport activity, fmol/min per cell		
	A	B	C
Protocol 1	0.06 \pm 0.02	0.09 \pm 0.03	0.06 \pm 0.01
Protocol 2	3.04 \pm 0.30	3.98 \pm 0.53	3.03 \pm 0.11
Protocol 3	1.64 \pm 0.05	2.92 \pm 0.35*	1.65 \pm 0.10
(Inhibition [†])	(46.0 \pm 1.2%)	(28.1 \pm 0.6%)*	(45.2 \pm 0.9%)

Protocols 1-3 (Fig. 1) were compared in cells that had been acutely incubated (30 min) in low P_i (A), equilibrated for 120 min with low P_i (B), or equilibrated for 90 min in low P_i and subsequently resuspended in fresh low P_i buffer for 30 min (C).

**P* < 0.01 vs. A.

[†]Inhibition = [1 - (protocol 3/protocol 2)] \times 100.

parallel incubations were performed on cells equilibrated with low P_i in the presence or absence of $^{32}P_i$.

Insulin (protocol 2) stimulated 3-*O*-methylglucose uptake ≈ 50 -fold above basal activity (protocol 1) (3.02 ± 0.08 vs. 0.06 ± 0.01 fmol/min per cell) (Fig. 2). In the absence of adenosine receptor stimulation—i.e., removal of adenosine by adenosine deaminase and addition of the adenosine receptor antagonist 8DPCPX ($1 \mu M$) to block the binding of the deaminase-resistant adenosine receptor agonist PIA (protocol 3)—isoproterenol inhibited transport activity by 64% (1.11 ± 0.06 fmol/min per cell). This inhibitory response was suppressed by the presence of adenosine and PIA (3.21 ± 0.22 fmol/min per cell) (protocol 4).

The corresponding subcellular distribution of total glucose transporters, GLUT1 plus GLUT4, was determined by cytochalasin B (CB) binding, and the distribution of the individual transporters was determined by Western blotting (Fig. 3). Insulin increased cytochalasin B binding in the plasma membranes 5.6-fold (2.50 ± 0.62 to 14.0 ± 1.24 pmol/mg of protein), which comprised a doubling in GLUT1 and a 3.7-fold increase in GLUT4. Correspondingly, insulin decreased cytochalasin B binding to the intracellular membranes by 55% (27.5 ± 0.62 to 12.8 ± 0.31 pmol/mg), which constituted a 74% decrease in GLUT1 and a 33% decrease in GLUT4. These effects of insulin on transporter(s) distribution were unaltered by isoproterenol in the presence of PIA and adenosine (protocol 4). However, exposure to isoproterenol in the absence of adenosine receptor stimulation (protocol 3) produced a small but significant decrease in the number of GLUT4 ($P < 0.05$ in paired *t* test but not significant in ANOVA in these three experiments). In an additional series of 15 separate experiments comparing protocol 3 with protocol 2, isoproterenol induced a decrease in GLUT4 concentration in plasma membranes to $86.1 \pm 2.3\%$ ($P < 0.01$ in analysis of variance) of that observed following protocol 2, and a corresponding increase in the intracellular membranes. The distribution of GLUT1 remained unaffected (Fig. 3A).

As previously reported (14), no ^{32}P was found in GLUT1 under any of the above conditions (data not shown). By contrast, GLUT4 was phosphorylated under all conditions in both plasma and intracellular membranes (Fig. 4). Thus, to assess whether any changes in phosphorylation occurred as a consequence of the various incubation conditions, it was necessary to account for the GLUT4 concentration in each of

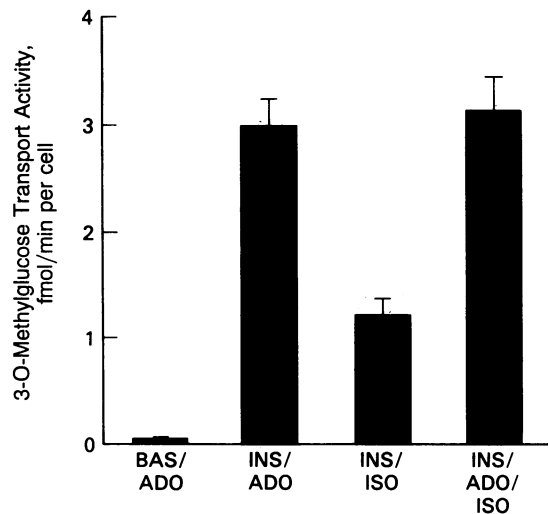


FIG. 2. Effects of insulin and isoproterenol on 3-*O*-methylglucose transport activity. Quadruplicate 200- μ l aliquots were removed for 3-*O*-methylglucose transport activity determinations from cell incubations subjected to the protocols outlined in Fig. 1. Data represent mean \pm SEM of three separate experiments.

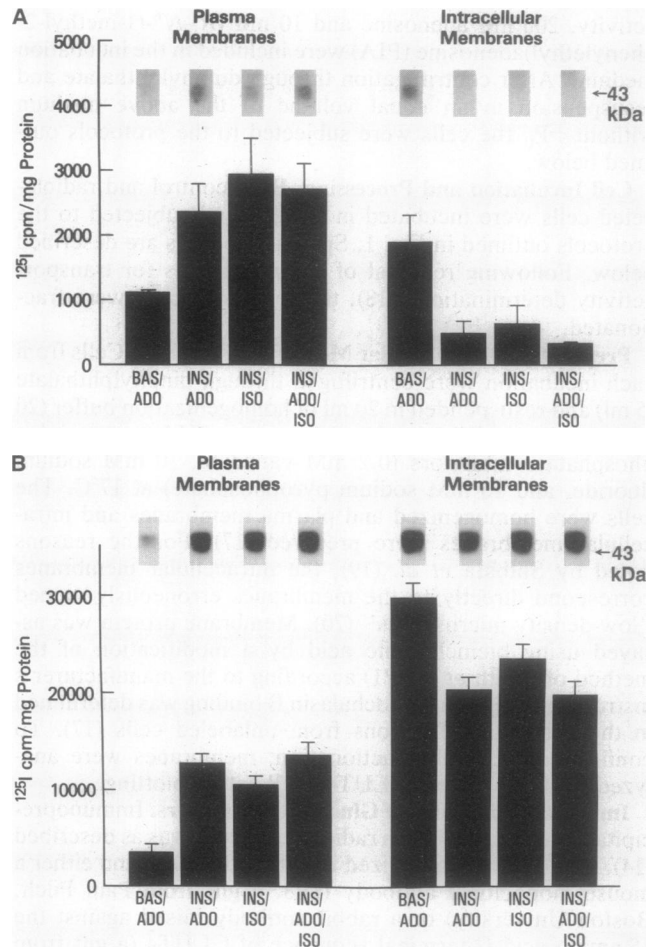


FIG. 3. Effects of insulin and isoproterenol on the subcellular localization of GLUT1 and GLUT4. Plasma membranes and intracellular membranes were prepared from adipose cells preincubated with $^{32}P_i$ for 90 min and subsequently exposed to protocols 1–4 (see Fig. 1). GLUT1 and GLUT4 were immunoprecipitated from the respective membrane fractions; 20% of the immunoprecipitate was subsequently subjected to SDS/PAGE and Western blot analysis for the presence of the respective proteins. Insets show typical autoradiographs of GLUT1 (A) and GLUT4 (B) immunoprecipitated from plasma membranes and intracellular membranes prepared from cells incubated as described above. The blots were quantitated by excising the corresponding bands and measuring the bound ^{125}I -protein A by γ counting. Data represent mean \pm SEM of three separate experiments.

the immunoprecipitates. This was achieved (Fig. 5) by computing the ratio of ^{32}P incorporated/mg of membrane protein to the ^{125}I -protein A/mg of membrane protein bound to GLUT4 in the Western blot assay. There was no significant difference in the level of phosphorylation of GLUT4 in the plasma or intracellular membranes prepared from basal or insulin-stimulated cells (protocols 1 and 2) and plasma membranes prepared from protocols 3 and 4. In contrast, isoproterenol increased the phosphorylation of GLUT4 transporters only in the intracellular membranes by 40% (1.27 ± 0.11 in protocol 2 vs. 1.77 ± 0.08 in protocol 3 and 1.88 ± 0.01 arbitrary units in protocol 4; $P < 0.01$).

An independent assessment of A-kinase activation under the various protocols was provided by monitoring the phosphorylation state of perilipin and hormone-sensitive lipase (Fig. 6). Perilipin exhibited basal phosphorylation in cells from protocol 1 or 2 and migrated on SDS/PAGE as a 62-kDa protein. However, this protein migrated as a highly phosphorylated 65/67-kDa species under protocols 3 and 4. The disappearance of the 62-kDa species under protocols 3 and 4

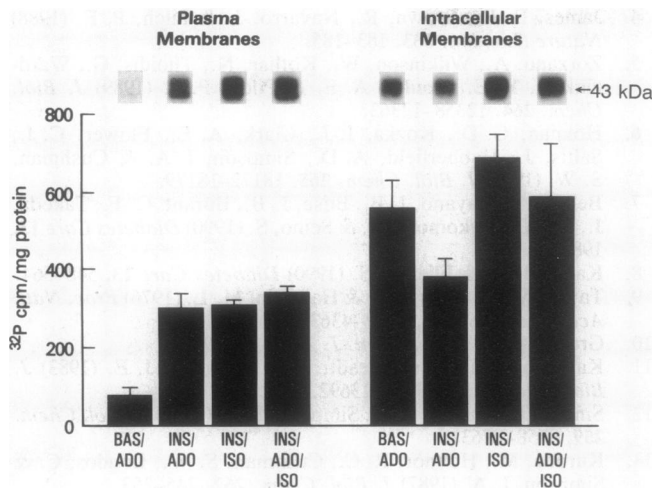


FIG. 4. Incorporation of ³²P_i into GLUT4. Insets show a typical autoradiograph of ³²P-labeled GLUT4 immunoprecipitated from plasma membranes and intracellular membranes prepared from cells exposed to protocols 1–4 (see Fig. 1). Phosphorylation was quantitated by excision and Cerenkov counting of the appropriate bands. Data represent mean ± SEM of three separate experiments.

is evidence of a substantial activation of A-kinase. Further confirmation of A-kinase activation was provided by detection of radiolabeled hormone-sensitive lipase as an 84-kDa protein in the same fat extracts.

Based on the specific activity of the ATP and the assumptions that (i) the cytochalasin B binding data are a measure of the total number of transporters in the plasma membrane and (ii) GLUT4 is 10-fold more abundant than GLUT1 in insulin-stimulated plasma membranes (5, 6), the extent of GLUT4 phosphorylation corresponds to 0.1–0.2 mol/mol in the insulin-stimulated intracellular membranes. This value increases to 0.2–0.3 mol/mol in the intracellular membranes prepared from cells subjected to protocol 3 or 4.

In contrast to transport activity (Table 1), washing had no effect on the phosphorylation state of GLUT4 in either plasma membranes or intracellular membranes prepared from cells exposed to insulin with or without isoproterenol (data not shown).

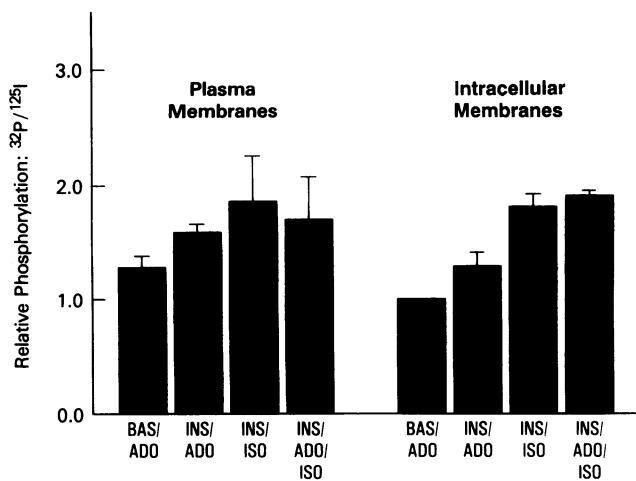


FIG. 5. Effects of insulin and isoproterenol on relative incorporation of ³²P_i into GLUT4, assessed by expressing the ratio of total ³²P incorporated in the respective membranes (Fig. 4) to the total GLUT4 concentration present in the corresponding membranes (Fig. 3B).

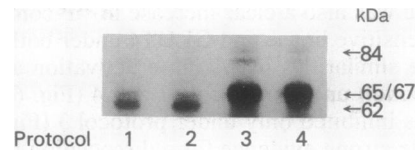


FIG. 6. Effect of insulin, adenosine, and isoproterenol on the phosphorylation state of perilipin and hormone-sensitive lipase. Perilipin and hormone-sensitive lipase were extracted from the fat cakes obtained from the respective incubations following homogenization of the ³²P-labeled cells and centrifugation to pellet the plasma membranes (see *Methods*). The extracted proteins were separated by SDS/PAGE and detected by autoradiography.

DISCUSSION

Previously, we suggested (13) that the modulation of insulin-stimulated glucose transport activity by ligands which interact with receptors that stimulate or inhibit adenylyl cyclase occurred by a mechanism that was independent of A-kinase activity. Notably, PIA reversed the transport inhibition by isoproterenol whereas the A-kinase activity remained stimulated (13). By contrast, as outlined above, recent studies by James and colleagues (15, 16) provided evidence that isoproterenol regulates transport activity by an A-kinase-mediated phosphorylation of GLUT4. We therefore undertook the present study to define the relationship among A-kinase activation, transport activity, and the extent of GLUT4 phosphorylation.

Confirming the observations of James *et al.* (15), GLUT4, in contrast to GLUT1 (14), is constitutively phosphorylated (0.1–0.2 mol/mol) (Fig. 4) in basal and insulin-stimulated cells irrespective of subcellular location. Phosphorylation is enhanced by 40% in intracellular membranes prepared from cells exposed to insulin in combination with isoproterenol (Fig. 5).

Our first indication that some discordance might exist between phosphorylation of GLUT4 and transport modulation came from comparing the effects of the P_i equilibration protocol on transport activity and GLUT4 phosphorylation (Table 1). Isoproterenol inhibition of transport was significantly blunted by the preincubation, whereas the GLUT4 phosphorylation and A-kinase activation remained unchanged. While the underlying cause of the diminished isoproterenol response remains unknown, the response was restored by washing the cells, a finding that highlights the need to compare all parameters simultaneously.

Substantial evidence for a dissociation between transport activity (Fig. 2) and GLUT4 phosphorylation (Fig. 5) comes from a direct comparison of isoproterenol action in the presence (protocol 4) and absence (protocol 3) of adenosine receptor stimulation. Since isoproterenol and adenosine produce opposing effects on both adenylyl cyclase (30) and transport activity (9), one might reasonably infer that adenosine opposition of the isoproterenol action on transport is secondary to a lowering of cAMP and thus A-kinase activity. However, adenosine receptor stimulation only partially inhibits adenylyl cyclase, and conditions exist under which strong adenosine effects on transport activity are evident in the face of sufficient cAMP to activate A-kinase. Indeed, these are the conditions of protocol 4. Confirmation that A-kinase was stimulated under this protocol is provided by the behavior of perilipin in SDS/PAGE (Fig. 6). Moreover, this procedure permits one to assess both kinase activity and glucose transporter phosphorylation in the same radiolabeled cells. As expected, perilipin was quantitatively phosphorylated by isoproterenol action absent adenosine receptor restraint (protocol 3). Similarly, the data are unambiguous under protocol 4; i.e., the A-kinase was sufficiently activated to shift all of the perilipin from the 62-kDa to the 65/67-kDa

form. There was also a clear increase in ^{32}P content of both hormone-sensitive lipase and GLUT4 under both protocols. Despite the similarities in A-kinase activation and GLUT4 phosphorylation under protocols 3 and 4 (Fig. 6), transport activity was inhibited only under protocol 3 (Fig. 1). These data provide strong evidence for a dissociation between the isoproterenol effects on transport activity and cAMP formation.

Further, the increase of GLUT4 phosphorylation in response to isoproterenol (0.1–0.2 to 0.2–0.3 mol/mol) is significant only in intracellular membranes and seems incompatible with the 50–60% inhibition of transport activity induced by the β -adrenergic agonist. Moreover, the extent of phosphorylation may be overestimated, as ATP specific activity is based on the total ATP pool, whereas the fraction that serves as the phosphoryl donor may be more rapidly turning over and consequently of higher specific radioactivity than the total ATP pool (31); certainly this is the case in muscle (26). However, it is conceivable that a small proportion of GLUT4 molecules are responsible for mediating transport activity and that the combination of phosphorylation with the absence of adenosine leads to decreased transport activity. Of perhaps greater significance is the observation, first made by James *et al.* (15), that isoproterenol produces a greater increase in phosphorylation of intracellular GLUT4 transporters. This finding, together with the observation that the phosphatase inhibitor okadaic acid promotes the phosphorylation of GLUT4 from 0.2 to 0.6 mol/mol, has led to the suggestion that the A-kinase-stimulated phosphorylation may impair the recycling of GLUT4 (32).

That adenosine and isoproterenol might modulate transport activity by a mechanism independent of A-kinase should not be surprising. The list of membrane proteins and ion channels regulated by receptors and their associated guanine nucleotide-binding proteins is expanding rapidly and now includes phospholipases as well as Na^+ , Mg^{2+} , Ca^{2+} , and K^+ channels (33). It is not unreasonable to suggest that the glucose transporters represent a further addition to this list. There are intriguing similarities between the adenylyl cyclase molecule and the glucose transporter(s) that might suggest a similar modulation. Both are integral proteins with 12 membrane-spanning domains, both possess high-affinity sites for forskolin, and both show mutually antagonistic responses to isoproterenol and adenosine.

We thank M. J. Zarnowski, S. R. Richards, D. R. Yver, and T. Davies-Hill for expert technical assistance. We thank Dr. A. Canfield (Hoffmann-LaRoche) for providing the anti-GLUT4 antibody, Dr. P. Pilch for kindly providing the 1F8 antibody and many constructive discussions, and Dr. S. Vannucci for critical reading of the manuscript.

- Cushman, S. W. & Wardzala, L. J. (1980) *J. Biol. Chem.* **255**, 4758–4762.
- Suzuki, K. & Kono, T. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 2542–2545.
- Simpson, I. A. & Cushman, S. W. (1986) *Annu. Rev. Biochem.* **55**, 1059–1089.
- James, D. E., Brown, R., Navarro, J. & Pilch, P. F. (1988) *Nature (London)* **333**, 183–185.
- Zorzano, A., Wilkinson, W., Kotliar, N., Thoidis, G., Wardzinski, B. E., Rouho, A. E. & Pilch, P. F. (1989) *J. Biol. Chem.* **264**, 12358–12363.
- Holman, G. D., Kozka, I. J., Clark, A. E., Flower, C. J., Saltis, J., Habberfield, A. D., Simpson, I. A. & Cushman, S. W. (1990) *J. Biol. Chem.* **265**, 18172–18179.
- Bell, G. I., Kayano, J. B., Buse, J. B., Burant, C. F., Takeda, J., Lin, D., Fukumoto, H. & Seino, S. (1990) *Diabetes Care* **13**, 198–208.
- Kahn, B. B. & Flier, J. S. (1990) *Diabetes Care* **13**, 548–564.
- Taylor, W. M., Mark, M. & Halperin, M. L. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 4359–4363.
- Green, A. (1983) *Biochem. J.* **212**, 189–195.
- Kashiwagi, A., Heucksteadt, T. P. & Foley, J. E. (1983) *J. Biol. Chem.* **258**, 13685–13692.
- Smith, U., Kuroda, M. & Simpson, I. A. (1984) *J. Biol. Chem.* **259**, 8758–8763.
- Kuroda, M., Honnor, R. C., Cushman, S. W., Londos, C. & Simpson, I. A. (1987) *J. Biol. Chem.* **262**, 245–253.
- Joost, H. J., Weber, T. M., Cushman, S. W. & Simpson, I. A. (1987) *J. Biol. Chem.* **262**, 11261–11267.
- James, D. E., Hiken, J. F. & Lawrence, J. C., Jr. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 8368–8372.
- Lawrence, J. C., Jr., Hiken, J. F. & James, D. E. (1990) *J. Biol. Chem.* **265**, 2324–2332.
- Weber, T. M., Joost, H. G., Simpson, I. A. & Cushman, S. W. (1988) in *The Insulin Receptor*, eds. Kahn, C. R. & Harrison, L. C. (Liss, New York), Vol. 2, pp. 171–187.
- Karnieli, E., Zarnowski, M. J., Hissin, P. J., Simpson, I. A., Salans, L. B. & Cushman, S. W. (1981) *J. Biol. Chem.* **256**, 4772–4777.
- Shibata, Y., Flanagan, J. E., Smith, M. M., Robinson, F. W. & Kono, T. (1987) *Biochim. Biophys. Acta* **902**, 154–158.
- Cushman, S. W. & Wardzala, L. J. (1980) *J. Biol. Chem.* **255**, 4758–4762.
- Smith, P. K., Krohn, R. I., Hermanson, G. T., Mazilia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. & Klenk, D. C. (1985) *Anal. Biochem.* **150**, 76–85.
- Haspel, H. C., Birnbaum, M. J., Wilk, E. W. & Rosen, O. M. (1985) *J. Biol. Chem.* **260**, 7219–7225.
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
- Greenberg, A. S., Egan, J. J., Wek, S. A., Garty, N. B., Blanchette-Mackie, E. J. & Londos, C. (1991) *J. Biol. Chem.* **266**, 11341–11346.
- Egan, J. J., Greenberg, A. S., Chang, M.-K. & Londos, C. (1990) *J. Biol. Chem.* **265**, 18769–18775.
- Beavo, J. A., Bechtel, P. J. & Krebs, E. G. (1974) *Methods Enzymol.* **30**, 299–308.
- Egan, J. J., Chang, M.-K. & Londos, C. (1988) *Anal. Biochem.* **175**, 552–561.
- Hopkirk, T. J. & Denton, R. M. (1986) *Biochim. Biophys. Acta* **885**, 195–205.
- Lawrence, J. C., Jr., & James, C. (1984) *J. Biol. Chem.* **259**, 7975–7982.
- Londos, C., Cooper, D. M. F. & Rodbell, M. (1981) *Adv. Cyclic Nucleotide Res.* **14**, 163–171.
- Mayer, S. E. & Krebs, E. G. (1970) *J. Biol. Chem.* **245**, 153–160.
- Lawrence, J. C., Jr., Hiken, J. F. & James, D. E. (1990) *J. Biol. Chem.* **265**, 19768–19776.
- Birnbaumer, L. (1990) *Annu. Rev. Pharmacol. Toxicol.* **30**, 675–705.