Effect of Depletion of Bicarbonate or Phosphate Ions on Insulin Action in Rat Adipocytes

FURTHER CHARACTERIZATION OF THE RECEPTOR-EFFECTOR SYSTEM*

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In the preceding paper (Shechter, Y., and Ron, A. (1986) J. Biol. Chem. 261, 14945-14950) we have shown that in fat cells, prepared and maintained in an isotonic buffer (pH 7.4) containing neither phosphate nor bicarbonate anions (Buffer A), the dose-response curve to insulin shifted to the right by about 2 logarithms and insulin binding affinity or capacity was only slightly decreased. In the current paper we demonstrate that progressive loss of insulin binding, either by treatment with trypsin or preincubating the cells with isoprotexanol, correlates well with the reduced ability of the cells to elicit maximal lipogenesis in response to insulin. We further demonstrate in the "new" system that: (a) the dissociation of labeled insulin from fat cells is not accelerated by the inclusion of unlabeled insulin in the medium; (b) termination of lipogenesis in Buffer A occurs immediately; (c) ligand-induced receptor internalization is grossly defective; and (d) insulin is unable to stimulate lipogenesis at 15°C.

The data support the hypothesis that in the new experimental system all measurable binding sites are linked to a coupling mechanism. Each site behaves as an independent, separate entity and there are no sites to site interactions. This leads to a linear relationship between binding and bioactivation, lack of negative or positive cooperativity, accelerated rate of termination, defective internalization, a shift to the right in the dose-response curve to insulin, and a lack of insulin response at a lower temperature. In more general terms, the study indicates that all measurable insulin receptors are chemically homogeneous in their potential capability to be coupled to an insulin effector (biologically relevant) system, and they do so under particular experimental conditions.

In rat adipocytes, insulin is believed to trigger all its biological actions via high affinity class of receptor sites having an apparent \( K_d \) value of about 3 nM (1). Rat fat cells, however, are maximally stimulated at 0.02-0.04 nM insulin (1-6). Therefore, it was assumed that limited occupancy (i.e. 1-2%) of the total high affinity sites is sufficient to elicit the maximal responses of insulin while most of the binding sites were defined as "spare receptors" (1, 2, 6). There remains no proof, however, that the receptors measured are chemically homogeneous or that they are all functionally linked to a coupling mechanism (reviewed in Ref. 7). This is an especially valid issue as Scatchard plots of insulin-receptor binding of intact cells or plasma membranes are ordinarily concave (8-10), suggesting heterogeneity of binding sites or negative cooperativity (10). The insulin receptor itself is a heterotetramer of the form (\( \alpha \beta \gamma \delta \)), which can bind two molecules of insulin (reviewed in Ref. 11). Highly purified (Triton-soluble) insulin receptors exhibit a curvilinear Scatchard plot. Thus, the "negatively cooperative" mode of insulin binding may exist within the two sides of the heterotetrameric receptor molecule itself (12).

In the preceding study (13) we have described that, in fat cells maintained in an isotonic buffer (pH 7.4) containing neither phosphate nor bicarbonate anions (Buffer A), the dose-response curve to insulin has been shifted to the right by about 2 logarithms. Insulin binding affinity and capacity was only slightly changed (13). Thus, in this experimental system the 2-logarithm "discrepancy" between insulin binding data and biological activation are expected to disappear. This further implies that in Buffer A all receptor sites are functionally linked to a coupling mechanism and that all "heterogenous" receptors are becoming "chemically homogenous" with respect to the above mentioned feature.

In this paper we further study the modified form of the KRB1 buffer that has been traditionally used in the insulin-responsive fat cell system. Among several features, we demonstrate that indeed in the "new" experimental system all insulin binding sites are linked to a coupling mechanism, and that the cells are defective (in a reversible manner) in their ability to produce ligand-induced receptor internalization. Attempts are made to assess the relationship between biological induced signal transduction and ligand induced receptor internalization.

EXPERIMENTAL PROCEDURES

Materials—Porcine insulin was purchased from Eli Lilly. \(^{14}C\)Glucose (4-7 mCi/mol) and Na\(^{125}I\) were obtained from New England Nuclear. Collagenase type I (134 units/mg) was obtained from Worthington. Buffer A that was used throughout this study contained: 10 mM HEPES (pH 7.4), 120 mM NaCl, 1.4 mM CaCl\(_2\), 0.2 mM KCl, 1.2 mM MgSO\(_4\), and 0.1% bovine serum albumin. When supplemented with bicarbonate, the pH was corrected to 7.4 with HCl. Anti-porcine insulin (raised in guinea pig) is the product of BioYeda Ltd., Rehovot, Israel.

Methods—Fat cells were prepared from male Wistar rats (100-140 g) essentially according to Rodbell (14). The cells were digested for 20 min at 37°C in Buffer A containing 2 mg/ml of collagenase.¹²

¹ The abbreviations used are: KRB, Krebs-Ringer bicarbonate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylenebis(oxyethylenenitrito)tetracetic acid.

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Insulin was prepared to a specific activity of 250,000 ± 30,000 cpm/ng, according to Ref. 15. Only preparations that demonstrated specific binding to liver membranes greater than 80% of total binding were used throughout this study. Binding to fat cells was terminated by removing 140-μl aliquots and centrifuging the cells through a layer of silicone fluid (16). Lipogenesis assays were carried out under the specified conditions of the individual experiments according to Moody et al. (17).

Rate and level of receptor internalization were estimated by adding an aliquot of 125I-insulin (2 ng, 250,000 cpm/ng) to freshly prepared adipocytes (0.22 × 10^6 cells in 0.2 ml of the indicated buffer). An aliquot of anti-insulin serum of high titer was added prior to, and at various time intervals after, the addition of the labeled hormone. The cells were further incubated for 2 min at 22°C and then separated from the medium by centrifugation through a layer of silicone fluid (16). All figures and tables in this study are the result of at least three separate experiments. Each point in the figures represents the mean ± S.E. All assays were run in duplicate.

RESULTS AND DISCUSSION

Initial studies were aimed at justifying directly our main working hypothesis which states that in the new experimental system all binding sites are functionally coupled to an insulin-effector system. This implies that any manipulation which reduces the number of sites would be reflected in a parallel decrease in the maximal response obtainable at high insulin concentrations. To analyze this point we chose two experimental approaches. In the first one, the cells were subjected to increasing concentrations of trypsin which destroys insulin binding (2). Trypsin alone exhibits insulin-like action in fat cells maintained in KRB buffer (18) but does not, at any concentration of trypsin examined, have insulinomimetic effects in adipocytes maintained in Buffer A (Fig. 10, Ref. 13). Therefore, we could analyze directly the relationship between binding and lipogenesis. Progressive loss of insulin binding correlated well with the ability of the cells to elicit maximal lipogenesis in response to high concentrations (2 μg/ml) of insulin (Fig. 1). In KRB buffer, rat adipocytes in which 80–90% of their binding sites were destroyed by trypsin were still capable of producing maximal responses when challenged with a high dosage of the hormone (i.e. Ref. 19).

In an alternative approach, cells (Buffer A) were preincubated with isoproterenol. This treatment reduces insulin receptor numbers by a β2-adrenergic receptor, cAMP-mediated mechanism, as recently shown by Pessin and co-workers (20). In our experiments, pretreatment with isoproterenol reduced the binding capacity of the cells by 25 ± 2% (Table I) and subsequently reduced the extent of lipogenesis (at high insulin concentrations) by 24 ± 4% (Fig. 2). It, therefore, seems by these two approaches that in our new experimental system, all the measurable binding sites are coupled to an effector system, namely (in the more general sense), all plasma membrane binding sites of the rat adipocyte are chemically homogenous in their potential capability to be coupled to an insulin effector (biologically relevant) system, and they do so under specific experimental conditions.

An experimental system in which all sites are functionally linked, is expected to behave differently from one in which they are not so linked, with respect to several binding and biological parameters. With respect to binding parameters the rate of insulin binding to cells maintained in either Buffer A or KRB buffer at 22°C is illustrated in Fig. 3. With the exception of higher plateau obtained in KRB buffer, the rate constants of association in both buffers are essentially the same. Biomolecular rate constants of 1.1 ± 0.1 × 10⁷ M⁻¹ s⁻¹ and 1.0 ± 0.1 × 10⁷ M⁻¹ s⁻¹ are estimated in KRB buffer and Buffer A, respectively. The dissociation of labeled insulin as a function of time at 22°C in both buffers is given in Fig. 4. The dissociation patterns and rates are nearly the same. In KRB buffer, however, the inclusion of cold insulin in the diluting buffer accelerates the rate of insulin dissociation as previously reported (10). Such acceleration was not observed in Buffer A (Fig. 4). De Meyts et al. (10, 21) interpreted this acceleration as reflecting site to site interactions in the negative cooperative manner. If this interpretation is correct, the data obtained in Buffer A suggests a lack of receptor-receptor interactions in the new experimental system. This is already strongly supported by the data illustrated in Figs. 1 and 2 and

![Fig. 1. Correlation between insulin binding and insulin-dependent maximal rate of lipogenesis in Buffer A. Suspension of adipocytes (equally divided in vials) were subjected to the indicated concentrations of trypsin for 15 min at 37°C. Reaction was terminated by addition of soybean trypsin inhibitor (3-fold excess). Aliquots were then withdrawn and the extent of lipogenesis in Buffer A in the presence of 2 μg ml⁻¹ of insulin (O), and specific insulin binding capacity determined (●). Binding was assayed in Buffer A-5 mM NaHCO₃ with 1 nm ¹²⁵I-insulin in the presence and absence of 5 μM unlabeled hormone. Dashed line represents basal level of lipogenesis.](image1)

![Fig. 2. Effect of isoproterenol pretreatment on maximal lipogenesis in the Buffer A system. Adipocytes in Buffer A (3 × 10⁶ cells/ml) were preincubated for 30 min at 37°C without (O) or with isoproterenol (5 μg/ml, ▲) and then subjected to lipogenesis at increasing concentrations of insulin. Basal- and insulin-stimulated lipogenesis were 1,300 ± 100 and 12,600 ± 300 cpm/3 × 10⁵ cells/h, respectively.](image2)

<table>
<thead>
<tr>
<th>Table I</th>
<th>Binding capacity of adipocytes after pretreatment with isoproterenol</th>
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<tbody>
<tr>
<td>Cells were preincubated and maintained in Buffer A throughout the experiment.</td>
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<tr>
<td>Specifically bound ¹²⁵I fmol/10⁶ cells⁰</td>
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<tr>
<td>Control cells</td>
<td>23 ± 2</td>
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<tr>
<td>Isoproterenol-treated cells</td>
<td>17 ± 2</td>
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</tbody>
</table>

⁰ Binding was performed for 30 min at 22°C with labeled insulin (1 nM) in the presence and absence of 5 μM unlabeled hormone.

¹ The cells were preincubated with isoproterenol at 2 μg ml⁻¹ for 30 min at 37°C, prior to assaying the cells for lipogenesis (Fig. 2) and insulin binding.
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**Fig. 3.** Rates of binding of $^{125}$I-insulin to isolated adipocytes at 22 °C in KRB and Buffer A. Fat cells (1.5 x 10^6 cells/ml), maintained in either KRB buffer (C) or Buffer A (O), were incubated with 0.4 x 10^-6 M $^{125}$I-insulin. At the indicated time intervals, 140-μl aliquots were removed and centrifuged through a layer of silicone fluid. Nonspecific binding (obtained by including 5 μM unlabeled insulin for each time point) was subtracted from the total binding for each time point.

**Fig. 4.** Rates of dissociation of insulin in both buffer systems in the presence and absence of cold insulin. Suspension of adipocytes (3 x 10^6 cells/ml) were incubated for 20 min at 22 °C with 2 nM $^{125}$I-insulin in the indicated buffers. The medium was then removed and the cells were diluted 20-fold with buffer alone or buffer containing unlabeled insulin (20 μg ml^-1), aliquots were then withdrawn and the amount of radioactivity associated with the cells determined at the indicated time points. *Significantly different from the same time point obtained in the absence of cold insulin (p < 0.01).

**Fig. 5.** Time course of lipogenesis in both buffers at 29 and 37 °C. Lipogenesis assays were performed under the conditions specified in the figure. Reactions were terminated at the indicated time intervals by addition of Bray scintillation fluid and shaken immediately.

**Fig. 6.** Termination of lipogenesis in both buffer systems. The extent of formation of 14C-labeled lipids as a function of time was determined over 70 min in the absence of insulin (O) or its presence (C). Final concentrations of insulin were 2 ng ml^-1 in KRB buffer or 200 ng ml^-1 in Buffer A. After 20 min (indicated by the arrows), anti-insulin serum (4 μl) or EGTA (final concentration 1.6 mM) were added to the KRB or Buffer A system, respectively.

**Fig. 7.** Rate of insulin internalization in Buffer A and Buffer A-25 mM NaHCO₃ at 22 °C. Fat cells (1.1 x 10^6 cells/ml) in Buffer A (O) or Buffer A-25 mM NaHCO₃ (O) were incubated with $^{125}$I-insulin (1.5 nM, 250,000 cpm/ng). Anti-insulin serum (5 μl) was added at different times after the addition of the labeled hormone. Aliquots (140 μl) were then withdrawn, and the amount of radioactivity associated with the cells was determined.

will be further substantiated below.

For a variety of biological parameters, the rate of initiation and termination of insulin-mediated lipogenesis have been estimated. Lipogenesis in response to insulin followed after a similar lag period in both buffer systems (Fig. 5), 0.8 ± 0.1 min and 4 ± 0.2 min at 37 and 29 °C, respectively. On the other hand, termination in Buffer A is a much faster process

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defective in the Buffer A system and the latter process is interrelated to biological signal transduction, it is expected that at lower temperatures (where ligand-induced internalization is further inhibited; Refs. 22–24) the stimulating effect of insulin will disappear. This point is illustrated in Fig. 8. While in Buffer A-25 mM NaHCO$_3$ the effect of insulin is clearly obtainable at 15 °C (17% of the effect obtained at 37 °C), there was no significant insulin effect in Buffer A at this temperature (Fig. 8). It therefore seems that these two processes are interrelated.

In summary, we have characterized further our new insulin-responsive fat cell system in which the dose-response curve to insulin has been shifted to the right by 30 min in Buffer A-25 mM NaHCO$_3$ or Buffer A. The values $V_{\text{max}}$, $V_{\text{max}}$ obtained at 37 °C was taken as 100%. * Significant difference from the point obtained at 15 °C in KRB buffer ($P < 0.01$).

**FIG. 8. Effects of insulin at different temperatures in both buffer systems.** Lipogenesis assays were performed at the indicated temperatures in the absence and presence of 2 μg ml$^{-1}$ of insulin for 60 min in Buffer A-25 mM NaHCO$_3$ (C) or Buffer A (O). The values of $V_{\text{max}}$, $V_{\text{max}}$ obtained at 37 °C was taken as 100%. * Significant difference from the point obtained at 15 °C in KRB buffer ($P < 0.01$).