Guanosine nucleotides regulate hormone binding of insulin receptors

Eric R. MORTENSEN, Jonathan DRACHMAN and Guido GUIDOTTI

Department of Biochemistry and Molecular Biology, Harvard University, 7 Divinity Avenue, Cambridge, MA 02138, U.S.A.

Insulin receptors in turkey erythrocyte and rat adipocyte plasma membranes display non-linear hormone binding by Scatchard analysis. This result is consistent with evidence that the insulin-binding sites are heterogeneous and have at least two affinities for the hormone. Mild reduction of plasma membranes with dithiothreitol, before insulin binding, increased the fraction of hormone binding with high affinity without significantly changing the total number of receptor-binding sites. In the presence of guanosine 5'-[γ -thio]triphosphate, the amount of receptor with high affinity for insulin in the reduced membranes decreased to that present in the absence of reduction; the effect of the nucleotide was concentration- and temperature-dependent. This decrease in insulin binding was specific for guanine nucleotides.

INTRODUCTION

The insulin receptor is a dimeric membrane glycoprotein with the structure ($\alpha\beta$)₂. Each monomer is composed of an α chain ($M_r = 130\,000$) and a β chain ($M_r = 95\,000$) held together by disulphide bond(s), and is linked to another $\alpha\beta$ monomer by disulphide bonds between the α chains (Shoelson *et al.*, 1988; Finn *et al.*, 1990; Chiacchia, 1991). The α and β chains are derived from a single precursor, which undergoes post-translational proteolysis; the α chain is entirely extracellular and has the insulin-binding site, whereas the β subunit has a single putative transmembrane segment and tyrosine kinase activity (Czech, 1985; Rosen, 1987).

The properties of the insulin receptor with regard to insulin binding and signal transduction are complicated (Kahn, 1976). In the first place, the full biological effect of insulin can be achieved at variable levels of receptor occupancy: for instance, maximal stimulation of glucose oxidation by rat adipocytes occurs when 2–10% of insulin receptors are occupied (Kahn, 1976; Gammeltoft & Gliemann, 1973) while fractional occupancy of the receptors correlates linearly with stimulation of glycogen synthesis in hepatocytes (Gammeltoft *et al.*, 1978; Gammeltoft, 1984). Secondly, it is not clear whether one or two insulin molecules bind to the apparently symmetrical receptor dimer (Pang & Shafer, 1984; Sweet *et al.*, 1987; Swanson *et al.*, 1988); in the former case there is possibility that one site in the dimer is inactivated by negative co-operativity. In the third place, linear or negatively co-operative binding isotherms have been obtained, depending on the tissue source (Kohanski & Lane, 1983; Pang & Shafer, 1984), prior exposure of receptor to hormone (Stevens *et al.*, 1983; Corin & Donner, 1982), degree of purification and oxidation/reduction state of the thiol groups of the receptor. In the last-mentioned case, reduction of cell membranes with dithiothreitol (DTT) was reported to increase the affinity of a fraction of insulin receptors in rat liver and adipocyte membranes without reduction of Class I disulphides or dissociation of the receptor ($\alpha\beta$)₂ structure (Jacobs & Cuatrecasas, 1980; Schweitzer *et al.*, 1980; Harmon *et al.*, 1983). In contrast, other investigators have reported both a decrease and an increase in the affinity of the receptor for insulin coincident with reduction of the Class I

disulphides and production of $\alpha\beta$ monomers by DTT (Böni-Schnetzler *et al.*, 1986; Pike *et al.*, 1986; Sweet *et al.*, 1987).

Examination of the kinetics of insulin association with and dissociation from receptor (Lipkin *et al.*, 1986) and of the hormone dose/response curve for kinase activation (Pike *et al.*, 1984), and determination of the size of the receptor binding unit by radiation inactivation (Harmon *et al.*, 1983; Fujita-Yamaguchi & Harmon, 1988), have not provided satisfactory explanations for the heterogeneity in binding sites.

It has therefore remained unclear whether there are several populations of insulin receptors with distinct affinities for insulin in different tissues or a single group of receptors coupled in different ways to various metabolic activities.

One possibility for the heterogeneity in binding by the insulin receptor is the interaction with an affinity regulator, like a G-protein (Gilman, 1987; Cerione *et al.*, 1984). Indeed, there have been many results which point to such interactions in a circumstantial way [Houslay (1986), Ciaraldi & Maisel (1989) and references therein].

Recently, effects of guanosine 5'-[γ -thio]triphosphate (GTP[S]) on insulin binding and the tyrosine kinase activity of rat adipocyte insulin receptors have been reported (Davis & McDonald, 1990; Kellerer *et al.*, 1991). Furthermore, Baldini *et al.* (1991) have shown that addition of GTP[S] or guanosine 5'-[$\beta\gamma$ -imido]triphosphate (p[NH]ppG) to rat adipocytes permeabilized with α -toxin causes an insulin-like translocation of the GLUT 4 glucose transporter to the plasma membrane, suggesting the involvement of a G-protein in this process.

In the present work, mild reduction of the membranes of turkey erythrocytes and rat adipocytes with DTT was used to increase the fraction of insulin receptors with a high affinity for the hormone. GTP[S] was found to decrease the affinity for insulin specifically of the population of membrane-bound receptors with the high insulin affinity. The decrease in insulin binding occurred in a concentration-dependent fashion, with a $K_{0.5}$ (concn. giving 50% effect) for GTP[S] of approx. $7\ \mu\text{M}$; other guanine nucleotides had diminished ability to mimic the effect of the non-hydrolysable derivative, GTP[S]. This effect was temperature-dependent, and was completely abolished in fat-cell membranes when binding was done at 4 °C.

Abbreviations used: DTT, dithiothreitol; GTP[S], guanosine 5'-[γ -thio]triphosphate; p[NH]ppG, guanosine 5'-[$\beta\gamma$ -imido]triphosphate; p[NH]ppA, adenosine 5'-[$\beta\gamma$ -imido]triphosphate.

METHODS AND MATERIALS

Materials

Standard chemicals used were of the highest available grade from Mallinckrodt, Fisher Scientific Co. and Sigma Chemical Co. The radioactive compounds ^{125}I -insulin and ^{32}P were supplied by New England Nuclear. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was synthesized with the Promega Biotech Gammaprep synthesis system. GTP[S], p[NH]ppG and adenosine 5'-[γ -thio]triphosphate were obtained from Boehringer Mannheim Biochemicals. Membrane protein determination was by Bio-Rad Assay protocol.

Pig insulin was obtained from Eli Lilly, and BSA was obtained from Sigma.

Buffers and solutions

Low-carbonyl and peroxide-free Triton X-100 (Surfact-Amps X-100) was purchased from Pierce Chemical Co. and was kept under an inert atmosphere. Hepes (obtained from Sigma) was used as a buffer for all membrane solutions and suspensions at 50 mM, pH 7.8 (buffers were prepared with triethylamine rather than NaOH for control of total Na^+ concentration). Unless otherwise stated, the final Na^+ concentration was adjusted to 50 mM by addition of NaCl to buffers.

Turkey erythrocyte plasma membranes

Turkey blood was obtained from Raymond's Turkey Farm, Methuen, Massachusetts. Turkey erythrocyte plasma membranes were prepared by the procedure described by Ginsberg *et al.* (1976), with the modification by Aiyer (1983a) that sulphate was substituted for chloride in all buffers.

Rat adipocyte plasma membranes

Male CD rats (175–250 g) were used for preparation of adipocyte plasma membrane by differential centrifugation, by the method of Resh (1982). During the process of homogenization, a proteinase-inhibitor cocktail was added to this final concentration: phenylmethanesulphonyl fluoride 200 mM, leupeptin 1 $\mu\text{g}/\text{ml}$, antipain 2 $\mu\text{g}/\text{ml}$, benzamidin 10 mg/ml , aprotinin 10 trypsin-inhibitor units/ml, chymostatin 1 $\mu\text{g}/\text{ml}$, pepstatin 1 mg/ml . Membranes were stored at 50 mM-Hepes, pH 7.8, at -70°C before experiments.

DTT reduction of plasma membranes

Where indicated, samples of adipocyte or turkey erythrocyte plasma membrane were pelleted in Microfuge tubes in an Eppendorf Microfuge. The pellets were resuspended in 50 mM-Hepes (pH 7.8)/1 mM-EDTA/1 mM-DTT and incubated with rocking for 1 h at 27°C . Membranes were pelleted and washed three times with buffer without DTT and resuspended in the indicated buffer.

Insulin binding to plasma membranes

Thawed membranes (0.1 ml, containing 0.25–0.5 mg of plasma-membrane protein) were added to 1.1 ml of 50 mM-Hepes (pH 7.8)/5 mM- MgCl_2 /1 mM-EDTA/0.01% (w/v) BSA and incubated with 50 pM- ^{125}I -insulin at approx. 100 $\mu\text{Ci}/\mu\text{g}$ and increasing concentrations of unlabelled pig insulin from 0 to 27.41 nM for 2 h at 37°C , unless otherwise indicated. Non-specific insulin binding was measured in the presence of 2 μM unlabelled insulin. LiCl was incubated in control buffers for experiments evaluating the effect of GTP[S] on binding, as this nucleotide was commercially available only as the trilithium salt. Total insulin binding was measured in 0.5 ml samples as follows.

(a) **Turkey erythrocyte membranes.** Samples were added to 0.5 ml of ice-cold binding buffer in 1.5 ml polyethylene Microfuge tubes and centrifuged in a Beckman Microfuge for 3 min.

Supernatants were discarded, the surface of the membrane pellets was carefully rinsed with ice-cold binding buffer, and the tips of the Microfuge tubes were excised and counted for radioactivity in a Beckman 4000 γ -radiation counter (^{125}I counting efficiency, 50%).

(b) **Rat adipocyte membranes.** Filtration through Millipore GVWP Teflon filters was conducted as described by Cuatrecasas (1971). Filters were washed with 3–5 ml of ice-cold binding buffer and counted for radioactivity as above.

Data were analysed by a modification of the program LIGAND as described by Munson & Rodbard (1980) as modified by McPherson (1985) and obtained from Elsevier-BIOSOFT. Binding data are presented by the method of Scatchard (1949). Single-point measurement of specific insulin binding was conducted as above with 50 pM- ^{125}I -insulin with and without 2 μM unlabelled hormone.

Detergent extraction of plasma membranes

Triton X-100 (peroxide-free; Pierce Chemical Co.) (final concn. 1%, v/v), was added to membranes (protein concn. 12–15 mg/ml), in Hepes buffer at 3°C . The suspension was ultracentrifuged at 130000 g for 45 min at 3°C to yield a supernatant solution containing detergent-solubilized membrane proteins. The supernatants were collected and diluted with buffer to a detergent concentration of 0.2–0.25%.

Insulin binding to solubilized membranes

Solubilized membrane proteins (10–200 μg) were incubated with 50–100 pM- ^{125}I -insulin (with or without 1.25 μM unlabelled insulin), and the volume was adjusted to 1.2 ml with 50 mM-Hepes buffer. After either 1 h at 23°C or 16 h at 4°C , the samples were divided into duplicate 0.5 ml portions. To each tube was added 0.1 ml of bovine γ -globulin (5 mg/ml) as carrier. After mixing, 0.5 ml of ice-cold 25% PEG-6000 was added, and the tube was vortex-mixed and placed on ice for 20–30 min. The precipitate was spun down in a Beckman Microfuge for 4 min at 4°C . The supernatant was aspirated, and the pellet was gently rinsed twice with 50 mM-Hepes buffer. The radioactivity in the pellet was measured with a γ -radiation counter.

Cross-linking of insulin to the receptor

Di-*N*-hydroxysuccinimidyl suberate cross-linking of ^{125}I -insulin bound to turkey erythrocyte plasma membranes was performed as previously described (Aiyer, 1983b). Cross-linked membrane preparations were solubilized and submitted to SDS/PAGE without other modifications.

SDS/PAGE and autoradiography

SDS/PAGE analysis was carried out in the Laemmli (1970) system. Gel samples were prepared without boiling by addition of concentrated sample buffer. The separating gel was a linear or exponential 4–8%-acrylamide gradient. To prevent artifactual reduction of receptor in non-reducing gels, 5-fold-concentrated gel-sample buffer was made 10 mM with *N*-ethylmaleimide immediately before its addition to solubilized receptor samples. Reducing gel samples were made 5% (v/v) in β -mercaptoethanol.

Sucrose density gradients

Separation of insulin-receptor monomers and dimers was done by sucrose-density-gradient centrifugation as described by Mortensen *et al.* (1991).

RESULTS

In this paper we show that guanine nucleotides affect insulin binding to insulin receptors that have been exposed to mild

reducing agents. Accordingly, the effect of the reducing agents on the interaction of insulin with the receptor will be discussed first; then the ability of guanine nucleotides to inhibit insulin binding to the reduced receptor will be illustrated.

Effects of reduction on insulin-receptor binding

Since reduction of membranes was required to uncover the inhibition of insulin binding to its receptor by GTP[S], the effect of the reduction on the structure of the receptor was investigated.

Fig. 1 shows that reduction of turkey erythrocyte membranes or of solubilized membranes with DTT caused an increase in binding of 75 pM-insulin, followed by a decrease at concentrations of DTT greater than 10 mM. These results were unchanged whether or not DTT was removed before the addition of insulin, indicating that the hormone is not affected by the reduction. Scatchard analysis of the binding data, shown in Fig. 6, indicates that reduction caused an increase in the fraction of receptors with high affinity for insulin without significantly changing the total number of receptors. This result is similar to those obtained

Fig. 1. Stimulation of insulin binding to solubilized receptors (○) and intact membranes (●) of turkey erythrocytes

The samples were incubated with DTT for 20 min, then allowed to bind to 75 pM- 125 I-insulin for 1 h. The initial levels of binding were 4.8% for solubilized membranes and 9.3% for intact membranes (bound/total). Each point represents the average of duplicates. The specific binding was calculated as total binding minus non-specific binding (in the presence of 1.25 μ M-insulin). This result is representative of three experiments.

Table 1. Reversal of the effect of DTT by GSSG

Turkey erythrocyte membranes were treated with 1 mM-DTT for 20 min. After the excess DTT was washed away, the membranes were incubated with GSSG for 30 min and then with 75 pM- 125 I-insulin for 1 h at 23 °C. Incubation with GSSG alone did not alter insulin binding. Each value is the average of duplicate points. This result is representative of two experiments.

Conditions		Fraction of total 125 I-insulin bound
DTT (mM)	GSSG (mM)	
0	0	0.070
0	10	0.068
1	0	0.117
1	1	0.117
1	5	0.100
1	10	0.078

Fig. 2. Sucrose density gradients of DTT-treated insulin receptors

Turkey erythrocyte membranes were treated with (●, unreduced) 0, (▼) 1 mM- or (□) 10 mM-DTT for 30 min and then washed with buffer. The intact membranes were allowed to bind to 100 pM- 125 I-insulin for 1 h and then washed with ice-cold Hepes buffer to remove unbound insulin. The samples were solubilized in 3% Triton X-100 at 3 °C and loaded on 5–20%-sucrose density gradients. After centrifugation, the radioactivity of the fractions was determined by γ -radiation counting and plotted as a function of the percentage of the distance from the bottom of the gradient. The radioactivity was normalized to an average fraction size of 0.15 ml. This result is representative of four separate experiments.

by other investigations (Schweitzer *et al.*, 1980; Harmon *et al.*, 1983; Swanson *et al.*, 1988). Similar results were obtained when rat adipocyte membranes were reduced with 1 mM-DTT for 1 h at 25 °C; binding of 50 pM- 125 I-insulin increased 1.7-fold.

The effect of reduction on insulin binding by the receptor is reversible. After reduction with 1 mM-DTT, turkey erythrocyte membranes were exposed to various concentrations of GSSG to see if the low-affinity receptor could be regenerated. GSSG was chosen as the oxidant, because Schweitzer *et al.* (1980) have reported that it was effective in reversing DTT-induced effects in rat adipocytes. As shown in Table 1, GSSG did reverse the DTT effect on insulin binding; by itself, it did not significantly affect the basal level of insulin binding by the receptor.

One of the effects of reducing the insulin-receptor dimer with DTT is the production of receptor monomers (Aiyer, 1983a; Velicelebi & Aiyer, 1984). It was therefore decided to determine whether or not the change in insulin affinity of the receptor was correlated with the appearance of monomers. The situation with turkey erythrocyte membranes is complicated by the presence of receptor monomers in the native and unmanipulated membrane. The results in Fig. 2 show the effects of reduction of turkey erythrocyte membranes on the receptor monomer and dimer distribution; 1 mM- and 10 mM-DTT were used, followed by incubation with 100 pM- 125 I-insulin. The membranes were then solubilized and the solution was examined by sucrose-density-gradient centrifugation. The native membranes had both receptor dimers and monomers, but at the low insulin concentration the monomers predominated. Reduction with 1 mM-DTT caused a drastic increase in binding at the dimer position, without a great change in the material at the monomer position. The dimer peak was eliminated by reduction with 10 mM-DTT. The fact that the material at the receptor monomer position remains approximately constant under the various conditions suggests either that the receptor monomers are not affected by the

Fig. 3. Insulin cross-linking of native and reduced membranes

Intact turkey erythrocyte membranes were incubated with and without 1 mM-DTT and then thoroughly washed. The membranes were allowed to incubate with 5 nM- 125 I-insulin before cross-linking with di-*N*-hydroxysuccinimidyl suberate was done. The samples were analysed by SDS/PAGE on 4%-acrylamide non-reducing gels. The results of an autoradiograph of the gel are shown. Lanes 1 and 2 are for unreduced membranes: lane 1 in the presence and lane 2 in the absence of excess unlabelled insulin. Lanes 3 and 4 are for the reduced membranes: lane 3 in the presence and lane 4 in the absence of excess unlabelled insulin. The result is representative of three experiments.

reduction or that reduction inactivates the monomers while increasing the binding to the receptor dimers.

This conclusion is supported by examination of the amount of insulin cross-linked to the receptor. Turkey erythrocyte membranes were reduced with 1 mM-DTT, and then allowed to equilibrate with 5 nM- 125 I-insulin before exposure to the cross-linking agent di-*N*-hydroxysuccinimidyl suberate. Fig. 3 shows an autoradiograph of a gel on which the preparations were analysed by SDS/PAGE. Lanes 1 and 2 demonstrate the difference between non-specific and specific incorporation of radioactivity in the unreduced membrane. The most clearly labelled bands are dimer and monomer at a ratio of 1:3–4. Lanes 3 and 4 show the results of reduction with DTT. The dimer has become the predominant labelled species, whereas incorporation of radioactivity in the monomer is diminished. This experiment demonstrates that the increase in receptor binding is attributable to effects on the receptor dimer. This effect is observed even though the concentration of insulin is high enough to allow binding to at least 50% of the low-affinity receptors; this is a consequence of the fact that the cross-linking is not quantitative and, accordingly, more frequently samples the complexes that have longer lifetimes.

Since insulin-receptor monomers are not present in the native membranes of rat adipocytes, one could inquire directly whether or not the effects of DTT on insulin binding are related to the conversion of receptor dimers into monomers by the reducing agent. This question was addressed by determination of the size of the receptors that bound low concentrations of insulin after

the exposure to DTT; monomers and dimers were separated by sucrose-density-gradient centrifugation. Thus rat adipocyte plasma membranes were reduced with various concentrations of DTT. After removal of the reducing agent, the membranes were incubated with 100 pM- 125 I-insulin either before or after solubilization with Triton X-100, and the preparation was resolved into receptor dimers and monomers by sucrose-density-gradient centrifugation. Fig. 4 illustrates the amounts of insulin bound to receptor monomers and dimers. Fig. 4(a) shows the results obtained when solubilization of the receptor preceded insulin binding; in this case reduction caused an increase in insulin binding only to the receptor monomer. On the other hand, if insulin binding preceded solubilization of the membrane, there was an increase in insulin bound to both receptor dimers and monomers at the lowest concentration of DTT (Fig. 4b). At higher concentrations of DTT, the receptor was converted into monomers. The presence of high-affinity dimers in the latter case might be due to insulin-dependent association of monomers (Chiacchia, 1988) or to a difference in the ability of DTT to reduce insulin-bound and free receptors. The result demonstrates, however, that the DTT-dependent increase in the affinity of the receptor is independent of the state of the Class I disulphide bonds between $\alpha\beta$ monomers.

There appears to be a discrepancy in the magnitude of the effect of reduction on insulin-receptor binding, depending on whether or not the receptors are subjected to sucrose-density-gradient centrifugation (Figs. 2 and 4, compared with Fig. 1 and the text above concerning it). This illusion is caused by the dissociation of some of the ligand from the low-affinity receptors during the centrifugation, resulting in the appearance of a larger relative amount of the high-affinity species. This situation does not arise when membranes are used to measure binding.

We conclude that the increase in insulin affinity brought about by reduction of the membranes with DTT is unrelated to the monomer-dimer status of the insulin receptor. Indeed, there is no evidence that native receptor monomers and dimers differ in their insulin affinity.

Effect of guanine nucleotides on insulin-receptor binding

Fig. 5 shows the effect of GTP[S] on the specific binding of insulin to membranes with or without prior reduction. In Fig. 5(a) is shown binding of 50 pM- 125 I-insulin to native and reduced turkey erythrocyte membranes as a function of increasing concentrations of GTP[S]. Fig. 5(b) shows the same experiment performed with rat adipocyte plasma membranes. The nucleotide had little or no effect on specific insulin binding in membranes which had not been previously reduced with DTT. Although GTP[S] caused a dose-dependent inhibition of insulin binding in both turkey and fat-cell membranes after reduction, its effect was most notable on the adipocyte membranes (Fig. 5b), owing to the greater increase in binding seen in response to the reduction. GTP[S] at 100 μ M effected a 40% decrease in specific binding of insulin to membranes previously reduced with 1 mM-DTT. Addition of nucleotide completely eliminated the increase in binding seen in response to reduction. This latter observation was constant, despite variations in the magnitude of the increase in binding produced by DTT with different membrane preparations (results not shown). The variability in the magnitude of the increase in binding produced by DTT reduction of adipocyte membranes has been noted previously (Swanson *et al.*, 1988). Unfortunately, neither addition of bacitracin to the binding buffer (Hammons *et al.*, 1982) nor insolation of fat-cell membranes in the presence of KCN or phosphate (Shechter & Ron, 1986) were useful in decreasing the variability in the effect of DTT on the receptors (results not shown).

The concentration-dependence of the effect of GTP[S] on the

Fig. 4. Effect of DTT on the insulin affinity of receptor monomers and dimers

Rat adipocyte plasma membranes were reduced with (●) 0, (▲) 1 mM-, (■) 5 mM- and (+) 10 mM-DTT for 60 min at 25 °C. The reducing agent was removed and each sample divided into two. For one aliquot of each sample, the membrane was solubilized and then allowed to bind to 50 pM-¹²⁵I-insulin (a); for the other, binding to insulin preceded solubilization of the membrane (b). Membranes were solubilized with 1% peroxide-free Triton X-100, and solubilized receptors were then applied to sucrose density gradients. Results shown are ¹²⁵I radioactivity (c.p.m./fraction) displayed as a function of the percentage of the gradient volume; 100% represents the top of the gradient.

DTT-dependent high-affinity insulin binding yielded a $K_{0.5}$ of 7.7 μ M. As GTP[S] was added to binding tubes from 100-fold-concentrated stock solutions, technical limitations of solubility prevented extension of the nucleotide dose/response curve to higher concentrations. Although ionic-strength effects or non-specific complexing with nucleotide were not expected to represent a likely complication in this experiment, p[NH]ppA was also used at the highest concentration of GTP[S] as a control to show that the effect seen was specific to GTP[S] (Fig. 5b).

Guanine nucleotides decrease binding to high-affinity receptor

Fig. 6 shows Scatchard plots of insulin binding to control and reduced turkey erythrocyte membranes in the absence and presence of GTP[S]. Owing to the marked nonlinearity of the Scatchard plot, data were most appropriately modelled by a two-site model of hormone binding to low- and high-affinity sites. Reduction of turkey erythrocyte membranes results in an increase in the fraction of receptors showing high-affinity binding from approx. 0.137 to 0.459. Analysis of binding to reduced receptors showed the K_d of the high-affinity site to be 1.28×10^{-10} M, whereas the low-affinity site had a K_d of 2.45×10^{-9} M. Native receptors had high- and low-affinity sites with estimated K_d values of 1.08×10^{-10} M and 1.18×10^{-9} M respectively. Statistical analysis predicted the following distribution of high- (R_H) and low- (R_L) affinity sites: unreduced membranes, $R_H = 122.6$

fmol/mg, $R_L = 772.6$ fmol/mg; reduced membranes, $R_H = 432.2$ fmol/mg, $R_L = 498.9$ fmol/mg. The number of total binding sites was unchanged by reduction; the estimated sites were 895.2 ± 111.0 fmol/mg and 922.1 ± 83.2 fmol/mg for the native and the reduced membranes respectively. Addition of GTP[S] to reduced membrane caused a decrease in binding to the component with high affinity for insulin produced by DTT reduction. The fraction of high-affinity sites decreased from 0.459 to 0.211 of total binding, with $R_H = 331.3$ fmol/mg and $R_L = 1.24$ pmol/mg and an apparent increase in total receptors to 1.57 ± 0.211 pmol/mg of protein. The large error in the determination of the number of low-affinity binding sites, however, was not incompatible with the possibility that there was no significant difference in total binding between these groups. The K_d of the high-affinity site was 1.58×10^{-10} M, and the low-affinity site had a K_d of 3.52×10^{-9} M.

The data were analysed with the assumption that allowing the values for the affinities and total sites to vary independently provided the best estimate for the parameters. It is clear, however, that the error in estimates for the K_d of the high-affinity site will be greater when these sites are only a small fraction of the receptors. By using the assumption that membrane reduction did not affect the values of K_d , but only increased the fraction of receptors displaying high affinity, a more accurate estimate of the K_d of the high-affinity sites were extracted from experiments

Fig. 5. Effect of GTP[S] on insulin binding to reduced and unreduced membranes

Specific binding of 50 pM-insulin to turkey erythrocyte (a) and rat adipocyte (b) plasma membranes was measured before ($\circ$, $\triangle$) and after ($\bullet$, $\blacktriangle$) reduction with 1 mM-DTT, as a function of increasing concentrations of GTP[S]. Data are presented as percentages of specific binding obtained with unreduced membranes in the absence of the nucleotide. Also shown in (b) is the effect of p[NH]ppA ($\blacksquare$), equimolar to the concentration of guanine nucleotide yielding a maximum decrease in binding, on reduced adipocyte plasma membrane.

using reduced membranes, where hormone binding was predicted to be roughly evenly divided between the two sites. With the estimated K_d of 128 pM for the high-affinity sites, an increase in the fraction of high-affinity sites with reduction from 0.161 to 0.459 was estimated. The presence of GTP[S] then reduces the fraction of high-affinity receptors to 0.192. Use of this model resulted in virtually no improvement in the statistics for fitting the model and in little change in the segregation of binding into two sites. Furthermore, it still resulted in a 2-fold increase in the predicted affinity of the low-affinity site after reduction. On the basis of the data, we cannot discriminate between these two models.

The possibility that GTP[S] was decreasing insulin binding to some non-specific binding sites rather than to insulin receptors was examined by cross-linking 125 I-insulin bound to the receptor, at a concentration of 75 pM, in the presence and absence of the guanine nucleotide. Only the 135 kDa α subunit was cross-linked to insulin and the label was displaced by unlabelled hormone. When the cross-linking was done in the presence of 10 μ M- and 100 μ M-GTP[S], the 125 I radioactivity bound to the α subunit decreased to 90 % and 75 % respectively of the amount in the absence of the nucleotide. These values agree well with the results of the binding studies.

Specificity of the effect of guanine nucleotides

Results obtained with turkey erythrocyte membrane receptors prompted an examination of the effect of guanine nucleotides on the binding of insulin to the plasma membrane of rat adipocytes,

Fig. 6. Scatchard plots of insulin binding to turkey erythrocyte plasma membranes

Plasma membranes were divided into three groups; one group was incubated without DTT and two were incubated with 1 mM-DTT. One reduced membrane sample was divided into binding tubes containing final concentrations of 100 μ M-GTP[S] and 300 μ M-LiCl. The other reduced and non-reduced membrane samples were bound to insulin in buffers containing only 300 μ M-LiCl. Shown are plots of insulin bound (in pmol/mg of membrane protein) as a function of the ratio of bound to free insulin (B/F). (a) Unreduced membranes; (b) reduced membranes; (c) reduced membranes in the presence of GTP[S].

a hormone-responsive tissue. Other nucleotide analogues were examined to evaluate the specificity of this effect for guanine nucleotides. Rat adipocyte plasma membranes were reduced with 1 mM-DTT as described in the Methods and materials section. After removal of reductant, membranes were bound to 125 I-insulin in the absence or presence of a 100 μ M concentration of a variety of nucleotide analogues. Fig. 7 shows that p[NH]ppG and GTP were less effective in mimicking the GTP[S] effect. Nucleotides other than guanine analogues were largely ineffective in decreasing binding. The fact that binding remained essentially unchanged from control in the presence of other nucleotides decreases the probability of some non-specific effect of GTP[S] on binding.

Temperature-dependence of guanine nucleotide effect

The effect of GTP[S] on insulin binding showed a dramatic temperature-dependence. High-affinity binding in adipocyte plasma membranes was best demonstrated by incubation with labelled hormone at 3 $^{\circ}$ C, which increases the half-time of dissociation and decreases proteolysis from potential membrane contaminants. Fig. 8, however, shows that the effect of GTP[S] on the high-affinity receptor was virtually abolished at this temperature. As a consequence of this temperature-dependence of the nucleotide effect, we have been unable to evaluate binding to receptor at temperatures which would decrease the experimental noise and improve an estimation of total binding and receptor affinity. This result implicates membrane fluidity as

Fig. 7. Effect of various nucleotides on insulin binding to the reduced receptor

Specific insulin binding to rat adipocyte plasma membranes reduced with 1 mM-DTT is shown. The membranes were incubated in the absence and in the presence of GTP[S] or a variety of other nucleotide analogues (all at 100 μ M) as indicated. All binding is shown as a percentage of control binding. Abbreviations: cAMP, cyclic AMP; ATP[S], adenosine 5'-[γ -thio]triphosphate.

influencing the effect of GTP[S]. This could be due to a requirement for receptor association/dissociation with a G-protein for alteration of the binding affinity.

Effect of guanine nucleotides on solubilized receptors

In order to determine the effect of the guanine nucleotides on solubilized receptors, it was necessary to concentrate the receptors, either before or after reduction, by lectin or insulin affinity chromatography; this procedure was necessary to obtain sufficient receptors to generate accurate data at low insulin concentrations. After concentration by these methods, the solubilized receptors were not affected by the presence of guanine nucleotides, irrespective of the procedure used for reduction. Similarly, Swanson *et al.* (1988) reported that purification of insulin receptors by lectin or insulin affinity chromatography prevented the stimulation of insulin binding by reduction with DTT.

We suggest that during chromatography the affinity regulator, G-protein, is removed from contact with the insulin receptor.

DISCUSSION

Two findings are reported in this paper. One is that mild reduction of preparations of the insulin receptor with DTT results in an increase in the binding of insulin to the receptor; this result has been obtained previously by other investigators. The other finding is that GTP[S] causes the disappearance of the high-affinity component of insulin binding uncovered by reduction; this result is novel.

Effect of DTT on insulin binding

Mild reduction of plasma membranes with DTT brings about an increase in the binding of insulin at low concentrations of ligand (Fig. 1). With turkey erythrocyte membranes, the effect is exclusively on receptor dimers (Figs. 2 and 3); with rat adipocyte membranes, however, both receptors dimers and monomers, the latter produced by the reduction, show the increase in insulin affinity (Fig. 4). This stimulation of binding is reversible; GSSG

Fig. 8. Effect of temperature on the inhibition of insulin binding by GTP[S]

Rat adipocyte plasma membranes were reduced with 1 mM-DTT, and then the binding of 50 pM-¹²⁵I-insulin was measured at different concentrations of GTP[S]. The three curves displayed represent binding done at 37 °C for 3 h (■), at 22 °C for 3 h (●) and at 3 °C for 12 h (▲). Conditions chosen were found to optimize control specific binding. Each curve is normalized to binding in the absence of nucleotide at the individual temperature as 100 % of control. Also included is a single point of insulin binding to membrane at 37 °C for 3 h in the presence of a concentration of p[NH]ppA equivalent to the highest GTP[S] concentration tested (▽).

brings about a decrease in insulin binding (Table 1). The results shown in Fig. 6 indicate that the reduction increases the proportion of receptors with a high affinity for insulin without affecting the total number of receptors; this result explains the increase in binding obtained at low concentrations of insulin.

The central question is whether the effect of reducing agents on the affinity of the insulin receptor is a consequence of the reduction of the insulin receptor itself or of an associated molecule. This question does not appear to have a simple answer, principally because the results obtained by different investigators are diverse and appear to be contradictory. There is, however, a simple answer.

We suggest that the results in the literature and those described here can be explained by the following hypothesis. (1) Judicious reduction of membrane-bound or solubilized, but unpurified, receptors can lead to an increase in the fraction of receptors with high affinity, principally due to the effect of the reducing agent on an affinity regulator, which we consider to be a G-protein; the formation of receptor $\alpha\beta$ monomers by the reduction is unrelated to the increase in affinity. (2) Purified receptors are no longer associated with the G-protein. (3) Reduction of purified receptors generates receptor monomers with decreased affinity for insulin, or with an affinity intermediate between that of the high-affinity and the low-affinity sites of the receptor dimers; these effects depend on the state of the purified receptor and the number of receptor disulphide groups reduced and are unrelated to those observed with membrane-bound receptors.

This hypothesis is supported by two types of results. One is that in general mild reduction of membranes leads to an increase in high-affinity insulin binding without formation of receptor monomers. Indeed, reduction of turkey erythrocyte, rat liver and adipocyte membranes with low concentrations of DTT increases

insulin binding at low insulin concentrations (Jacobs & Cuatrecasas, 1980; Schweitzer *et al.*, 1980; Massague & Czech, 1982; Harmon *et al.*, 1983; Swanson *et al.*, 1988; the present paper). The other result is that, in general, reduction of purified receptors causes a decrease (Pike *et al.*, 1986; Böni-Schnetzler *et al.*, 1986, 1987; Swanson *et al.*, 1988) or no change (Sweet *et al.*, 1987) in insulin affinity. [Sweet *et al.* (1987) actually suggested that reduced but not dissociated ($\alpha\beta$)₂ dimers are nearly identical with non-reduced dimers with regard to insulin binding, whereas $\alpha\beta$ receptor monomers have a single class of binding sites with affinity intermediate between that of the high- and low-affinity sites of the dimers. Examination of the results of gel filtration, however, indicates that the presumed non-dissociated dimers are actually monomers, whereas the presumed monomers are probably aggregates of the α and β chains (Fig. 5 of Morrison *et al.*, 1988).]

One can ask why the results in the literature on the effects of reduction on the structure and function of the insulin receptor are so variable. One reason is that the sensitivity of the insulin receptors to reduction is tissue- and species-specific. It is not valid to compare results obtained with receptors from turkey erythrocyte, rat adipocyte, rat liver and human placenta membranes, because the state of the receptors depends on the method of membrane preparation. Thus there are considerable amounts of receptor monomers in all these membranes, except for those of rat adipocytes, probably owing to the presence of reducing compounds in the cells (Aiyer, 1983a; Koch *et al.*, 1986; Fujita-Yamaguchi & Harmon, 1988; Treadway *et al.*, 1990; Mortensen *et al.*, 1991). It is apparent that the properties of these receptor monomers are different from those of the receptor dimers (Treadway *et al.*, 1990; Mortensen *et al.*, 1991; Li *et al.*, 1991). Since native receptor monomers are inactivated by reduction (Fig. 3), their presence in membrane preparations will blunt the increase in insulin binding caused by reduction, as is observed with human placenta (Jacobs & Cuatrecasas, 1980; Swanson *et al.*, 1988) and rat liver (Schweitzer *et al.*, 1980) membranes.

Another complication is that reduction produces receptor monomers, and the properties of insulin-receptor monomers are highly variable. Thus, endogenous monomers have an insulin affinity similar to (Koch *et al.*, 1986) or greater than (Fujita-Yamaguchi & Harmon, 1988) that of the dimers, whereas monomers obtained by reduction have been shown to have higher (Fig. 4), lower (Pike *et al.*, 1986; Böni-Schnetzler *et al.*, 1986, 1987; Swanson *et al.*, 1988), or unchanged (Sweet *et al.*, 1987) insulin affinity, depending on the degree of purification of the receptors. There is therefore confusion about the effects of reduction, because the influence of the monomers on the results has been unclear. We suggest that the insulin affinity of native receptor monomers is identical with that of the dimers; the reported decreases in affinity are due to partial denaturation of the receptor.

Although it cannot be excluded that DTT has access to different receptor disulphide groups in purified receptors as compared with membrane-bound ones, the results presented here suggest that the target of the reduction by DTT in membranes is not the insulin receptor itself but another membrane protein that is lost during the purification. The likely scenario is that the affinity of the receptor is not influenced by the presence of the Class I disulphide bonds; a variable fraction of the receptors interacts with a factor in the membrane, which increases the receptor's affinity for insulin, and reduction increases the amount of active factor, resulting in an increase in affinity of additional receptors.

Effect of GTP[S] on insulin binding

The results in Fig. 5–8 show that the GTP[S] decreases hormone

binding by the insulin receptors of turkey erythrocyte and rat adipocyte plasma membranes if the membranes had been reduced with DTT. In turkey erythrocytes, this decrease in binding is shown to result from the loss of high-affinity insulin-binding sites produced by reduction, a result consistent with the existence of heterogeneous binding sites. The amount of ¹²⁵I-insulin specifically cross-linked to the receptor decreased with increasing concentration of GTP[S], suggesting that in the presence of the nucleotide there was a decrease in insulin binding to the receptor. This effect was specific for guanine nucleotides (Fig. 7) and was eliminated if binding was performed at 3 °C (Fig. 8). This result suggests, but does not establish, a role for membrane fluidity in permitting the association of receptor and membrane-bound regulatory proteins.

There are several recent studies on the interaction of guanine nucleotides with the insulin receptor. Burdett *et al.* (1990) found that 100 μ M-GTP[S] was without effect on insulin binding by rat liver plasma membranes; this result is not surprising, in view of the requirement for mild reduction to elicit the effect of GTP[S]. On the other hand, Davis & McDonald (1990) have reported that high concentrations of GTP[S] (0.5–1 mM) inhibited both insulin binding and the tyrosine kinase activity of solubilized insulin receptors from rat adipocyte membranes; it was suggested that the effect of the nucleotide might not be dependent on conventional G-proteins, because there was no evidence for their presence in partially purified preparations of the receptor. Kellerer *et al.* (1991) have shown that insulin activated the binding of GTP[S] to adipocyte membranes and that 10 μ M-GTP[S] decreased the affinity of the membranes for insulin.

We propose that a G-protein is involved in the effect of guanine nucleotides on the affinity of the insulin receptors; the fraction of receptors with a high affinity for insulin would represent the receptors coupled to the G-proteins in the nucleotide-free state. The hypothesis is that during membrane preparation there is oxidation and inactivation of the G-proteins (Winslow *et al.*, 1987); mild reduction re-activates the G-proteins and allows them to interact with the insulin receptors, increasing the fraction with a high affinity for insulin. Addition of GTP[S] reverses the effect of reduction by causing dissociation of the G-protein/receptor complex. The fact that perturbation of the membrane is required, in some instances, to demonstrate the interaction of a receptor with G-proteins has been reported previously (Florio & Sternweis, 1985).

According to this view, the observation that insulin effects correlate linearly with receptor occupancy in liver cells and do not display the 'spare receptor' phenomena seen in fat-cells may reflect a tissue-specific difference in the stoichiometry between insulin receptors and affinity modulator/receptor effector units (Gammeltoft, 1984).

The supposition that G-proteins have a role in signal transduction from the insulin receptor is suggested by the results presented in the Introduction that guanine nucleotides, toxins and G-proteins have effects on insulin action. The results described in this paper and the recent evidence (Baldini *et al.*, 1991) that exposure of permeabilized rat adipocytes to GTP[S] caused translocation of glucose transporters to the plasma membrane in a way comparable with that effected by insulin provide support for this notion.

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