Adenosine Receptor Down-regulation and Insulin Resistance Following Prolonged Incubation of Adipocytes with an A₁ Adenosine Receptor Agonist*

(Received for publication, February 24, 1987)

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Adenosine, via interaction with A₁ adenosine receptors, increases insulin sensitivity and inhibits lipolysis in adipocytes. To investigate regulation of this system, adipocytes were incubated for up to 72 h with the nonmetabolizable adenosine receptor agonist, N⁶-phenylisopropyl adenosine (PIA). Adenosine receptors were measured by the binding of [H]-hydroxyphenylisopropyl adenosine to membranes. PIA down-regulated adenosine receptors, decreasing the number of binding sites with no change in affinity.

Adipocytes were incubated for 48 h without or with 100 nM PIA to down-regulate the A₁ receptors by approximately 60%. The cells were washed, and lipolysis and glucose transport were assessed. The ability of PIA to inhibit lipolysis was markedly attenuated in the down-regulated cells. Furthermore, the EC₅₀ of insulin was increased ~3-fold in the PIA-treated cells. [H]-Insulin binding to the PIA-treated cells was unchanged, demonstrating that the decreased insulin sensitivity is not due to decreased insulin receptor binding.

Pertussis toxin catalyzed ADP-ribosylation of a 41-kDa protein thought to be the α-subunit of Gi. This 41-kDa protein was decreased in membranes from cells treated with PIA, with a maximal 50% loss. This suggests that Gi is down-regulated and that loss of both the A₁ adenosine receptor and Gi are involved in the metabolic changes observed after PIA treatment.

Adenosine has been implicated as an important endogenous regulator of adipose tissue metabolism. Thus, physiological concentrations of adenosine increase the sensitivity of adipocyte glucose transport and oxidation to stimulation by insulin (1, 2). This increased insulin sensitivity occurs without changes in insulin binding (3), suggesting that adenosine increases the efficiency of coupling of insulin receptors to insulin action. Adenosine is also a potent inhibitor of lipolysis (4, 5), and it has been suggested that lipolysis in vivo is normally under regulation by adenosine and other inhibitory agents (6).

The effects of adenosine outlined above are thought to be mediated via specific cell-surface adenosine receptors. Based on the order of potency of various agonists, adenosine receptors can be subdivided into two classes: A₁ receptors, which are usually inhibitory to adenylyl cyclase, and A₂ receptors, which usually stimulate adenylyl cyclase (7, 8). Rat adipocytes possess primarily receptors of the A₁ class, coupled to inhibition of adenylyl cyclase (9, 10) by the GTP-dependent regulatory protein, Gₛ (11, 12). While it is fairly clear that the inhibitory effect of adenosine on lipolysis is secondary to inhibition of adenylyl cyclase (4, 5), the mechanism by which adenosine increases insulin sensitivity is not understood.

The purpose of this study was to determine first whether adenosine receptors can, like many other receptors, be down-regulated in vitro by chronic exposure to an agonist. Second, studies were performed to investigate whether Gi is lost during adenosine receptor down-regulation. Finally, the metabolic consequences of adenosine receptor down-regulation, with respect to insulin sensitivity and lipolysis, have been evaluated. To investigate these questions, a primary culture system, developed by Marshall and co-workers (13, 14), has been utilized to incubate adipocytes for up to 72 h with the nonmetabolizable adenosine analog, PIA.¹

MATERIALS AND METHODS

Dulbecco’s modified Eagle’s medium was purchased from Gibco. Fetal calf serum was from Armour. Glyceraldehyde-3-phosphate dehydrogenase, and pyruvate kinase were from Sigma. Pertussis toxin was from List Biological Laboratories (Campbell, CA). [α-³²P]NAD was from ICN Radiochemicals, Inc. All other reagents were from previously published sources (1, 15–17).

Primary Culture of Rat Adipocytes—Adipocytes were isolated from epididymal fat pads of male Sprague-Dawley rats, weighing 100–210 g, by the method of Rodbell (18). The cells were maintained in primary culture as described by Marshall and co-workers (13, 14). Adenosine deaminase (1 µg/ml) was added to the culture medium to maintain the extracellular adenosine concentration at a low constant level. The activity of the enzyme was measured by incubating aliquots of the incubation medium in 50 mM Hepes, pH 7.6, plus 0.15 mM adenosine and following the decrease in absorbance at 260 nm. The adenosine deaminase activity decreased by less than 10% during 72-h incubations (data not shown).

Isolation of Adipocyte Membranes—Following incubation for various times, adipocytes were washed three times in Dulbecco’s modified Eagle’s medium containing 20 mM Hepes, pH 7.4, and 1% bovine serum albumin, followed by one wash in homogenizing buffer (250 mM sucrose, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 20 mM Hepes, pH 7.4). The cells were then homogenized by vigorous mixing in 16 × 100-mm glass test tubes on a vortex mixer (19). The homogenate was centrifuged for 5 min at 1,000 × g, and the supernatant was centrifuged for 20 min at 16,000 × g. The pellet was suspended in 154 mM NaCl, 10 mM MgCl₂, 50 mM Hepes, pH 7.6, for adenosine receptor studies or in 100 mM Hepes, 40 mM thymidine, 5 mM MgCl₂, pH 7.6, for toxin-catalyzed ADP-ribosylation. Protein concentration of the membrane suspensions was determined by the method of Bradford (20) using a kit from Bio-Rad and using bovine γ-globulin as a standard.

¹ The abbreviations used are: PIA, (-)-N⁶-phenylisopropyl adenosine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HIPA, (-)-N⁶-p-hydroxyphenylisopropyl adenosine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EC₅₀, concentration causing a half-maximal effect.

* This work was supported by New Investigator Research Award DK 38719 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Measurement of A1 Adenosine Receptors—Adenosine receptors were determined by the binding of \(^{125}\text{I}-\text{HPIA}\), an A1 adenosine receptor agonist (21–23), to the crude membrane preparations as follows. Membranes (approximately 20 \(\mu\)g of protein) were incubated in 154 mM NaCl, 10 mM MglCl, 50 mM Hepes, pH 7.5, adenosine deaminase (10 \(\mu\)g/ml), and 0.2 nM \(^{125}\text{I}-\text{HPIA}\) plus unlabeled HPIA (0–100 nM) in a final volume of 70 \(\mu\)l. After 2.5 h at 37°C the membranes were precipitated as follows. Bovine \(\gamma\)-globulin solution (20 \(\mu\)l of 12.5 mg/ml in 50 mM Hepes, pH 7.5) was added, followed by 500 \(\mu\)l of ice-cold 15% (w/v) polyethylene glycol. Duplicate 200-\(\mu\)l samples were transferred to plastic 300-\(\mu\)l microtubes (W. Sarstedt & Co.) containing 50 \(\mu\)l of 10% polyethylene glycol and centrifuged for 5 min. The tips of the microtubes, containing the precipitate, were cut and counted in a \(\gamma\)-counter. Non-specific binding was determined in the presence of 10 nM HPIA and was typically about 10% of total binding (1% of total radioactivity). All data have been corrected for non-specific binding. Preliminary experiments (not shown) revealed that these conditions for measurement of A1 adenosine receptors were optimum with respect to incubation time, temperature, adenosine deaminase concentration, and stopping procedure.

Photochemical Cross-linking of Adenosine Receptors—Details of this technique have been published (17). Briefly, membranes (80 \(\mu\)g of protein) were incubated with 1 nM \(^{125}\text{I}-\text{HPIA}\) for 2.5 h at 37°C. \(^{125}\text{I}-\text{HPIA}\) was then covalently cross-linked to the receptors by use of the photoactive heterobifunctional compound, N-5-azido-2-nitrobenzoxoxygenimine. The labeled membranes were analyzed by SDS-PAGE (10% gels) and autoradiography.

ADP-ribosylation of G—The 41-kDa pertussis toxin substrate thought to be the \(\alpha\)-subunit of G (24, 25) was quantitated as follows. Membranes (5 \(\mu\)g) were incubated for 1 h at 37°C in a total volume of 100 \(\mu\)l containing 50 mM Hepes, 2.5 mM MgCl2, 20 mM thymidine, 5 \(\mu\)Ci of \([\text{\textsuperscript{32P}}]\text{NAD}\), 1 mM ATP plus 0.5 \(\mu\)g of pertussis toxin (preactivated by incubating with 25 mM dithiothreitol for 30 min at room temperature). The membranes were centrifuged (20,000 \(\times\) g for 20 min) and analyzed by SDS-PAGE (26) using 10% gels. The gels were dried, and autoradiography was performed using preflashed Kodak X-AR-5 film in x-ray cassettes containing Kodak X-OMAT regular intensifying screens at -70°C for 1–3 days.

Glucose Transport Assay—Following cell culture, adipocytes were washed three times in 127 mM NaCl, 5 mM KCl, 4.2 mM NaHCO\(_3\), 1.3 mM CaCl\(_2\), 0.5 mM KH\(_2\)PO\(_4\), 0.5 mM MgCl\(_2\), 0.5 mM MgSO\(_4\), 20 mM Hepes, pH 7.4, plus 1% bovine serum albumin. Uptake of 2-deoxy\([\text{\textsuperscript{3}H}\]glucose was used as an index of the rate of glucose transport (27) as previously described (16).

Insulin Binding Studies—Iodination of insulin and measurement of insulin-insulin binding to intact adipocytes were performed as previously described (15, 16).

Lipolysis Assay—Lipolysis was measured by following glycerol release as follows. Adipocytes were washed and suspended in the same buffer used for the glucose transport assay (see above). Cells (approximately 100,000 in 0.5 ml) were incubated for 1 h at 37°C. The cells were centrifuged (50 g for 1 min), and 400 \(\mu\)l of buffer was withdrawn from below the cell layer and precipitated with 40 \(\mu\)l of 5 M HClO\(_4\). The samples were centrifuged (2,000 \(\times\) g for 5 min), and the supernatants were neutralized with 10 M KOH. Precipitated KClO\(_4\) was removed by centrifugation (2,000 \(\times\) g for 5 min), and glycerol was assayed enzymatically (28).

Cell Counts—Adipocytes were counted manually by determining the number of cells in 10-\(\mu\)l aliquots of the cell suspensions, after dilution as necessary. The cells were counted in a “hanging drop” on an inverted microscope slide (29). At least 250 cells were counted for each cell preparation. None of the treatments altered cell number.

Results

Down-regulation of Adipocyte Adenosine Receptors—Experiments were performed to determine whether A1 receptors can be down-regulated by an A1 agonist. Adipocytes spontaneously release adenosine into the incubation medium (5, 30). Therefore, the strategy employed was to incubate the cells with adenosine deaminase (to keep the adenosine concentration in the medium low) and then test the effect of addition of a nonmetabolizable A1 adenosine receptor agonist, PIA (31). Adipocytes were incubated for up to 72 h with 0–1000 nM PIA. After washing the cells, adenosine receptors were evaluated by measuring binding of \(^{125}\text{I}-\text{HPIA}\) to a crude plasma membrane fraction (see “Materials and Methods”).

Fig. 1 shows \(^{125}\text{I}-\text{HPIA}\) binding to membranes isolated from adipocytes that had been treated for 48 h with 0–1000 nM PIA. PIA caused a dose-related loss of \(^{125}\text{I}-\text{HPIA}\) binding. The highest concentration of PIA caused approximately 80% loss of binding. The half-maximally effective concentration of PIA was approximately 16 nM.

The data in Fig. 2 demonstrate that the PIA-induced loss of binding is time-dependent. This experiment was performed in the presence of a submaximally effective concentration of PIA (100 nM). Loss of binding was detectable after 6 h and half-maximal after about 24 h.

In the above experiments, receptor binding measurements were performed using a “tracer” concentration of \(^{125}\text{I}-\text{HPIA}\). Therefore, the findings could be explained by either a decrease in receptor number or an affinity change. To distinguish between these possibilities, membranes were prepared from cells treated with or without 100 nM PIA for 48 h. Equilibrium binding of \(^{125}\text{I}-\text{HPIA}\) was then determined in the presence of 0–10 nM unlabeled HPIA, and competition curves were constructed (Fig. 3). Binding was markedly lower in the membranes from PIA-treated cells at all concentrations of HPIA.
cells, and $1Z51$-HPIA binding was determined in the presence of $0-10$ nM PIA for adipocytes. HPIA binding to membranes from control and PIA-treated shows the data as a Scatchard plot.

To investigate possible structural changes in the receptor after PIA treatment, $1Z51$-HPIA was covalently cross-linked to the membranes in the presence of $0-10$ nM HPIA, as described under "Materials and Methods." The autoradiography. See "Materials and Methods" for details. The parallel plots for the control and treated cells, indicating that the PIA-induced loss of $1Z51$-HPIA binding is due to a decrease in receptor number, rather than affinity. Values, calculated from the slope of the plots, was approximately $0.6$ nM in both groups of cells.

Scatchard analysis of the binding data (Fig. 3, inset) gave parallel plots for the control and treated cells, indicating that the PIA-induced loss of $1Z51$-HPIA binding is due to a decrease in receptor number, rather than affinity. $B_{max}$ values, calculated from the intercept on the abscissa of the Scatchard plots, were $251$ and $110$ fmol/mg protein, and the $K_D$, calculated from the slope of the plots, was approximately $0.6$ nM in both groups of cells.

To investigate possible structural changes in the receptor after PIA treatment, $1Z51$-HPIA was covalently cross-linked to membranes from control and treated cells. The labeled receptors were analyzed by SDS-PAGE and autoradiography (Fig. 4). This technique revealed specific labeling of a 38-kDa protein, as we have previously reported (17). Receptors in membranes from PIA-treated cells showed the same relative mobility as those from control cells, indicating that the receptors remaining after PIA treatment are not structurally altered. The intensity of labeling was decreased, as would be expected from the binding studies.

**Effect of Chronic PIA Treatment on Lipolysis**—Adenosine and other $A_1$ agonists, such as PIA, are potent inhibitors of lipolysis in adipocytes. To determine whether the PIA-induced loss of adenosine receptors, documented above, causes reduced sensitivity of lipolysis to PIA, the experiments shown in Fig. 5 were performed. Adipocytes were incubated as before, in the absence or presence of $100$ nM PIA for $48$ h. They were then washed, and the rate of glycerol release was measured.

The basal rate of lipolysis was approximately $35\%$ higher in the PIA-treated cells than in the controls (see legend to Fig. 5). This may have been due to decreased sensitivity of the cells to endogenous adenosine. However, this is difficult to evaluate since it is also possible that rates of adenosine release are different in the two groups of cells. To overcome this problem, the cells were incubated with adenosine deaminase to remove endogenous adenosine. The rate of glycerol release was still slightly higher in the PIA-treated cells. More importantly, lipolysis in the PIA-treated cells was much less sensitive to inhibition by PIA than in control cells. Thus, the dose-response curve was shifted to the right, such that the half-maximally effective concentration of PIA (indicated by arrows on Fig. 5) was almost 6-fold higher in the cells that had been treated with PIA for $48$ h. This finding indicates that down-regulation of adipocyte adenosine receptors leads to a decrease in the sensitivity of the cells to PIA.

**Insulin Action in PIA-treated Cells**—Both adenosine and PIA increase the sensitivity of adipocyte glucose utilization to stimulation by insulin by an unknown mechanism (1, 3). This occurs without changes in insulin binding to the cells (3). Therefore, it was of interest to determine whether down-regulation of adenosine receptors would alter insulin sensitivity. The effect of insulin on glucose transport (measured by the 2-deoxyglucose uptake technique) was used to evaluate insulin action in control and PIA-treated cells (Fig. 6). Glucose transport assays were performed both in the absence and in the presence of adenosine deaminase (Fig. 6, a and b, respectively). These assays were done in control and PIA-treated cells in the absence and presence of adenosine deaminase (10 $\mu$g/ml) and PIA as indicated. The basal rate of lipolysis (i.e., in the absence of adenosine deaminase) was $205$ in the controls and $275$ in the treated cells. The data are mean $\pm$ S.D. for three separate experiments, each performed in duplicate. Differences that are statistically significant (student's $t$ test) are indicated by: *, $p < 0.05$; **, $p < 0.02$; †, $p < 0.005$. 

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**FIG. 3.** Competition curve and Scatchard analysis of $1Z51$-HPIA binding to membranes from control and PIA-treated adipocytes. Adipocytes were incubated without (●) or with (○) $100$ nM PIA for $48$ h. A crude membrane fraction was isolated from the cells, and $1Z51$-HPIA binding was determined in the presence of $0-10$ nM HPIA, as described under "Materials and Methods." The inset shows the data as a Scatchard plot.

**FIG. 4.** Photoaffinity cross-linking of $1Z51$-HPIA to control and PIA-treated adipocytes. Membranes were isolated from control and PIA-treated adipocytes ($100$ nM PIA, $48$ h). $1Z51$-HPIA was covalently cross-linked to the membranes in the presence (lanes 1 and 3) or absence (lanes 2 and 5) of a large excess of unlabeled HPIA ($10$ $\mu$M). The membranes were then analyzed by SDS-PAGE and autoradiography. See "Materials and Methods" for details. The dashes on the left side of the figure show the molecular mass of standard marker proteins, as revealed by Coomassie Blue staining. The dash on the right shows the position and $M_t$ of the specifically labeled band.

**FIG. 5.** Effect of adenosine receptor down-regulation on lipolysis. Adipocytes were incubated for $48$ h in the absence (●) or presence (○) of $100$ nM PIA. The cells were washed, and glycerol release was measured over a $1$-h period in the presence of adenosine deaminase ($10$ $\mu$g/ml) and PIA as indicated. The basal rate of lipolysis $(\mu$mol/l, $10^8$ cells) was almost 6-fold higher in the cells that had been treated with PIA for $48$ h. This finding indicates that down-regulation of adipocyte adenosine receptors leads to a decrease in the sensitivity of the cells to PIA.
Adenosine Receptor Down-regulation and Insulin Sensitivity

The dose-response curve for insulin's effect on glucose transport was markedly shifted to the right. The EC₅₀ of insulin was 0.17 ng/ml in the absence of adenosine deaminase, see "Materials and Methods" for details. Data are means ± S.D. of three separate experiments, each performed in triplicate. Differences between control and treated cells that are statistically significant (student's t test) are indicated by: *, p < 0.05; **, p < 0.02; †, p < 0.01.

Glucose transport was stimulated by insulin in both control and PIA-treated cells (Fig. 6). When endogenous adenosine was allowed to accumulate (i.e. in the absence of adenosine deaminase, Fig. 6a), maximal insulin-stimulated transport was equal in the two groups of cells. However, the basal rate of transport was lower in the PIA-treated cells. In addition, the dose-response curve for insulin's effect on glucose transport was markedly shifted to the right. The EC₅₀ of insulin was 0.17 ± 0.02 ng/ml in control cells and 0.47 ± 0.13 ng/ml in the PIA-treated cells. This finding indicates that down-regulation of adipocyte adenosine receptors leads to decreased basal glucose transport rates and decreased insulin sensitivity.

To determine whether the effect of PIA treatment observed in Fig. 6a is due to different rates of adenosine release from the cells, glucose transport measurements were also performed in the presence of adenosine deaminase (Fig. 6b). Adenosine deaminase caused a shift to the right in the insulin dose-response curve, by removing endogenous adenosine, as has previously been reported (1). However, the difference between EC₅₀ values of insulin for control and PIA-treated cells was still evident (1.7 ± 0.1 and 5.0 ± 0.7 ng/ml in control and treated cells, respectively). This indicates that the decreased insulin sensitivity after PIA treatment cannot be explained on the basis of differences in adenosine release but is inherent to the cells. In addition to the rightward shift in the dose-response curve, the maximally insulin-stimulated glucose transport rate was lower in PIA-treated cells when measured in the presence of adenosine deaminase. The reason for this difference is not clear.

Insulin binding was assessed by measuring binding of a "tracer" concentration of ¹²⁵I-insulin (0.3 ng/ml) to control and treated adipocytes. These measurements were performed at 16 °C to ensure that cell-associated radioactivity represents binding of insulin to cell-surface receptors, rather than insulin internalization (32). Control cells bound 35.1 ± 3.6 pg/10⁶ cells, and treated cells bound 31.5 ± 1.5 pg/10⁶ cells (mean ± S.D. of three experiments, each performed in triplicate). It is clear that insulin binding is not altered by PIA treatment, demonstrating that the decreased insulin sensitivity of PIA-treated adipocytes is not secondary to changes at the level of insulin binding to the cells.

G₁ in PIA-treated Cells—To investigate whether PIA treatment alters the concentration of G₁ in the membranes, adipocytes were incubated for 48 h with 0–1000 nM PIA. This incubation with PIA induced adenosine receptor down-regulation as described above. Membranes were isolated as before, incubated with activated pertussis toxin and [³²P]NAD, and then analyzed by SDS-PAGE and autoradiography. Fig. 7 demonstrates that this technique labeled a 41-kDa protein, which is thought to be the α-subunit of G₁ (24, 25). The intensity of labeling of this protein was decreased by PIA pretreatment in a dose-dependent fashion (Fig. 7A). Photodensitometry demonstrated approximately a 45% decrease in labeling of this protein in cells treated with 300 nM PIA (Fig. 7B). The time course of down-regulation of the 41-kDa pertussis toxin substrate is shown in Fig. 8. PIA-induced down-regulation was time-dependent, with approximately a 50% loss after 3 days. These findings suggest that G₁ is down-regulated along with the adenosine receptor.

DISCUSSION

The findings demonstrate that prolonged incubation of adipocytes with an A₁ adenosine receptor agonist down-regulates both the adenosine receptor and a 41-kDa pertussis toxin substrate thought to be the α-subunit of G₁. PIA also causes marked metabolic alterations in the cells. Recently,
were incubated without autoradiography. The graphs show results of photodensitometry of pertussis toxin and \[^{32}\text{P}\]NAD and then analyzed by SDS-PAGE and adenosine receptor, although the effect appeared to be small. These findings suggested that PIA could down-regulate the effects of chronic administration of PIA to rats apparently due to a reduced number of binding sites, with no change in affinity.

Hoffman et al. (33) and Parsons and Stiles (34) reported effects of chronic administration of PIA to rats in vivo. Both groups found approximately a 30% reduction in \(\alpha\) adenosine receptors in adipocyte plasma membranes from treated rats. These findings suggested that PIA could down-regulate the adenosine receptor, although the effect appeared to be small. The current findings demonstrate that in a direct in vitro system, PIA can cause up to 80% down-regulation of adenosine receptors after 48 h. The PIA-induced loss of adenosine receptors was time- and concentration-dependent and was apparently due to a reduced number of binding sites, with no change in affinity.

Agonist-induced down-regulation of cell-surface receptors has been extensively documented. The mechanism is generally considered to involve internalization of the receptor-ligand complex and subsequent degradation of the receptor within the cell (see Ref. 35 for review). Relatively rapid down-regulation (minutes to a few hours) is usually thought to represent sequestration of the receptor from the cell surface, as has been observed, for example, with the frog erythrocyte \(\beta\)-adrenergic receptor (36, 37). In many systems, for example, the adipocyte insulin receptor, most of the receptors are rapidly recycled back to the cell surface, and a net loss of receptors is not seen for several hours (13, 38). In the experiments described here, receptors were measured in a crude membrane fraction, with no attempt to distinguish between cell-surface and sequestered receptors. We found similar down-regulation of adenosine receptors when the experiments were performed on detergent-solubilized extracts of whole cells (data not shown). Furthermore, receptor loss was not observed until at least 6 h of incubation with PIA. Hence, it is likely that the observed receptor loss is due to a decrease in total cellular receptors, resulting from endocytotic internalization and degradation. However, other potential mechanisms for down-regulation such as receptor inactivation at the cell surface or shedding of receptors into the incubation medium cannot be excluded.

\(\beta\)-Adrenergic receptors in some cells are structurally altered following exposure to agonists. Thus, the turkey erythrocyte \(\beta\)-adrenergic receptor migrates somewhat slower on SDS-PAGE if the cells are pre-exposed to \(\beta\)-agonists. This is thought to be due to phosphorylation of the receptor (39). Photoaffinity cross-linking demonstrated that the relative mobility of adenosine receptors from PIA-treated cells was unaltered. However, receptor phosphorylation often occurs without changes in electrophoretic mobility, and so we cannot rule out the possibility that the adenosine receptor is phosphorylated.

Pertussis toxin has been reported to ADP-ribosylate a number of proteins in the 39–45-kDa range. In adipocytes, under appropriate conditions, the toxin is thought to label primarily the 41-kDa \(\alpha\)-subunit of \(G_i\) (34, 40). In the current studies a single 41-kDa protein was ADP-ribosylated, and hence this is assumed to be the \(\alpha\)-subunit of \(G_i\), but other possibilities cannot be totally excluded. This 41-kDa protein was down-regulated by PIA. Previous studies on down-regulation of G-protein-linked receptors have failed to demonstrate concomitant loss of the G-protein. For example, down-regulation of opiate receptors (which inhibit adenylyl cyclase via \(G_i\)) in neuroblastoma x glioma NG108-15 hybrid cells occurred without loss of \(G_i\) (41). Similarly, \(\beta\)-adrenergic receptor down-regulation has been reported to occur without concomitant internalization of \(G_i\) (37). However, Parsons and Stiles (34) also reported apparent loss of \(G_i\) in adipocytes from PIA-treated rats. \(G_i\) down-regulation may be unique to the adenosine receptor system, or it could be a function of the cell type utilized in these studies.

As described above, one possibility is that the mechanism of adenosine receptor down-regulation is endocytosis and intracellular degradation. This raises the possibility that \(G_i\) is internalized along with the adenosine receptor. Interestingly, \(G_i\) was down-regulated by only about 45% under conditions where about 80% adenosine receptor loss occurred. Furthermore, the dose-response relationships were quite different (compare Figs. 1 and 7). There are several possible explanations for these quantitative differences. For example, it is possible that there are many more \(G_i\) molecules than adenosine receptors or that there are different populations of \(G_i\) associated with the different receptor types responsible for inhibition of adenylyl cyclase. Another possibility is that the quantitative kinetics of \(G_i\) internalization, recycling, and degradation differ from those of the adenosine receptor or that the mechanisms of receptor and \(G_i\) down-regulation are totally different.

An important finding in the present study is that the sensitivity of adipocytes to insulin can be decreased by prolonged incubation with an adenosine receptor agonist in vitro. Although the mechanism by which this decreased insulin sensitivity occurs is not clear, \(^{125}\text{I}\)-insulin binding to the cells was unaltered. This suggests that insulin receptors are unaffected by PIA treatment. The insulin receptor is known to have associated with it a tyrosine-specific protein kinase, which may be involved in the mechanism by which insulin stimulates glucose transport (42, 43). We cannot rule out the possibility that the insulin receptor kinase was altered by prolonged PIA treatment. However, the simplest explanation for the findings is that PIA decreased insulin sensitivity by down-regulating the adenosine receptor/\(G_i\) system. This hypothesis is consistent with the known ability of both adenosine and PIA to acutely increase adipocyte insulin sensitivity (1, 3). Surprisingly, the decreased insulin sensitivity of the PIA-treated cells was also evident when glucose transport was measured in the presence of adenosine deaminase. This suggests that the status of the adenosine receptor/\(G_i\) system can influence insulin sensitivity even in the absence of adenosine. One possible explanation is that \(G_i\) may be involved in mediating effects of other inhibitory receptors. This could also explain the observation that the rate of lipolysis in the presence of adenosine deaminase was higher in the PIA-treated cells.
Insulin resistance is a common finding associated with conditions such as obesity and type II (noninsulin-dependent) diabetes. Insulin resistance is considered to arise either from insulin receptor abnormalities or deficiencies, from poorly defined "post-receptor defects," or from a combination of problems (44, 45). Furthermore, it has been suggested that abnormalities in the insulin receptor kinase may be involved in insulin resistance in some situations (46, 47). The findings presented here suggest a further potential mechanism by which insulin sensitivity, at least in adipose tissue, could be altered, i.e. via abnormalities in the adenosine receptor and/or G,.

Acknowledgments—I would like to thank Maristella Partin for excellent technical assistance and LaDeane Bramer for preparing the manuscript.

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