Elevation of the Number of Cell-surface Insulin Receptors and the Rate of 2-Deoxyglucose Uptake by Exposure of 3T3-L1 Adipocytes to Tolbutamide*

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Sulfonylurea compounds are hypoglycemic agents which by unknown mechanisms alter the amount of insulin receptor and the rate of glucose utilization in tissues exposed to the drugs. In this study the effects on insulin binding and uptake of 2-deoxyglucose by 3T3-L1 adipocytes were assessed after maintaining cell monolayers for 1–3 days in medium containing different concentrations of the sulfonylurea, tolbutamide. The amount of insulin bound by treated monolayers gradually increased to values 150–250% of those of control monolayers after 2–3 days of exposure to 1.5 mm tolbutamide. Such increases in insulin binding capacity arose primarily from an increase in receptor number and not from an alteration in the affinity of the receptor for insulin. Concomitant with the changes observed for the insulin receptor, tolbutamide-treated monolayers expressed 1.5–2-fold higher rates of uptake of 2-deoxyglucose relative to control monolayers at concentrations of insulin between 0 and 10^{-10} M. This study thus demonstrates the responsiveness of adipocytes to tolbutamide and also establishes the usefulness of 3T3-L1 cells as a model system in which to study the mechanism of tolbutamide action, both as it relates to the use of sulfonylurea compounds in clinical applications and as possible probes for perturbing and studying relatively uncharacterized regulatory pathways controlling receptor level and biological responses to insulin.

Administration of sulfonylurea drugs, alone or in combination with insulin therapy and diet control, has been shown to ameliorate hyperglycemia and restore insulin sensitivity in some patients (1–3). The initial effects of sulfonylurea administration appear to arise from acute stimulation of the rate of insulin release from the pancreas, thus providing a sufficient increase in the concentration of circulating insulin to reduce plasma glucose concentrations (1, 2, 4). Some patients continue to demonstrate reduced plasma glucose concentrations and increased insulin binding to isolated cells after chronic treatment with sulfonylurea agents (3, 5, 6). Thus, these compounds possess the interesting additional property of increasing glucose uptake and receptor number in peripheral tissues after prolonged exposure when the concentrations of plasma insulin have subsided to pretreatment levels. Similar findings have been reported in studies with animals exposed for prolonged intervals to various sulfonylurea agents (4, 7).

An understanding of the mechanism of action of sulfonylurea drugs can provide both a better rationale for their clinical use or disuse and possibly important insight into unknown mechanistic aspects of the regulation of receptor concentration and distribution within the cell.

The demonstrated effects of sulfonylurea agents on cellular receptor level and glucose utilization in vitro have been variable. Sulfonylurea drugs do elevate the level of insulin receptors expressed by certain cells in culture (8). However, other comprehensive studies with lymphocyte, mammary carcinoma, and hepatoma cell lines exposed to a variety of sulfonylurea agents demonstrated no alterations in insulin receptor level (9). Although the sulfonylurea, tolazamide, did not affect the receptor level for rat adipose tissue in organ culture, it did elevate the rate of insulin-stimulated 2-deoxyglucose uptake (10).

The variability of observed effects of sulfonylurea compounds in vitro on receptor level and glucose utilization may reflect the specificity of tissue response, the type of sulfonylurea utilized, or the inability to duplicate the extended intervals of exposure of cells to sulfonylureas attained in vivo. Although sulfonylurea agents can be shown to have rapid short-term effects on some cellular processes, such as ion flux (11), longer intervals of exposure appear to be required to observe their effects on insulin receptor levels and on the rate of glucose uptake in vitro.

We have investigated the effects that different concentrations and time of exposure to the sulfonylurea, tolbutamide, have on the rate of basal and insulin-stimulated uptake of 2-deoxyglucose, and on the level of insulin receptor expressed by 3T3-L1 adipocytes. Consistent with the chronic effects of sulfonylurea drugs on extrapancreatic tissues in vivo, this study demonstrates that exposure of 3T3-L1 adipocytes to tolbutamide for intervals in excess of 2 days induces both a change in surface insulin receptor number and the characteristics of glucose uptake. The magnitude of these responses to tolbutamide by 3T3-L1 adipocytes is larger than that normally observed in other in vitro systems and should facilitate further mechanistic studies of tolbutamide action in vivo.

MATERIALS AND METHODS

Cell Culture—Undifferentiated 3T3-L1 preadipocytes were cultured in 3.5-cm dishes (Falcon) with Dulbecco's modified Eagle's
medium (25 mM glucose) containing 10% (v/v) fetal calf serum (Gibco and KC Biological) as described (12). At 2 days post-confluence, the monolayers were exposed to fresh medium (1.5 ml) containing 10% fetal calf serum, 0.5 mM isobutylmethylxanthine, 1 mM dexamethasone, and 10 µg/ml of insulin (day 0). On day 2, the isobutylmethylxanthine and dexamethasone were removed, and the medium was replaced every 2 days with medium containing 10% fetal calf serum and 10 µg/ml insulin. By day 6, >90% of the cells expressed the adipocyte phenotype.

For earlier experiments presented in Figs. 1–3, the monolayers were washed several times with phosphate-buffered saline prior to replacing the medium with Dulbecco’s modified Eagle’s medium (25 mM glucose) containing 10% fetal calf serum with or without tolbutamide. For the experiments presented in Figs. 4–7, a more extensive wash protocol was employed to ensure rapid attainment of basal glucose transport levels, and efficient reversal of the insulin induced state of receptor down-regulation (13). Prior to each experiment, the monolayers were washed with 2 × 1.5 ml of Dulbecco’s modified Eagle’s medium (25 mM glucose) containing 1% (v/v) fetal calf serum and then incubated at 37°C (5% CO2, 95% air) for 20 min intervals. The medium was replaced with fresh medium prior to each 20 min incubation. After the last wash, the monolayers were incubated for 3 days with the concentrations of tolbutamide. On the 3rd day the duplicate monolayers maintained in the absence of sulfonylurea bound approximately 0.028 pmol of insulin/10^6 cells under these assay conditions. Exposure of 3T3-L1 adipocytes for 3 days to 0.5, 1.0, or 2.5 mM tolbutamide increased the amount of insulin bound to levels 1.7-, 2.1-, and 2.5-fold those of control values, respectively. A loss of cells from the monolayer was observed at the highest concentration of tolbutamide tested, and suggests that decreased cell viability (25% drop in cell number/dish) with 5 mM tolbutamide may have contributed to the fall in insulin binding observed at this concentration. The experiment presented in Fig. 1 is representative of three separate experiments which demonstrated a maximal effect on insulin binding by exposure of the cells to concentrations of tolbutamide between 1–3 mM. For this range of tolbutamide concentrations the mean of the ratios of the amount of insulin bound by tolbutamide-treated and untreated monolayers (treated/untreated) was 1.87 ± 0.38 (S.D., n = 6). The 95% confidence limits for the ratio of insulin binding between tolbutamide-treated and untreated adipocytes were calculated by dividing the ratio of insulin binding between tolbutamide-treated and untreated monolayers by the number of measurements made.

**Materials and Methods**

**Drug Effects on Insulin Receptor Number and Sugar Uptake**

**Results**

**Effects of Tolbutamide on the Amount of Surface Insulin Receptor Expressed by 3T3-L1 Adipocytes**—The results presented in Fig. 1 demonstrate a tolbutamide-dependent increase in 125I-insulin binding to 3T3-L1 adipocytes. Control monolayers maintained in the absence of sulfonylurea bound approximately 0.028 pmol of insulin/10^6 cells under these assay conditions. Exposure of 3T3-L1 adipocytes for 3 days to 0.5, 1.0, or 2.5 mM tolbutamide increased the amount of insulin bound to levels 1.7-, 2.1-, and 2.5-fold those of control values, respectively. A loss of cells from the monolayer was observed at the highest concentration of tolbutamide tested, and suggests that decreased cell viability (25% drop in cell number/dish) with 5 mM tolbutamide may have contributed to the fall in insulin binding observed at this concentration. The experiment presented in Fig. 1 is representative of three separate experiments which demonstrated a maximal effect on insulin binding by exposure of the cells to concentrations of tolbutamide between 1–3 mM. For this range of tolbutamide concentrations the mean of the ratios of the amount of insulin bound by tolbutamide-treated and untreated monolayers (treated/untreated) was 1.87 ± 0.38 (S.D., n = 6). The 95% confidence limits for the ratio of insulin binding between tolbutamide-treated and untreated adipocytes were calculated by dividing the ratio of insulin binding between tolbutamide-treated and untreated monolayers by the number of measurements made.

**Cell Number Determinations**—The number of cells contained in each dish was determined by incubating the monolayers for 2–3 h at 37°C in 2 ml of Krebs-Ringer phosphate buffer, pH 7.4, lacking calcium and magnesium, and containing 1% (w/v) bovine serum albumin, 25 mM glucose, and 5 mM EDTA. The number of cells released from duplicate dishes was then determined using a hemocytometer or Coulter counter. The values of insulin binding or 2-deoxyglucose uptake determined per dish were normalized to cell number. The 95% confidence limits for the ratio of insulin binding between tolbutamide-treated and untreated monolayers were calculated by dividing the ratio of insulin binding between tolbutamide-treated and untreated adipocytes by the number of measurements made.

**Conclusion**

The results presented in Fig. 1 demonstrate a tolbutamide-dependent increase in 125I-insulin binding to 3T3-L1 adipocytes. Control monolayers maintained in the absence of sulfonylurea bound approximately 0.028 pmol of insulin/10^6 cells under these assay conditions. Exposure of 3T3-L1 adipocytes for 3 days to 0.5, 1.0, or 2.5 mM tolbutamide increased the amount of insulin bound to levels 1.7-, 2.1-, and 2.5-fold those of control values, respectively. A loss of cells from the monolayer was observed at the highest concentration of tolbutamide tested, and suggests that decreased cell viability (25% drop in cell number/dish) with 5 mM tolbutamide may have contributed to the fall in insulin binding observed at this concentration. The experiment presented in Fig. 1 is representative of three separate experiments which demonstrated a maximal effect on insulin binding by exposure of the cells to concentrations of tolbutamide between 1–3 mM. For this range of tolbutamide concentrations the mean of the ratios of the amount of insulin bound by tolbutamide-treated and untreated monolayers (treated/untreated) was 1.87 ± 0.38 (S.D., n = 6). The 95% confidence limits for the ratio of insulin binding between tolbutamide-treated and untreated adipocytes were calculated by dividing the ratio of insulin binding between tolbutamide-treated and untreated monolayers by the number of measurements made.

**Fig. 1.** Altered binding of 125I-insulin to 3T3-L1 adipocytes exposed to varying concentrations of tolbutamide. Differentiated monolayers were incubated for 3 days with the concentrations of tolbutamide presented in the figure. The medium was replaced on the 2nd day of incubation with fresh medium and the appropriate concentrations of tolbutamide. On the 3rd day the duplicate monolayers were assayed for insulin binding capacity using 0.5 nm 125I-insulin as described under "Materials and Methods." The results are expressed as the mean ± range of the two determinations. The values in parentheses represent the ratio (treated/untreated) of the amount of insulin bound by tolbutamide-treated and untreated monolayers.
confidence interval for the mean ratio was 1.47–2.27.  

**Kinetics for the Alteration by Tolbutamide of Insulin Binding to 3T3-L1 Adipocytes**—To determine the kinetics by which tolbutamide alters the level of 125I-insulin binding to the cell surface of 3T3-L1 adipocytes, differentiated cells were treated with or without 1.5 mM tolbutamide. The binding of insulin to 3T3-L1 adipocytes was measured (using 0.5 nM 125I-insulin) as described under "Materials and Methods" at intervals between 0 and 96 h of exposure to tolbutamide. The results from one of two similar experiments are presented in Fig. 2. The amount of 125I-insulin bound by tolbutamide-treated cells rose significantly within 24 h. This rise, however, was only slightly greater than that observed in the control monolayers and is attributed predominantly to the reversal of the "down-regulated" state of cell-surface insulin receptors present prior to removal of insulin. The level of receptor measured in control monolayers decreased gradually during the following 2 days in culture. In contrast, exposure of 3T3-L1 cells to 1.5 mM tolbutamide increases the level of 125I-insulin binding to values 1.3-fold those of control after 24 h of exposure, and in this experiment binding attained levels 2.2- and 2.7-fold those of control values after 48 and 96 h, respectively.

**Effects of Tolbutamide on the Isotherms for 125I-Insulin Binding to 3T3-L1 Adipocytes**—To assess whether changes in receptor number or affinity were responsible for the observed increase in insulin binding after exposure of 3T3-L1 cells to tolbutamide, monolayers were incubated in the presence or absence of 1.5 mM tolbutamide for 3 days. Specific insulin binding was then measured at eight different insulin concentrations. One set of binding isotherms obtained from tolbutamide-treated and untreated monolayers is presented in Fig. 3. At saturation, control monolayers bound approximately 0.6 pmol of insulin/10^6 cells. This is approximately 2-fold the level of receptor measured previously for down-regulated cells maintained in the presence of high levels of insulin (16–18), and is consistent with the anticipated increase in receptor number following the removal of insulin from the medium. As shown by the values in parentheses (Fig. 3), relative to control monolayers, tolbutamide-treated monolayers bound 1.6–2.0-fold the amount of insulin at all concentrations of insulin tested. The possibility of small tolbutamide-induced alterations in affinity of the receptor for insulin therefore cannot be excluded due to the observed variation in the ratio of binding of insulin to untreated and treated monolayers at nonsaturating concentrations of insulin. At saturating concentrations of insulin, tolbutamide-treated monolayers bound approximately 1.2 pmol of insulin/10^6 cells compared to 0.6 pmol of insulin/10^6 cells for untreated monolayers. Thus, in this experiment 3T3-L1 adipocytes exposed to tolbutamide expressed a level of insulin receptors on the cell surface which is 2-fold that of control monolayers, and 4-fold that of down-regulated cells.

The extrapolated values for insulin binding at saturating concentrations of insulin were obtained from 13 similar sets of isotherms. The mean ± standard deviation, and (95% confidence interval) for insulin binding to control monolayers were: 0.58 ± 0.18 (0.47–0.69) pmol of insulin bound/10^6 cells. The corresponding values for insulin binding to tolbutamide-treated monolayers were: 0.97 ± 0.35 (0.76–1.18) pmol of insulin bound/10^6 cells. The mean, standard deviation, and 95% confidence interval for the ratio of insulin binding (treated/untreated) were 1.67 ± 0.32 (1.48–1.87). When the means, paired ratios (treated/untreated), or paired differences (treated minus untreated) were compared, tolbutamide-treated monolayers were found to bind a statistically greater insulin than untreated monolayers (p < 0.002).

**Antagonistic Effects of Insulin and Tolbutamide on the Expression of the Insulin Receptor on the Cell Surface**—To determine what effects the continued presence of high levels of insulin (a persistent down-regulated state, see Ref. 13) would have on the ability of tolbutamide to elevate receptor levels, differentiated 3T3-L1 monolayers were treated with either no additions, 1.5 mM tolbutamide, 10 µg/ml insulin, or 1.5 mM tolbutamide plus 10 µg/ml insulin. At the intervals presented in Fig. 4 the plates were washed to remove added insulin, and 125I-insulin binding was measured (using 5 nM 125I-insulin). Similar results were obtained from two separate experiments.

The gradual rise in receptor level (insulin binding) in control monolayers to amounts approximately twice those of insulin-treated monolayers confirms a previous demonstration (13) of the ability of this cell line to regulate its cell-surface receptor concentration when insulin is added to or removed from the culture media. When 3T3-L1 adipocytes were cultured in the absence of unlabeled insulin, tolbutamide

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**Fig. 2.** Kinetics of the alteration of insulin binding to 3T3-L1 adipocytes during exposure to tolbutamide. Washed monolayers were incubated in the presence (Ⅲ), or absence (Ⅱ), of 1.5 mM tolbutamide for intervals between 0 and 96 h. The insulin binding capacity of each monolayer was determined after each interval using 0.5 nM 125I-insulin as described under "Materials and Methods." The values in parentheses represent the ratio (treated/untreated) between the amount of insulin bound by tolbutamide-treated and untreated monolayers. The assays were performed in duplicate and the results expressed as the mean ± range of the two determinations.

**Fig. 3.** Isotherms for 125I-insulin binding to tolbutamide-treated and untreated 3T3-L1 adipocytes. To assess whether tolbutamide was affecting insulin binding through alterations of receptor affinity or receptor number, monolayers of 3T3-L1 adipocytes were incubated for 3 days in the presence (Ⅲ), or absence (Ⅱ), of 1.5 mM tolbutamide. The medium was replaced with fresh medium (±1.5 mM tolbutamide) on the 2nd day of incubation. On the 3rd day the insulin binding capacity of the treated and untreated monolayers was determined at insulin concentrations of 0.15, 0.60, 1.5, 3, 15, 40, 200, and 500 nM as described under "Materials and Methods." Values in parentheses represent the ratio (treated/untreated) of 125I-insulin bound to tolbutamide-treated and untreated monolayers at identical concentrations of free insulin. Each point represents the mean of duplicate determinations.
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Fig. 4. Demonstration of the ability of tolbutamide to elevate the level of receptor in 3T3-L1 adipocytes both in the presence and absence of insulin. After 24 h of incubation of 3T3-L1 adipocytes in medium without insulin, the medium was replaced (at 0 h) with medium containing either no additions (○), 1.5 mM tolbutamide (■), 10 μg/ml insulin (△), or 10 μg/ml insulin and 1.5 mM tolbutamide (●). After the intervals of incubation presented in the figure, insulin binding was measured using 5 nm 125I-insulin as described under “Materials and Methods.” The values in parentheses represent the ratio (treated/untreated) of 125I-insulin binding to tolbutamide-treated and untreated monolayers cultured in the absence (●, ○), or presence (△, △) of unlabeled insulin. Duplicate assays were performed and the results presented as the mean ± range of the two determinations.

Increased the measured binding of 125I-insulin to values 130–140% of those observed in control cells. Treatment of the monolayers with insulin and tolbutamide also increased the level of 125I-insulin binding to 140% of that of monolayers exposed to insulin alone. Although the percentage by which tolbutamide elevated 125I-insulin binding was similar in either insulin-treated or untreated monolayers, the absolute value of the increase was less in insulin-treated compared to untreated monolayers (0.3 pmol/10^6 cells versus 0.7 pmol/10^6 cells).

Concentration Dependence of the Effect of Tolbutamide on the Basal Rate of Uptake of 2-Deoxyglucose by 3T3-L1 Adipocytes—As chronic exposure of 3T3-L1 monolayers to tolbutamide markedly altered the level of surface receptor, it was of interest to determine if tolbutamide would also alter the rate of glucose utilization and/or the sensitivity of this process to insulin. Differentiated 3T3-L1 adipocytes exhibit both rapid and long-term control of basal and maximal insulin-stimulated rates of glucose utilization (13, 19, 20). Under our culture conditions, as the monolayers were maintained in high levels of insulin to promote differentiation, the levels of both basal and insulin stimulated 2-deoxyglucose uptake were high in newly differentiated monolayers. Upon removal of insulin from the medium, concomitant dramatic losses in both the basal and insulin-stimulated rates of 2-deoxyglucose uptake were observed within the first several hours. Steady-state levels of both basal and insulin-stimulated rates of 2-deoxyglucose uptake were achieved during the ensuing 24 h when the monolayers were maintained in insulin-free medium (data not presented). The fall in transport activity was not simply a reversal of the insulin-stimulated state, as both basal and maximal stimulated rates fell over the 24-h period, and may have arisen from a reduction in the amount of total available transporter activity in addition to dissociation of insulin from the receptor. These processes were reversible, as prolonged exposure of the monolayers to insulin gradually increased the maximal stimulated rate of glucose uptake far above that rate observed for the acute transport activation process normally occurring within minutes of exposure of the cells to insulin (data not shown). Under conditions of chronic exposure to insulin, reduction of the level of cell-surface insulin receptors and a shift of the dose-response curve for sugar uptake to higher concentrations of insulin also occurs (13). Therefore, analysis of tolbutamide effects on glucose uptake were undertaken at intervals greater than 24 h after the removal of the insulin present in the media during differentiation, and insulin-stimulated rates were measured shortly after acute (30 min) exposure of the monolayers to insulin.

Thus, to determine the concentration dependence of the effects of tolbutamide on 2-deoxyglucose uptake by 3T3-L1 adipocytes, monolayers were incubated for 24 h in Dulbecco’s modified Eagle’s medium (25 mM glucose) containing 10% fetal calf serum and the concentrations of tolbutamide presented in Fig. 5. After 24 h of incubation in the presence or absence of tolbutamide, the monolayers were assayed for their basal rates of uptake of 2-deoxyglucose. As was observed for tolbutamide’s effects on insulin binding, basal rates of 2-deoxyglucose uptake by 3T3-L1 adipocytes were raised to values 160–180% of control rates by exposure to concentrations of tolbutamide between 0.8–2.5 mM.

Effect of Tolbutamide on the Dose-response Relationship between Insulin Concentration and the Rate of Uptake of 2-Deoxyglucose—The previous experiments indicated that tolbutamide is capable of elevating both surface receptor number and the basal rate of 2-deoxyglucose uptake in cultured 3T3-L1 adipocytes. To determine how the dose-response relationship for insulin-stimulated 2-deoxyglucose transport might be altered by exposure of 3T3-L1 adipocytes to tolbutamide, monolayers were incubated without insulin in the presence or absence of 1.5 mM tolbutamide for a period of 1 day (Fig. 6A) or 3 days (Fig. 6B). The monolayers were then assayed for 2-deoxyglucose uptake in the presence of the indicated concentrations of insulin as described under “Materials and Methods.”

Fig. 5. Altered rates of 2-deoxyglucose uptake by 3T3-L1 adipocytes after exposure to different concentrations of tolbutamide. Monolayers of 3T3-L1 adipocytes were washed with Dulbecco’s modified Eagle’s medium (25 mM glucose) containing 1% fetal calf serum as described under “Materials and Methods.” The medium was replaced with identical medium containing 10% (v/v) fetal calf serum and no insulin. After incubation for 24 h, the medium was again replaced with identical medium lacking insulin and containing either 0, 0.8, 1.6, 2.4, or 3.2 mM tolbutamide. After an additional 24 h of incubation, the rate of 2-deoxyglucose uptake was measured. Each assay was performed in duplicate and the results presented as the mean ± range of the two determinations. The values in parentheses represent the ratio (treated/untreated) of the rate of 2-deoxyglucose uptake in treated and untreated monolayers.
After 1 day (Fig. 6A), elevated rates of basal transport appear in tolbutamide-treated monolayers. These differences are evident in assays using concentrations of insulin approaching $10^{-10}$ M. At higher concentrations of insulin the differences are statistically insignificant. After 3 days of exposure to tolbutamide (Fig. 6B), 1.9-fold differences in basal rates of 2-deoxyglucose uptake are observed in tolbutamide-treated monolayers. In this experiment the rates of 2-deoxyglucose uptake were significantly higher than control cells throughout the range of insulin concentrations tested ($0-10^{-6}$ M).

The experiment presented in Fig. 6B was repeated using cells grown and maintained under identical conditions only using a different lot of fetal calf serum. The results are presented in Fig. 6C. The rates of 2-deoxyglucose uptake were approximately $0.7-1.5$ nmol/min/10$^6$ cells lower than those presented in Fig. 6B at all insulin concentrations tested. Tolbutamide nevertheless maintained its ability to stimulate the rate of 2-deoxyglucose uptake by 2-fold both in the absence and at low concentrations of insulin ($10^{-12}-10^{-11}$ M). In the experiment presented in Fig. 6C, some effect of tolbutamide exposure on the rate of 2-deoxyglucose uptake could also be observed at high insulin concentrations ($10^{-8}-10^{-6}$ M), but this was inconsistently observed in repeat experiments. To obtain a statistical comparison of the effects of tolbutamide on the rate of 2-deoxyglucose uptake in the two different sera, the data for rates of 2-deoxyglucose uptake observed in the two plateau regions of the dose-response curve, one at low ($0-10^{-11}$ M) and one at high ($10^{-8}-10^{-6}$ M) concentrations of insulin, were analyzed. Five experiments with cells maintained in the serum used in Fig. 6B (serum A) and six experiments with cells maintained in the serum used in Fig. 6C (serum B) have been performed.

Table I shows that at insulin concentrations below 0.01 nM, tolbutamide clearly stimulates the rate of 2-deoxyglucose uptake in either serum. At high insulin concentrations (>10 nM) the effect of tolbutamide is significant only when cells were maintained in serum A and not when maintained in serum B. Similar results were obtained when statistical analyses of both the differences and the ratios of paired values were performed (data not shown).

**Table I**

| Serum | Insulin Concentration | 2-Deoxyglucose Uptake | 95% Confidence Interval | Rejection of Equality of Means |
|-------|-----------------------|-----------------------|-------------------------|------------------------------|
|       | $1.5$ mM | nmol/min/10$^6$ cells |                         |                              |
| A     | $0-10^{-11}$ | $1.33 \pm 0.34^a$ | 1.23-1.43 | $< 0.001$ |
|       | $10^{-8}-10^{-6}$ | $2.22 \pm 0.63^a$ | 2.02-2.41 | $< 0.001$ |
| B     | $0-10^{-11}$ | $0.29 \pm 0.19^a$ | 0.24-0.34 | $< 0.001$ |
|       | $10^{-8}-10^{-6}$ | $0.44 \pm 0.18^a$ | 0.39-0.49 | $< 0.001$ |

*a S.D., n = 44.  
*b S.D., n = 50.  
*c S.D., n = 54.  
*d S.D., n = 42.  
*e S.D., n = 40.
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FIG. 7. Kinetics for the development of enhanced rates of basal and insulin-stimulated uptake of 2-deoxyglucose in 3T3-L1 adipocytes exposed to tolbutamide. Monolayers were washed with medium containing 1% fetal calf serum and incubated for 24 h in medium containing 10% fetal calf serum (v/v) to remove insulin and return the monolayers to basal rates of 2-deoxyglucose uptake. The medium was replaced with fresh medium containing 10% fetal calf serum and either no additions or 1.5 mM tolbutamide. On each of the following 3 days of incubation, both control monolayers and those incubated with 1.5 mM tolbutamide were washed and assayed for both basal and insulin-stimulated (10^{-7} M insulin) rates of 2-deoxyglucose uptake as described under “Materials and Methods.” (A) Basal rates of 2-deoxyglucose uptake in control (○) and tolbutamide-treated (●) monolayers. (B) Insulin-stimulated rates of 2-deoxyglucose uptake in control (○) and tolbutamide-treated (●) monolayers, measured in the presence of 10^{-7} M insulin. The lot of fetal calf serum utilized in these experiments was identical to that used in Fig. 6C (serum B). Results are presented as the mean ± range of duplicate determinations.

DISCUSSION

3T3-L1 adipocytes have proven to be a useful model system with which to continue an investigation of the mechanism of action of the hypoglycemic agent, tolbutamide, as chronic exposure to tolbutamide resulted in significant alterations of both surface receptor levels and the characteristics of sugar uptake in this cell line.

Concentrations of tolbutamide of 1 mM or greater increased the amount of receptor detectable on the cell surface of 3T3-L1 adipocytes by insulin binding isotherms to values 150–250% of those of control. This process was not rapid and reflected the results of previous in vivo studies which demonstrated alterations in tissue receptor number after chronic exposure to tolbutamide (3–7). 3T3-L1 adipocytes in culture required 2–3 days of exposure to tolbutamide to express maximal increases in insulin binding and receptor number. Other studies with isolated adipocytes have previously demonstrated no effect of sulfonylurea treatment on the level of insulin binding or receptor number (10). The ability to demonstrate such effects with 3T3-L1 adipocytes in this study may reflect the difference in the structure of the sulfonylureas employed in the two studies (tolazamide versus tolbutamide).

Previous relationships established between receptor number and the sensitivity to insulin of glucose uptake in 3T3-L1 adipocytes suggested a possible precedent for relating the effects of tolbutamide on receptor number to its effects on 2-deoxyglucose uptake which would be consistent with the chronic hypoglycemic action of sulfonylurea agents observed in vivo. When 3T3-L1 preadipocytes differentiate into adipocytes in culture, this process is accompanied by an approximate 10-fold increase in the level of insulin receptor, primarily through an increase in the rate of receptor synthesis (16, 17). This increase in receptor number is paralleled by a marked increase in the sensitivity of glucose uptake by the cells to insulin (19). Removal of insulin present in the medium after differentiation of 3T3-L1 preadipocytes into adipocytes further elevates the level of insulin receptors by 2-fold. This process likewise is characterized by an increase in the sensitivity of glucose uptake to insulin in such up-regulated cells (13). From these results it was tempting to predict a priori that an increase in the capacity of 3T3-L1 adipocytes to bind insulin after exposure to tolbutamide would increase their sensitivity of glucose uptake to insulin, increase glucose utilization at submaximal stimulatory concentrations of insulin, and mimic the hypoglycemic action of the drug observed in vivo. In an effort to test this potential, the rate of glucose uptake in insulin binding capacity to enhanced glucose uptake, the effects of tolbutamide on the dose-response relationships between insulin concentration and 2-deoxyglucose uptake were analyzed. The results of five experiments identical to that presented in Fig. 6B demonstrated that, at all concentrations of insulin, the rates of 2-deoxyglucose uptake were higher in tolbutamide-treated cells, but there was no significant alteration of the concentration of insulin required for half-maximal stimulation of 2-deoxyglucose uptake relative to control monolayers. However, as there was a reasonably constant difference in rates of uptake of approximately 0.8 nmol/min/10^6 cells between tolbutamide-treated and control monolayers at each concentration of insulin tested (Fig. 6B), tolbutamide appeared to be activating a component of the uptake process that was independent of insulin-activated uptake. This alternate interpretation, however, was not entirely supported by the results of the six additional experiments identical to that presented in Fig. 6C, which utilized a different lot of fetal calf serum. In Fig. 6C no differences in uptake rates were observed between tolbutamide-treated and untreated monolayers at insulin concentrations between 10^{-10} and 3 × 10^{-8} M insulin. Although some effect of tolbutamide could be observed at high insulin concentrations (10^{-8}–10^{-6} M) in the particular experiments presented in Fig. 6C and 7B, the results of the six repeat experiments indicated that, when measured at high insulin concentrations, the effect of tolbutamide on the uptake of 2-deoxyglucose was statistically insignificant when the cells were maintained in the lot of serum identical to that used in Fig. 6C. The reason for the differences observed between the dose-response curves obtained from cells maintained in the two different lots of serum (A and B) are unknown. A comparison of the results obtained with the two sera can be summarized as follows: (a) at low insulin concentrations statistically significant higher rates of uptake were observed in tolbutamide-treated relative to control monolayers with either serum; (b) at high insulin concentrations statistically significant higher rates of 2-deoxyglucose uptake arising from tolbutamide treatment were observed only in serum A; (c) in general, for untreated monolayers the rates of 2-deoxyglucose uptake were approximately 1.2 nmol/min/10^6 cells lower in cells maintained in serum B when compared to cells maintained in serum A (this relative difference in rates was ob-
served at both high and low insulin concentrations); and (d) the two serum environments did not appear to markedly affect the ability of insulin to maximally alter the rate of 2-deoxy-
glucose uptake. When the rates of uptake were compared for the two plateau regions at low and high insulin concentrations (0–10^{-10} M and 0–10^{-10} M, respectively), the increase in the rate of uptake arising from the higher insulin concentration was between 1.1 and 1.2 nmol/min/10^6 cells regardless of the serum utilized or the presence or absence of previous exposure to tolbutamide.

It should be emphasized that the serum-related differences observed in transport rates arose from previous growth and maintenance of the cell monolayers in the different lots of serum. The serum was not present during the assay of 2-deoxyglucose uptake. However, regardless of the serum lots utilized and the associated rates of basal transport, tolbutamide consistently increased by 1.5–1.7-fold the rates of basal 2-deoxyglucose uptake and the rate of uptake observed at physiological insulin concentrations between 10^{-12} and 10^{-10} M. Tolbutamide also increased the rate of uptake of 2-deoxyglucose measured at insulin concentrations of 10^8 M or greater in some preparations of cells, but this effect was less consistently observed and appears to be modulated by other unidentified serum factors. Thus, the observed effects of tolbutamide on glucose uptake and complex uptake cannot be described simply by a shift of the dose-response curve to lower insulin concentrations arising from higher receptor levels as would be predicted by the previous examples (13, 19), nor do these effects appear to arise solely from an increase in basal transport rates.

What then, is the mechanism by which tolbutamide elevates both insulin receptor levels and rates of glucose uptake in 3T3-L1 adipocytes? As mentioned previously, whether tolbutamide alters 2-deoxyglucose uptake in 3T3-L1 cells in a manner which is dependent or independent of the simultaneous increase in the number of insulin receptors is unknown. The ability of another sulfonlurea, tolazamide, to elevate insulin-stimulated glucose uptake in the absence of increased insulin binding would suggest the lack of such a dependence (10). It is interesting to note that the active concentration range in which tolbutamide maximally alters both the rate of 2-deoxyglucose uptake measured at low insulin concentrations and surface receptor number in 3T3-L1 adipocytes is quite similar (1–3 mm, Figs. 1 and 5). Furthermore, the respective changes in the rate of 2-deoxyglucose uptake and receptor number in tolbutamide-treated monolayers relative to control monolayers show similar kinetics for expression (Figs. 2 and 7A) as well as similar relative changes (approximately 1.7-fold). The similarity in the kinetics, concentration dependence, and magnitude of the changes in receptor number and the rate of 2-deoxyglucose uptake suggest that a common mechanism might be responsible for the effects of tolbutamide on the two processes of regulation of receptor number and transporter activity.

Regulation of cell-surface insulin receptor number and some forms of activation of glucose transport by insulin operate through a control of the cellular distribution of the respective receptor and transporter protein and thus potentially would share similar pathways of internalization and recycling of the transporters or receptors to and from external and internal cellular sites (21, 22). Tolbutamide could act by interrupting the normal traffic of both transporter and receptor proteins between internal and cell-surface sites in a fashion that could increase their steady-state surface concentration and thereby simultaneously increase the level of cell-surface receptor and the rate of glucose uptake. Tolbutamide has been suggested to inhibit internalization of some types of cell-surface molecules (23), and could presumably be inhibiting the internalization of both insulin receptors and glucose transporter units via a common mechanism. Whatever the mechanism, insulin appears to antagonize the effects of tolbutamide on receptor level (see Fig. 4), possibly by increasing the rate of receptor internalization and shifting the distribution of receptor to internal sites. Such antagonism of insulin-induced down-regulation of the insulin receptor by the sulfonlurea, glyburide, has been previously noted in human fibroblasts (8).

The effects of tolbutamide on insulin receptor level and rates of glucose uptake in 3T3-L1 adipocytes were observed at concentrations of 1 mM or greater. How do these concentrations relate to those observed clinically? Patients receiving a dosage of 0.5 g of tolbutamide daily demonstrate lower plasma concentrations of tolbutamide (0.15 mm) than utilized in these studies (24). Those patients, however, receiving 2–3 g of tolbutamide daily (3) would approach plasma concentrations of tolbutamide similar to those concentrations utilized in this study, thus suggesting a clinical relevance for the in vitro effects of tolbutamide demonstrated with 3T3-L1 adipocytes.

In summary, chronic exposure of 3T3-L1 adipocytes to tolbutamide has been demonstrated to increase both the number of insulin receptors as well as the rate of glucose uptake measured at low insulin concentrations. Both processes would contribute towards the hypoglycemic action of the drug observed in vivo, especially when tissues such as muscle and liver are also affected, as previously demonstrated (4, 7). The value of utilizing the 3T3-L1 adipocyte system for studying the mechanism of tolbutamide action in vitro is derived from: (a) the demonstration that the effects on glucose transport and receptor level are larger than those previously observed in other in vitro systems, and (b) the current availability of methods previously applied to this cell line which permit a measurement of altered rates of receptor internalization (25), synthesis (17, 26, 27), degradation (17, 18), and their effects on total receptor number and distribution. We are currently measuring the effects of tolbutamide on these processes to assess which are affected by chronic exposure of the cell to tolbutamide.

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