MOLECULAR PHARMACOLOGY, 22:614-618

Modulation of Insulin Sensitivity by Adenosine

Effects on Glucose Transport, Lipid Synthesis, and Insulin Receptors of the Adipocyte

HANS-GEORG JOOST[1] AND HANS-JÜRGEN STEINFELDER

Institut für Pharmakologie und Toxikologie der Universität Göttingen, 3400 Göttingen, Federal Republic of Germany

Received March 15, 1982; Accepted July 27, 1982

SUMMARY

Insulin action and insulin binding in isolated rat fat cells incubated with adenosine or adenosine deaminase were studied. Adenosine enhanced the effects of insulin on glucose transport and glucose metabolism. The nucleoside shifted the concentration-response curves of insulin-stimulated D-[3-^{3}H]glucose incorporation into total lipids, and of D-[U-^{14}C]glucose conversion to fatty acids to smaller insulin concentrations. In addition, the maximal response of the fatty acid synthesis was increased. Insulin sensitivity and maximal response to insulin of the glucose transport system, as assessed by the rate of uptake of 2-deoxyglucose and 3-*O*-methylglucose, were increased by adenosine. The adenosine derivative N^6-phenylisopropyladenosine similarly enhanced deoxyglucose transport in the presence of insulin. However, insulin binding was not affected by adenosine. The results suggest that adenosine modulates insulin action at a step distal from the insulin receptor, and before, or at, the glucose transport system.

INTRODUCTION

Isolated adipocytes spontaneously release adenosine which in turn may modulate the metabolic activity of the cells (1, 2). The nucleoside, as well as several N^6-substituted analogues (3), reduces lipolysis by lowering cyclic AMP levels of the fat cells (4). In addition, adenosine modifies the effects of insulin on adipocyte metabolism, sensitizing the cell to the action of the hormone. In the absence of adenosine, insulin fails to inhibit catecholamine-stimulated lipolysis, and is less potent in stimulating the oxidation of glucose (5, 6). Recently, adenosine receptors have been identified and characterized in plasma membranes of adipocytes (7). Therefore, it has been concluded that adenosine plays a significant role as an endogenous regulator of adipocyte metabolism.

The present study was designed to investigate further the modulation of insulin action by adenosine. In order to identify the site of action of adenosine, three different levels of insulin action were studied: the binding of insulin to its receptor, the effect of insulin on glucose transport, and its effect on lipid synthesis. Adenosine deaminase was used to remove spontaneously released adenosine in the control experiments.

The results indicate that glucose transport is the earliest insulin-dependent step of cell metabolism modulated by adenosine. It is thus suggested that the nucleoside may modify the coupling process between insulin receptors and glucose transport.

MATERIALS AND METHODS

Chemicals. Adenosine, phloretin, and bovine serum albumin (Fraction V) were obtained from Serva (Heidelberg, Federal Republic of Germany). N^6-Phenylisopropyladenosine (as a gift) and adenosine deaminase (from calf intestine) were from Boehringer (Mannheim, Federal Republic of Germany). Crystalline porcine insulin was supplied as a gift from the Hoechst AG (Frankfurt, Federal Republic of Germany). ^{125}I-Insulin (average specific activity 800 Ci/mmole) was purchased from Behringwerke AG (Frankfurt, Federal Republic of Germany). All other radiochemicals (D-[3-^{3}H]glucose, D-[U-^{14}C]glucose, 2-deoxy-D-[1-^{3}H]glucose, and 3-*O*-methyl-D-[1-^{3}H]glucose) were obtained from the Radiochemical Centre (Amersham, England). Unlabeled deoxyglucose and methylglucose were obtained from Sigma Chemical Company (St. Louis, Mo.), and crude bacterial collagenase (Type IV, Lot 49P207) from Worthington Biochemicals (Freehold, N. J.). The scintillation cocktail for water-soluble samples (Instagel) was purchased from Packard (Frankfurt), and the toluene-based cocktail (Quickszint) from Zinsser (Frankfurt). All other reagents (analytical grade) were obtained from Merck AG (Darmstadt, Federal Republic of Germany).

Animals. Male albino Wistar rats (body weight 150–200 g) bred in our institute were used throughout. The animals had free access to food and water.

Preparation of fat cells. Isolated fat cells were prepared by the method of Rodbell (8). Approximately 1 g of adipose tissue was incubated in 5 ml of Krebs/Ringer/

[1] With the technical assistance of Christoph Schmitz-Salue.

0026-895X/82/060614-05$02.00/0

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer containing 5 mg collagenase, albumin (40 mg/ml), and 1 mM glucose. The incubation vial (polypropylene) was kept in a shaking water bath (two strokes per second) at 37° for 60 min, and gently shaken by hand for an additional 5 min in order to complete the isolation. The cells were filtered through nylon mesh (pore width 0.3 mm) and washed five times with fresh buffer. An aliquot of the resulting fat cell suspension was fixed with osmium tetroxide (9) and counted in a Fuchs-Rosenthal counting chamber. Total lipid volume was determined by centrifugation of an aliquot in a hematocrit tube (10). The suspension was adjusted to the desired fat cell concentration and immediately distributed into the incubation vials which contained the agents under investigation. All incubations were carried out with Krebs/4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer containing 1% defatted albumin.

Lipid synthesis. The incorporation of D-[3-^{3}H]glucose into total lipids was measured as described by Moody *et al.* (11). A fat cell suspension (1 ml) containing 70–100 $\times 10^3$ cells was incubated for 60 min in polypropylene scintillation vials in the presence of 0.3 μCi of tracer glucose and 1 mM unlabeled glucose. The incubation was stopped by the addition of the toluene-based scintillation cocktail. The vials were shaken for 120 min to extract total lipids and allowed to equilibrate overnight; radioactivity was counted without separation of the two layers (11). Blanks containing cells and radioactivity were included in each experimental series, and the reaction was stopped immediately after mixing without prior incubation. The data were calculated and expressed as nanomoles of [^{3}H]glucose incorporated, regardless of the metabolic fate of the tritium atom, and therefore do not represent the total amounts of glucose incorporated.

Fatty acid synthesis was investigated in a separate series of experiments, using uniformly labeled [^{14}C]glucose. Cells (70–100 $\times 10^3$) were incubated for 60 min in 1 ml of buffer containing 0.15 μCi of [U-^{14}C]glucose and 1 mM unlabeled glucose. The samples were extracted for 120 min with 5 ml of toluene, the toluene layer was separated and evaporated to dryness, and 1 ml of ethanol/potassium hydroxide (12) was added. The samples were heated in a water bath (80°) and allowed to evaporate; 1 ml of 1 N hydrochloric acid was added to the dry residue. Fatty acids were extracted with 2 ml of toluene, and the radioactivity in an aliquot of the toluene layer was determined. The data were corrected for blanks and calculated on the basis of the specific activity as nanomoles of [^{14}C]glucose incorporated.

Deoxyglucose uptake. The rate of 2-deoxyglucose uptake was determined as described by Olefsky (12) with minor modifications. Samples containing 200–300 $\times 10^3$ cells/200 μl were incubated for 30 min with the indicated concentrations of insulin, adenosine, or adenosine deaminase in glucose-free buffer. The samples were equilibrated to room temperature (22°) and were added to 5 μl of buffer containing 0.25 μCi of [^{3}H]deoxyglucose (final concentration 0.15 mM). The assay was stopped after 3 min by rapid centrifugation of the cells through a layer of silicone oil. The cells were floated to the top of the vials, and were removed with the aid of a disposable pipette tip. Scintillation cocktail was added (Instagel) and radioactivity was determined. In control experiments it was assured that the rate of deoxyglucose uptake was linear over a period of 5 min. To correct the data for radioactivity trapped within the cell layer or unspecifically bound by the cells, blanks which contained 1 mM phloridzin to inhibit carrier-mediated transport were included in each series and subtracted from each value.

3-O-Methylglucose uptake. Transport of methylglucose was determined by the previously described method of Whitesell and Gliemann (13) with minor modifications. Approximately 400 $\times 10^3$ cells/200 μl were incubated for 30 min at 37° in the presence of insulin, or other agents under investigation. After equilibration to room temperature (22°), 200 μl of each sample were added to 5 μl of buffer containing 0.6 μCi of [^{3}H]methylglucose (final concentration 0.15 mM). Transport of the glucose analogue was stopped after 4 sec by addition of 8 ml of ice-cold phloretin solution (1 mM). Silicone oil was added, and the samples were centrifuged within 2 min after the termination of transport. Blanks were included which had been added to the stopping solution prior to the addition of the radioactivity, and all data were corrected accordingly. Timing of the transport assay was performed with the acoustic signals of a stopwatch. Control experiments revealed that the uptake approached equilibrium within 20 sec. After 4 sec, 16% of the water space was filled in unstimulated cells, and 38% of equilibrium uptake was reached in insulin-stimulated cells.

Insulin binding. The analysis of insulin binding was performed as previously described (14). Briefly stated, 200–300 $\times 10^3$ cells/200 μl were mixed with 50 μl of Tris buffer containing 0.04 μCi of ^{125}I-labeled insulin. Buffer (50 μl) containing unlabeled insulin was added to each sample, and the vials were allowed to equilibrate at 22° for 45 min. The assay was terminated by centrifugation of the cells through a layer of dinonylphthalate. The cell layer was removed, and radioactivity was counted in a γ-scintillation counter. Average nonspecific binding, as assessed with samples containing a large excess of unlabeled insulin (10^{-5} M), was 15% of the total bound radioactivity.

Calculations and statistical analysis. Data were calculated and presented as means ± standard error, and differences were tested for statistical significance with the Wilcoxon matched-pairs signed-rank test or, where applicable, with the *U*-test of Wilcoxon, Mann and Whitney. The concentrations of insulin which gave rise to a half-maximal response were determined graphically in each separate experiment, and means ± standard error were calculated and presented.

RESULTS

The main finding of the present study was that adenosine increased the insulin sensitivity of glucose transport and glucose metabolism in adipocytes without any significant change in receptor binding. As compared with cells treated with adenosine deaminase, adenosine shifted the concentration-response curve of insulin-stimulated lipogenesis to lower insulin concentrations (Fig. 1). A significantly greater amount of [3-^{3}H]glucose was converted to lipids at submaximally stimulating insulin concentrations, whereas the basal response of the fat cells,

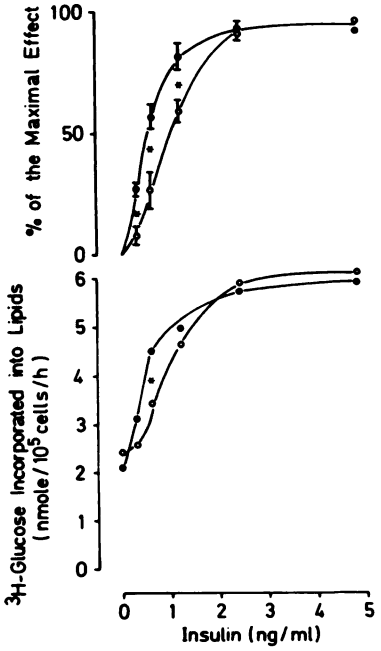

FIG. 1. *Effect of adenosine (10 μM; ●) on the insulin-stimulated incorporation of D-[3-^{3}H]glucose into total lipids*

Control experiments (○) were performed in the presence of adenosine deaminase (2.5 μg/ml). The data represent the means of eight experiments; *asterisks* symbolize significance ($2\ p < 0.01$). Insulin concentrations yielding half-maximal responses were 0.52 ± 0.04 ng/ml (adenosine) and 1.03 ± 0.9 ng/ml (adenosine deaminase) ($2\ p < 0.01$).

as well as the maximal response elicited by insulin, were not significantly affected.

The tracer hydrogen atom of [3-^{3}H]glucose is predominantly incorporated into the fatty acid moiety of lipids by NADPH, which is supplied by the pentose cycle (15). Therefore, we set up a separate series of experiments using [U-^{14}C]glucose as tracer (Fig. 2) in order to investigate whether the observed effect reflected the conversion of glucose carbon to fatty acids, or whether it was due to alterations of glucose degradation and NADPH generation in the pentose cycle. In these experiments adenosine decreased the insulin concentration which yielded a half-maximal response. In addition, the maximal effect of insulin on the conversion of glucose carbon to fatty acids was significantly enhanced.

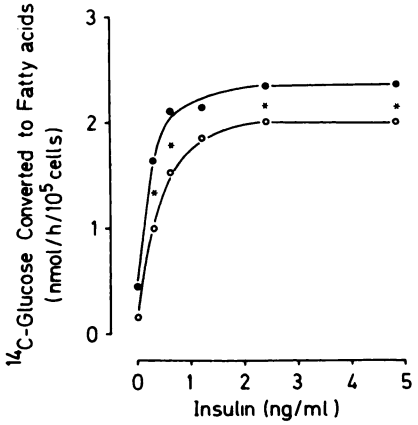

FIG. 2. *Effect of adenosine (10 μM; ●) on the insulin-stimulated conversion of D-[U-^{14}C]glucose to fatty acids*

Control experiments (○) were performed in the presence of adenosine deaminase (2.5 μg/ml). The data represent the means of eight experiments. Statistical significance ($2\ p < 0.01$, Wilcoxon's matched-pairs signed-rank test) is symbolized by *asterisks*. The concentrations of insulin yielding half-maximal responses were 0.24 ± 0.03 ng/ml (adenosine) and 0.46 ± 0.03 ng/ml (adenosine deaminase) ($2\ p < 0.001$).

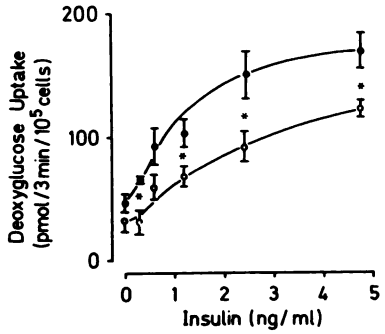

FIG. 3. *Effect of insulin on 2-deoxyglucose transport in the presence (10 μM; ●) and absence of adenosine (adenosine deaminase, 2.5 μg/ml; ○)*

The data represent the means of six experiments. *Asterisks* symbolize statistical significance ($2\ p < 0.05$). Insulin concentrations giving rise to half-maximal stimulation of rate of uptake were 0.96 ± 0.4 ng/ml (adenosine) and 1.44 ± 0.5 ng/ml (adenosine deaminase) ($2\ p < 0.05$).

Similar effects of adenosine were observed on insulin-stimulated glucose transport, as assessed by the determination of rates of uptake with the aid of the glucose analogues 2-deoxyglucose (Fig. 3) and 3-*O*-methylglucose (Fig. 4). The concentration-response curves revealed that the sensitivity of the cells, as well as the maximal response elicited by the hormone, were increased by adenosine. Even at a very high insulin concentration (36 ng/ml) the transport rate of cells treated with adenosine deaminase (5.15 ± 0.3) was lower than that of cells incubated in the presence of adenosine (6.16 ± 0.4 pmoles of methylglucose/4 sec/10^5 cells).

When compared with control cells incubated without added adenosine in buffer containing insulin (0.6 ng/ml), adenosine deaminase significantly reduced the rate of

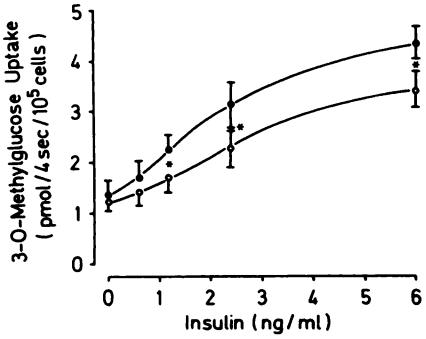

FIG. 4. *Effect of insulin on 3-O-methylglucose transport in the presence (●) and absence of adenosine (10 μM; ○; adenosine deaminase, 2.5 μg/ml)*

The data represent the means ± standard error of eight experiments. *Asterisks* symbolize statistical significance ($2\ p < 0.01$). The concentrations of insulin producing half-maximal stimulation of transport were 2.05 ± 0.16 ng/ml (adenosine) and 2.58 ± 0.23 ng/ml (adenosine deaminase) ($2\ p < 0.05$, Wilcoxon's matched-pairs signed-rank test).

uptake of deoxyglucose (53.1 ± 8 pmoles)/3 min/10^5 cells versus 78.4 ± 14 in the control cells ($p < 0.01$, Wilcoxon matched-pairs signed-rank test). Thus adipocytes incubated at such high cell concentrations (200–400 × 10^3/200 μl) apparently release sufficient amounts of adenosine to enhance the effect of insulin on glucose transport.

It should be noted that in the control series of these transport experiments an unexpected finding became apparent: considerably higher concentrations of insulin were necessary to accelerate glucose transport than to stimulate the conversion of glucose to lipids (Fig. 2). This decrease of insulin sensitivity was apparently inversely correlated to the cell concentrations used in the experiments. At higher cell concentrations, insulin sensitivity of adipocytes, as assessed by the concentrations of insulin yielding a half-maximal response, decreased in spite of the presence of adenosine. The possibility cannot be discounted that factors which, unlike adenosine, inhibit insulin action are released from the fat cells. Furthermore, degradation of insulin by the fat cells might contribute to this effect.

Since degradation of insulin by the fat cells might cause an apparent decrease of insulin sensitivity, the precipitability (trichloroacetic acid) of ^{125}I-labeled insulin after incubation under the conditions used in the transport assays was studied (400 × 10^3 cells/200 μl, 30-min incubation, 4.8 ng of insulin per milliliter). A fraction (17.5% ± 2.5%) of insulin was degraded by cells incubated in buffer only. Adenosine (18.7% ± 2.7%) as well as adenosine deaminase (17.8% ± 2.5%) failed to alter the degradation rate.

In order to demonstrate a concentration-dependent effect of adenosine on deoxyglucose transport, adipocytes were incubated in 20-fold lower cell concentration (20 × 10^3/200 μl) than in the previous experiments. Figure 5 shows that adenosine stimulated deoxyglucose uptake in a concentration range comparable to that inhibiting the theophylline-stimulated lipolysis.

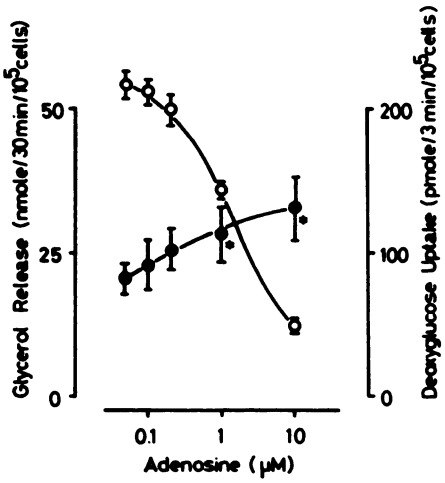

FIG. 5. *Concentration-response curves of the effects of adenosine on 2-deoxyglucose uptake (●) and on glycerol release (○) from fat cells*

The cell concentration was 20 × 10^3/200 μl. The data represent the means ± standard error of six experiments. Insulin (0.4 ng/ml) was present in the transport experiments, and *asterisks* symbolize significance ($p < 0.05$) of the stimulation. Lipolysis was stimulated by 0.6 mM theophylline.

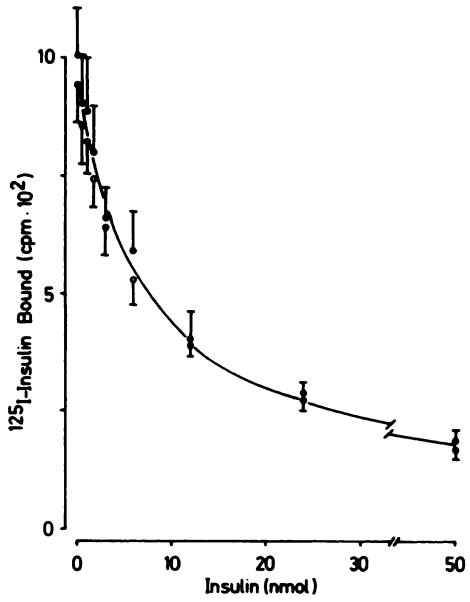

FIG. 6. *Lack of effect of adenosine on insulin binding to isolated fat cells*

The adipocytes had been preincubated for 60 min prior to the binding assay in the presence (10 μM; ○) and absence of adenosine (adenosine deaminase, 2.5 μg/ml; ○). The data represent the means of eight experiments and have not been corrected for nonspecific binding. The cell concentration per vial was 250,000/300 μl; total radioactivity was 68,000 cpm (150 pM tracer insulin).

Like adenosine, its derivative, N^6-phenylisopropyladenosine, significantly enhanced the effect of insulin (0.6 ng/ml) on deoxyglucose uptake. The transport rate of cells incubated in the presence of 50 nM of the adenosine derivative was 65.8 ± 15 pmoles/3 min/10^5 cells, compared with 39.5 ± 9 ($p < 0.05$) in the control cells. Both series had been incubated without the addition of adenosine deaminase.

In contrast to the observed enhancement of the metabolic effects of insulin, adenosine failed to produce significant effects on the receptor binding of insulin when added to the binding assay (data not shown), as well as when cells were preincubated for 60 min at 37° in the presence of the nucleoside (or adenosine deaminase, respectively) prior to the binding assay (Fig. 6).

DISCUSSION

The results presented above provide further evidence that adenosine sensitizes the fat cell to the action of insulin. Preliminary conclusions may be drawn concerning the possible site of action of the nucleoside. According to the hypotheses commonly maintained, insulin binding to its membrane receptor initiates an increase in glucose utilization, the first and largely limiting step of which is the rate of glucose transport. As adenosine failed to modify insulin-receptor binding and enhanced insulin action on glucose transport, the results strongly suggest a site of action distal from the insulin receptor, and before, or at, the glucose transport system.

This conclusion raises the question of whether adenosine modulates the coupling mechanism of insulin receptors and glucose transport or whether it affects the trans-

port system directly. The action of adenosine was apparently dependent on the stimulatory effect of insulin. Basal transport in the absence of insulin was only slightly stimulated (if at all) by adenosine. Thus a modulation of the coupling step between insulin receptors and glucose transport seems to be more likely than a direct action of adenosine on the glucose carriers.

The variety of effects produced by adenosine and its N^6-substituted derivatives in several biological systems (for review see ref. 16) largely reflects alterations of the cellular cyclic AMP levels (4–6, 17). For several years the nucleoside has been known to modulate membrane-bound adenylate cyclase (4). More recently, two distinct receptor types for adenosine have been identified (18): the purine (*P*) site, which mediates inhibition, and the ribose (*R*) site, which generally mediates activation of the adenylate cyclase. The predominant receptor type of the adipocyte is the *R* site, the stimulation of which, in contrast to other cell types, inhibits adenylate cyclase (19). The effects of adenosine observed in the present study may therefore reflect a decrease of cyclic AMP levels due to the inhibition of adenylate cyclase. However, one has to take into consideration that apparently not all effects of insulin are antagonized by high cyclic AMP levels. Unlike lipogenesis, glucose transport may be accelerated when cyclic AMP is elevated by catecholamines (20, 21). Therefore we hesitate to explain definitively the effects of adenosine on glucose transport by a reduction of cellular cyclic AMP levels.

REFERENCES

1. Schwabe, U., R. Ebert, and H. C. Erbler. Adenosine release from isolated fat cells and its significance for the effects of hormones on cyclic 3′,5′-monophosphate levels and lipolysis. *Naunyn-Schmiedebergs Arch. Pharmacol.* **276**:133–148 (1973).
2. Fain, J. N. Inhibition of adenosine cyclic 3′,5′-monophosphate accumulation in fat cells by adenosine, N^6-(phenylisopropyl)-adenosine, and related compounds. *Mol. Pharmacol.* **9**:595–604 (1973).
3. Trost, T., and K. Stock. Effects of adenosine derivatives on cyclic AMP accumulation and lipolysis in rat adipocytes and on adenylate cyclase in adipocyte plasma membranes. *Naunyn-Schmiedebergs Arch. Pharmacol.* **229**:33–40 (1977).
4. Fain, J. N., R. H. Pointer, and W. F. Ward. Effects of adenosine nucleotides on adenylate cyclase, phosphodiesterase, cyclic adenosine monophosphate accumulation, and lipolysis in fat cells. *J. Biol. Chem.* **247**:6866–6872 (1972).
5. Schwabe, U., P. S. Schönhöfer, and R. Ebert. Facilitation by adenosine of the action of insulin on the accumulation of adenosine 3′,5′-monophosphate, lipolysis, and glucose oxidation in isolated fat cells. *Eur. J. Biochem.* **46**:537–545 (1974).
6. Fain, J. N., and P. W. Wieser. Effects of adenosine deaminase on cyclic adenosine monophosphate accumulation, lipolysis, and glucose metabolism of fat cells. *J. Biol. Chem.* **250**:1027–1034 (1975).
7. Trost, T., and U. Schwabe. Adenosine receptors in fat cells. Identification by (−)-N^6-[^{3}H]phenylisopropyladenosine binding. *Mol. Pharmacol.* **19**:228–235 (1981).
8. Rodbell, M. Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. *J. Biol. Chem.* **239**:375–380 (1964).
9. Hirsch, J., and E. Gallian. Methods for the determination of adipose cell size in man and animals. *J. Lipid Res.* **9**:110–119 (1968).
10. Gliemann, J., K. Østerlind, J. Vinten, and S. Gammeltoft. A procedure for measurement of distribution spaces in isolated fat cells. *Biochim. Biophys. Acta* **286**:1–6 (1967).
11. Moody, A. J., M. A. Stan, M. Stan, and J. Gliemann. A simple free fat cell bioassay for insulin. *Horm. Metab. Res.* **6**:12–16 (1974).
12. Olefsky, J. M. Mechanisms of the ability of insulin to activate the glucose-transport system in rat adipocytes. *Biochem. J.* **172**:137–145 (1978).
13. Whitesell, R. R., and J. Gliemann. Kinetic parameters of transport of 3-*O*-methylglucose and glucose in adipocytes. *J. Biol. Chem.* **254**:5276–5283 (1979).
14. Joost, H. G., W. Arend, and S. Holze. Effects of tolbutamide on insulin binding to isolated fat cells of the rat. *Biochem. Pharmacol.* **31**:1227–1231 (1982).
15. Katz, J., and R. Rognstad. The metabolism of tritiated glucose by adipose tissue. *J. Biol. Chem.* **241**:3600–3610 (1966).
16. Schwabe, U., R. Ebert, and H. C. Erbler. Adenosine release from rat fat cells: effect on cyclic AMP levels and hormone actions. *Adv. Cyclic Nucleotide Res.* **5**:569–584 (1975).
17. Wieser, P. B., and J. N. Fain. Insulin, prostaglandin E_1, phenylisopropyladenosine and nicotinic acid as regulators of fat cell metabolism. *Endocrinology* **96**:1221–1225 (1975).
18. Londos, C., and J. Wolff. Two distinct adenosine-sensitive sites on adenylate cyclase. *Proc. Natl. Acad. Sci. U. S. A.* **74**:5482–5486 (1977).
19. Londos, C., D. M. F. Cooper, W. Schlegel, and M. Rodbell. Adenosine analogs inhibit adipocyte adenylate cyclase by a GTP-dependent process: basis for actions of adenosine and methylxanthines on cyclic AMP production and lipolysis. *Proc. Natl. Acad. Sci. U. S. A.* **75**:5362–5366 (1978).
20. Ludvigsen, C. L., Jarett, and J. M. McDonald. The characterization of catecholamine stimulation of glucose transport by rat adipocytes and isolated plasma membranes. *Endocrinology* **106**:768–790 (1980).
21. Häring, H. U., E. Biermann, and W. Kemmler. Coupling of insulin binding and insulin action on glucose transport in fat cells. *Am. J. Physiol.* **240**:E556–E565 (1981).

Send reprint requests to: Dr. H. G. Joost, Institut für Pharmakologie und Toxikologie, Robert-Koch-Strasse 40, 3400 Göttingen, Federal Republic of Germany.