Time-dependence of biological activity induced by covalent insulin–receptor complexes in rat adipocytes

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Lipogenesis in isolated adipocyte preparations is stimulated when photosensitive insulin derivatives are attached covalently to specific receptors. This response was compared quantitatively with that to reversibly associated insulin, and it was shown that both covalent and reversible insulin–receptor complexes behave very similarly. The extent of stimulation of lipogenesis was studied as a function of time. Cells were incubated in buffer for various times before addition to vials containing 0 (basal) or 10 ng of monocomponent insulin/ml (maximal) and [^3H]glucose. After 60 min, the toluene-soluble [^3H]lipids were measured. The maximal stimulation induced by reversibly bound insulin was virtually constant over a period of 4 h. In contrast, adipocytes to which $N^{\alpha}\text{B}^2$ -(2-nitro-4-azidophenylacetyl)-des-Phe $^{\text{B}1}$ -insulin had been covalently attached at the start of the experiment showed a loss of stimulation with time when incubated at 37 °C. This loss was decreased in the presence of lysosomotropic agents such as chloroquine at concentrations (approx. 200 μM) that had very little or no effect on the basal and maximal lipogenesis rates. A simple method was used to transform the measured rate of loss of stimulation into a rate of loss of effective units. A half-time of 80 min was calculated for the effective covalent insulin–receptor units in adipocytes at 37 °C at pH 7.4. This is very close to values reported by others for the internalization of covalent complexes in these cells, suggesting that this may be the causative event for the deactivation of the insulin–receptor unit. The inhibitory effect of chloroquine on the deactivation may indicate that the insulin–receptor complex can function even after internalization.

INTRODUCTION

A range of photoactivatable insulin derivatives have been shown to bind covalently and specifically to receptors in several cell and membrane preparations on irradiation of reversible complexes with u.v. light (Yip *et al.*, 1978, 1980; Jacobs *et al.*, 1979; Wisher *et al.*, 1980). Using isolated adipocytes, we have previously demonstrated that such covalent complexes are biologically effective (Brandenburg *et al.*, 1980; Diaconescu *et al.*, 1980; Wang *et al.*, 1983). More recently we were able to compare the effectiveness of covalent and reversible complexes, and to show that there was little difference between the two (Klotz *et al.*, 1984). As it was known that internalization of both reversible and covalent insulin–receptor complexes occurs in various cell types, including adipocytes (Berhanu *et al.*, 1982; Wang *et al.*, 1982), it was decided to examine the time-dependence of the biological response to covalently attached insulin and to compare this with the reported turnover rates for the insulin receptor in adipocytes. The decay of response, determined as stimulation of lipogenesis, had a half-life of approx. 80 min at 37 °C, and the photoaffinity-labelled insulin receptors are inactivated by a chloroquine- and temperature-sensitive endocytotic pathway.

MATERIALS AND METHODS

Materials

The preparation and chemical and biological characterization of the photoactivatable insulin derivative

$N^{\alpha}\text{B}^2$ -NAPA-des-Phe $^{\text{B}1}$ -insulin used in this study have been reported in detail elsewhere (Thamm *et al.*, 1980; Saunders *et al.*, 1980). Collagenase was from Worthington. Bovine serum albumin was from Sigma; before use it was dialysed against deionized water. Guinea-pig anti-(pig insulin) antibody (Lot no. 69.178.01) was generously given by Dr. L. Heding (Novo Therapeutisk, Copenhagen, Denmark). Monocomponent pig insulin was given by Novo, Copenhagen, Denmark. [^3H]Glucose was from Amersham, Braunschweig, Germany. Cell pools were maintained and washed in ultrafiltration chambers (47 mm internal diameter) containing an 8 μm -pore filter (Millipore), which was boiled in double-distilled water before use to remove soluble materials. The chambers were thermostatically controlled in water jackets.

Methods

Covalent binding of insulin to adipocytes. Epididymal fat tissue from three rats (150–180 g) was digested with collagenase (1 mg/ml) for 60 min at 37 °C and filtered through four layers of gauze to give an adipocyte suspension which was washed with buffer by centrifugation (Rodbell, 1964). The adipocytes were then divided into three pools, each 40 ml, in the ultrafiltration chambers A, B and C. Two 1 ml samples were removed from each chamber to measure the basal lipogenesis at the start of the experiment. Samples of a solution of the photoreactive insulin, sufficient to give a final concentration of 40–80 ng/ml per chamber, were added to chambers B and C. After 10 min at 37 °C chamber C was irradiated with the light from a black-glass-cladded HPK

Abbreviation used: NAPA, (2-nitro-4-azido)phenylacetyl-.

125 u.v. lamp (Philips) for 90 s from a distance of approx. 7 cm. Subsequently the cell pools in all three chambers were washed by passage of 250–300 ml of buffer (Whitesell & Gliemann, 1979) (at 37 °C) over a period of 20–30 min. These conditions were generally satisfactory for removing reversibly cell-bound as well as soluble insulin from the chamber, as was to be expected from the theoretical dilution factor of approx. 30 000 (Klotz *et al.*, 1984). Slight variations in the efficiency of the wash phases were minimized by the addition of sufficient guinea-pig anti-insulin antibody to bind 5 ng of insulin/ml per chamber. This antibody has no effect on the efficiency of the maximal stimulation achieved with 10 ng of insulin/ml or on the stimulation caused by covalently attached photoreactive insulin (Klotz, 1984).

Time dependence of lipogenesis. At various times after the start of the experiment ($t = 0$ after the irradiation and washing of the pools) two lots of triplicate 1 ml samples were removed from each cell pool and incubated with [3 H]glucose for 60 min at 37 °C in the presence or absence of 10 ng of insulin/ml in order to measure the basal and maximal rates of lipogenesis (Moody *et al.*, 1974). The incubations were stopped by addition of toluene-based scintillator and then shaking vigorously. After cooling to 10 °C overnight, toluene-soluble 3 H-labelled lipids were counted in a Packard 500C Tri-Carb scintillation counter. Typical results are shown in Fig. 2. Pools A (cells that had not been exposed to insulin) and B (cells exposed to insulin and then washed) gave similar results, which were averaged for further evaluation. In experiments where cells in pool B were obviously insufficiently washed, so that a residual insulin stimulation was observable, the results were discarded. The time-dependence of the maximal stimulation of pool C (already partially stimulated by the covalently attached insulin) by added insulin was not statistically different from that of pools A and B and for up to 3 h at 37 °C, and was not affected by the irradiation step. Basal lipogenesis increased slightly with time in pools A and B, to similar extents. The stimulation by covalently attached insulin declined in a quasi-linear fashion with time. The degree of stimulation of pool C brought about by the covalent attachment of the photoreactive insulin was calculated for each time as the ratio between the extent of stimulation of lipogenesis above basal in pool C and the maximal stimulation of lipogenesis at that time (i.e. the difference between the mean basal lipogenesis in pools A and B and the mean maximal lipogenesis in pools A, B and C). Results presented here are averages of six experiments. The same experimental design was used in experiments at 20 °C and with the addition of chloroquine, or other alkyl amines.

Time dependence of the effector system. The response of adipocytes to insulin is not linearly related to the concentration of insulin in the medium, nor to the number of insulin–receptor complexes, except within limits. As shown in Fig. 1, the log-dose–response curve for lipogenesis is sigmoid, but is quasi-linear between 20% and 80% of the maximal stimulation. As a reasonable approximation, between 20% and 80% maximal response, the response $R = m \log c + \log K$ (m = gradient, c = concn. of insulin). The value of m was determined from 33 dose–response curves as $70.9 \pm 2.9\%$. As only low extents of receptor occupancy are responsible for these degrees of stimulations, the occupancy,

$$w = c/(c + K_d) = c/K_d \text{ as } c \ll K_d.$$

Fig. 1. Typical correlation between response (R ; % of maximal) and log concentration of insulin for lipogenesis

Isolated rat adipocytes were incubated in Krebs–Ringer–Hepes buffer (Whitesell & Gliemann, 1979) with [3 H]glucose and different concentrations of insulin for 60 min at 37 °C with gentle shaking. After addition of toluene-based scintillator (10 ml), the extractable [3 H]lipids were counted for radioactivity in a Packard Tri-Carb 300CD scintillation counter. Results are plotted as R , % of maximal stimulation (achieved at 10 ng of insulin/ml); the basal lipogenesis in the absence of insulin is set at 0:

$$R = m \log c + \log K$$

Fig. 2. Typical results for lipogenesis in pools A, B and C as a function of time

Lipogenesis was measured as described in the Materials and methods section. Maximal lipogenesis was stimulated by addition of insulin at 10 ng/ml per pool. The basal lipogenesis in pools A and B ($\circ$) increases slightly over 180 min, although the maximal value ($\square$) stays constant. The stimulation of lipogenesis (R) caused by covalently bound photoreactive insulin (pool C, 'basal') ($\circ$) decreases with time. This value is determined as a percentage of the difference between the basal and the maximal values at a given time.

Hence for reversible systems $R = m \log (wK_d) + \log K$. This equation is equally valid for covalent occupancies, as it has been demonstrated that the relationship between amount bound and effect produced in this situation is a constant fraction of the relation for non-covalent

Fig. 3. Time-dependence of the response (R) to covalent insulin–receptor complexes

Cells were incubated either at 37 °C (●) or at 20 °C (○) after covalently attaching photoreactive insulin. After the indicated time periods, the stimulation of lipogenesis induced by covalent insulin–receptor complexes was determined as given in detail in the Materials and methods section. The values represent the means $\pm$ S.E.M. for six independent experiments at 37 °C and 20 °C respectively. ———, Linear regression after transformation to $\text{logit}(R)$; —, sigmoidal curve after re-transformation of the $\text{logit}(R)$ function to R , given as % response.

occupancies (Klotz *et al.*, 1984; Saunders *et al.*, 1984). If it is assumed that the effective covalent occupancy decreases in a monoexponential manner, then $w(t)/w(0) = e^{-kt}$. Naturally this is reflected in a change in response, so that $R(0) - R(t) = (m/2.3) kt$. The value of $[R(0) - R(t)]/t$ can be determined from Fig. 3 as 0.273%/min, so that

$$k = \frac{2.3 \times 0.273\% \cdot \text{min}^{-1}}{71\%} = 8.85 \times 10^{-3} \text{ min}^{-1}.$$

The half-time of the effective covalent complexes is given by $\ln 2/k$, and was calculated to be 78 min in a total of six independent experiments. A more complete description of the dose–response curve is given by $\text{logit}(R) = m' \log(c) + \log(K)$, where $\text{logit}(R) = \log[R/(1 - R)]$. This equation is linear over practically the whole dose–response curve. The turnover rate of the effector can be shown to be $[\text{logit } R(0) - \text{logit } R(t)]/(m't/2.3)$, which gives essentially the same values as the simpler transformation (above) between 20% and 80% maximal response. However, the logit equation predicts the time course of the response decay outside the limits adopted above, and is useful in experiments where the initial or final conditions are above 80% or below 20% maximal response.

RESULTS

The basal and maximal extents of lipogenesis from pools A, B and C in a typical experiment are shown in Fig. 2. The real maximal values in all three pools (stimulated with 10 ng of insulin/ml) were virtually identical and constant over the time of the experiment. This proves that the cell viabilities were comparable in pools which either had, or had not, been irradiated with u.v. light. The basal values in pools A and B rose slightly over the 3 h incubation period (Fig. 2). This provided a problem in defining the percentage at any given time. It

Fig. 4. Influence of chloroquine on the deactivation rate of covalent insulin–receptor complexes

Fat-cells, stimulated with covalently bound $N^{\alpha B2}$ -NAPA-des-Phe^{B1}-insulin, were incubated in Krebs–Ringer–Hepes buffer (Whitesell & Gliemann, 1979) without (●) or with (○) 200 μM -chloroquine. The half-life of the decay of response to the covalently bound photoreactive insulin was determined as described in the Materials and methods section. Values are the means $\pm$ S.E.M. for four independent experiments.

was most reasonable to set the measured basal value at 0%, although this value actually changed during the experiment. The method for calculating the apparent basal values for any time is described in the Materials and methods section. A supplementary problem at the start of these studies was that a transient slight additional increase in basal rate of lipogenesis was observed in pool B. This could be explained by the response to a decaying reversible occupancy of the adipocyte receptors with photoreactive insulin, an occupancy remaining after a not totally efficient wash procedure. The addition of anti-insulin antiserum greatly accelerated the decay of this effect. Antiserum was added thereafter routinely to all cell pools during the incubation phase. Its maximal capacity (5 ng/ml) was not enough to decrease the effect (i.e. maximal stimulation) of the insulin concentration (10 ng/ml) added to measure maximal lipogenesis. Previous experiments had demonstrated that this antiserum had no apparent influence on the response to covalently bound ^{125}I - $N^{\alpha B2}$ -NAPA-des-Phe^{B1}-insulin, but was able to neutralize completely the response to free concentrations of the same derivative (Klotz, 1984).

The 'basal' value of pool C was considerably higher than in pools A and B. This has been demonstrated previously to be the response to photoreactive insulins which have covalently bound to specific receptors on the adipocytes (Brandenburg *et al.*, 1980; Diaconescu *et al.*, 1980; Wang *et al.*, 1982). At the start of the experiment this response was typically 60–90% of the maximally stimulated lipogenesis. At later times this response was calculated as a percentage of the difference between the maximal response and the mean of the nearly equal basal responses observed in pools A and B. The response to covalently bound photoreactive insulin decreased with time, as shown in Fig. 3. The maximal response to

exogenous insulin remained constant. This was interpreted as a decay in the efficiency of the bound insulin. From the equations described in the Materials and methods section, it was possible to calculate the rate of decay of the response, and thus a half-time for the effective covalent insulin–receptor complexes. The rate was very reproducible [$k = 10, 7, 8, 8.5, 9.6, 10 (\times 10^{-3}) \text{ min}^{-1}$ in six experiments], with a mean of $8.85 \pm 0.49 (\times 10^{-3}) \text{ min}^{-1}$.

We are therefore confident in assigning a half-time in the range 74.5–82.5 min to the effective covalent complexes under the conditions of our experiments.

Effect of temperature or additives

Experiments were conducted to assess the effect of maintaining the cell pools during the initial incubation phase (before the 60 min lipogenesis period at 37 °C) at either 20 °C or in the presence of inhibitors such as alkylamines, dansylcadaverine and chloroquine. Maintaining the incubation temperature at 20 °C led to a virtual loss in decay of the response to covalently attached insulin in pool C (Fig. 3). This is strong evidence that internalization, which is greatly decreased at this temperature (Silverstein *et al.*, 1977), is involved in the decay of the response. The addition of inhibitors gave less clear results, because they also increased the basal lipogenesis and decreased the maximal lipogenesis in a concentration-dependent manner, as expected of a general metabolic poison. Effective concentrations were 2–10 mM for alkylamines and 10–100 μM for dansylcadaverine. In contrast, only at concentrations above 300 μM did chloroquine start to have effects on basal and maximal lipogenesis. At 100 μM and 200 μM , where no other effects were observed, chloroquine increased the half-life of the covalent effector system from 80 to approx. 100 and 156 min respectively (Fig. 4). The decreased rate of decay was observed in cells exposed to the drug for the whole wash and incubation period as well as in cells exposed to the drug only a few minutes before the wash and incubation periods.

DISCUSSION

It is now quite clear that insulin, and other ligands, can be internalized and degraded by receptor-mediated events (Gorden *et al.*, 1980; Olefsky *et al.*, 1982). Some studies have concluded that insulin–receptor complexes are metabolized more rapidly than are unoccupied receptors (Ronnett *et al.*, 1982). Moreover, a biochemical role has been assigned to the internalized insulin in certain tissues (Steiner, 1977; Goldfine, 1977). The rate of internalization of the adipocyte insulin receptor has been studied by a wide variety of methods. In Tris buffer, it seems that insulin receptors are not recycled, allowing one to determine the rate of loss from the cell surface. For this loss a half-time of 75 min has been determined (Marshall & Olefsky, 1981a,b), which is remarkably close to the rate of deactivation determined here. Wang *et al.* (1983) have shown that covalently bound ^{125}I -N⁶B²⁹-NAPA-insulin is translocated with the receptor from the plasma membrane of isolated adipocytes into a Golgi-enriched fraction. Approx. 30% of the complex was transferred in 30 min at 37 °C; in the same time we observe up to a 26% loss of effect. Heidenreich *et al.* (1983) reported a loss of photoaffinity-labelled insulin–receptor complexes from the cell surface of adipocytes, with a half-life of 1.4 h,

which is identical with the inactivation rate that we found. However, we were not able to determine the described biphasic nature of the curve, with a slower part after the initial phase of 60 min. Our interpretation of this discrepancy is that possibly recycled complexes are still labelled, but are no longer biologically active. We have demonstrated (Saunders *et al.*, 1984) that the efficacy of covalently linked insulin receptor complexes is drastically decreased by low pH (from 50% at pH 7.4 to 1% at pH 6.7). As chloroquine is able to stabilize the pH in lysosomes but also in prelysosomal (endocytotic) vesicles, the effect on the pH in this compartment may be an explanation for the partial inhibition of the deactivation process, and would indicate that the insulin–receptor complex can function even after internalization. From these results we suggest the following model for the inactivation of the effector unit (biologically effective insulin–receptor complexes): inactivation occurs immediately after internalization in endosomes by a pH-shift (acidification), which causes an accelerated dissociation of the active complex. In covalent insulin–receptor complexes complete dissociation is impossible, but lowering the pH leads to a movement of the covalently bound insulin away from the effective receptor-binding site, resulting in drastically decreased efficacy.

In summary, we have presented evidence here that the half-life of the effective insulin–receptor complex in isolated adipocytes at pH 7.4 and 37 °C is of the order of 80 min. Our assay was the ability of such complexes to stimulate lipogenesis. From these experiments, we are not able to define the cause of the loss of efficiency precisely. However, the virtual loss of decay of the response at 20 °C, as well as the influence of chloroquine on the half-life of the active effector system, are strong evidence that internalization is involved in this process. Furthermore, the remarkably close correspondence of molecular internalization rates determined by others for the insulin receptor in adipocytes suggests that internalization may be the causative event for the deactivation of the insulin–receptor unit.

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