Sulphydryl agents modulate insulin- and epidermal growth factor (EGF)-receptor kinase via reaction with intracellular receptor domains: differential effects on basal versus activated receptors

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Sulphydryl reagents have been shown to produce a variety of effects on insulin-receptor structure and function. However, localization of these effects to specific receptor domains has not been attempted. We have investigated this question with insulin- and epidermal growth factor (EGF)-receptors (both are receptor tyrosine kinases but have different sulphydryl/disulphide structures within the external domain), and the insulin receptor kinase (IRK) protein consisting solely of the insulin-receptor cytoplasmic domain and exhibiting constitutive kinase activity. Results showed a differential response between basal and activated receptors. The physiological reductant GSH stimulated basal receptor autophosphorylation, but was either without effect (EGF) or inhibited (insulin) activated receptors, and occurred without visible reduction of receptor structure. These

results contrast with those obtained with dithiothreitol which appears to activate phosphorylation in association with reduction of the extracellular insulin-receptor disulphides, but is without effect on the EGF receptor or the IRK protein. Alkylating agents *N*-ethylmaleimide (NEM) and iodoacetamide (IAM) had opposing effects on receptor autophosphorylation. However, only in the basal state was IAM able to protect receptors from the inhibitory effect of NEM. Our results suggest that complex sulphydryl interactions can occur within the cytoplasmic domain of insulin- and EGF-receptors to alter receptor kinase activity. The basal and activated state of receptors is not the same with respect to sulphydryl reagent action, possibly due to conformational change in the receptor induced by ligand (insulin, EGF) or constitutive (IRK) activation.

INTRODUCTION

The disulphide nature of the insulin receptor and the high cysteine content of the external domain are well documented (e.g. Zick, 1989). Profound, albeit conflicting, effects on both insulin-receptor structure and function have been shown by agents that can react with sulphydryls or disulphides. For instance, oxidants, some of which may react directly with the receptor, have been shown to stimulate insulin-like effects in whole cells (Czech, 1976; Ludvigsen and Jarett, 1982). However, oxidizing agents inhibit the isolated insulin-receptor tyrosine kinase, in direct contrast with their stimulatory effects on insulin action (Wilden and Pessin, 1987). The reductant dithiothreitol (DTT) has been shown by many groups to lead to progressive dissociation of the dimeric holoreceptor (Fujita-Yamaguchi and Kathuria, 1985; Sweet et al., 1987; Chiacchia, 1988). Initially, the Class I disulphides between two α -subunits are cleaved, resulting in receptor monomers. Under more extreme conditions the Class II disulphides between the α - and β -subunits are reduced to generate individual subunits (Massague and Czech, 1982). DTT treatment (at millimolar concentrations) has also been associated with stimulation of the insulin-receptor tyrosine kinase (Fujita-Yamaguchi and Kathuria, 1985; Wilden et al., 1986). However, while this correlates with the appearance of receptor monomers, whether the two are causally related has not been directly determined. Other reagents which block free thiol groups (alkylating agents, disulphide exchange reagents) have variable effects, although the major findings are inhibition of kinase activity (Chen et al., 1986; Wilden and Pessin, 1987). For instance, two alkylating agents, *N*-ethylmaleimide (NEM) and

iodoacetamide (IAM), have opposite effects, although both should block free sulphydryl groups (Wilden and Pessin, 1987; Li et al., 1991). Thus many reports show that the insulin receptor is susceptible to sulphydryl modification and dose-response curves suggest that the insulin-stimulated state is more sensitive (Wilden and Pessin, 1987). If, as has been proposed using antibodies (Herrera and Rosen, 1986; Perlman et al., 1989; Baron et al., 1990), the activated state of the insulin receptor has an altered conformation to the basal state, differential reactivity to sulphydryl agents may reflect these changes.

Very little work has examined the effect of sulphydryl modification on epidermal growth factor (EGF) receptor kinase. The EGF receptor shows much sequence similarity with the insulin receptor, especially within the kinase domain, and the extracellular domain has organizational similarities to the insulin receptor (Bajaj et al., 1987). However, although the extracellular domain of the EGF receptor has two cysteine-rich regions, it has no intermolecular disulphide bonds and exists, in the basal state at least, as a monomer (Cochet et al., 1988). Thus a comparison of the effects of sulphydryl-active agents on insulin- and EGF-receptors could be useful in defining common reactive groups and potential sites of action.

Most experiments to date have studied isolated receptors and thus the added reagent may act on both the extracellular and intracellular domains of the receptor. Results with DTT show that activation of insulin-receptor tyrosine kinase correlates with the appearance of receptor monomers, suggesting that its effect is extracellular. However, this may not be the case for other reagents. For example, the alkylating agent NEM appears to interact with the cytoplasmic domain of the insulin receptor as

Abbreviations used: EGF, epidermal growth factor; IRK, insulin receptor kinase; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; IAM, iodoacetamide; MEM, modified essential medium.

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determined by labelling studies (Finn et al., 1990). In order to investigate the site of action of various sulphhydryl agents we have utilized the insulin receptor kinase (IRK) protein (Ellis et al., 1988) purified from SF9 cells infected with a baculovirus vector, a constitutively active form of the insulin receptor with kinetics (Cobb et al., 1989) and autophosphorylation patterns (Tavaré et al., 1991) similar to those of the intact receptor. The IRK protein consists of the cytoplasmic domain of the receptor β -subunit and allows us to examine the effect of sulphhydryl-active agents solely on this domain, which contains no interchain disulphides (Cobb et al., 1989). In addition, for known, non-permeant reagents we initially compared results using whole cells with those from isolated receptors.

Is there a possibility that sulphhydryl-group modification could occur *in vivo*, perhaps modulating the insulin or EGF response? Such an effect has been proposed for T-cells (Kanner et al., 1992) where sulphhydryl oxidation inhibits receptor-mediated tyrosine phosphorylation of phospholipase C_γ . The ubiquitous cellular sulphhydryl oxidation/reduction system is glutathione (GSSG/GSH). GSH is the most prevalent intracellular thiol, important for the maintenance of cellular function and protection against cellular oxidation. Results from a variety of sources demonstrate quite profound changes in the levels of both tissue GSH and GSSG under conditions of fasting or stress (Gilbert, 1990) and also in diabetes, unrelated to alterations in food intake (McLennan et al., 1991). In addition, erythrocyte GSH appears to be elevated in some cancer patients, including those with breast cancer (Herbergs et al., 1992). The intracellular concentration of GSH is within the millimolar range; thus there exists the possibility that the tyrosine kinase activity of receptors could be modulated by changes in the level of GSH, but only through reaction at an intracellular site as it is cell impermeant (Meister and Anderson, 1983). The effect of GSH on either insulin- or EGF-receptor autophosphorylation has not been examined previously.

Our results with GSH suggest that it can alter the kinase activity of both insulin- and EGF-receptors. Unlike DTT, interaction is at an intracellular site and could therefore play some role *in vivo*. Furthermore, the basal and activated states of both insulin- and EGF-receptor kinase show a qualitatively different response to GSH. These latter results were confirmed and extended using additional sulphhydryl-active agents, and suggest that different cysteine residues may be modified in the basal and active states of insulin- and EGF-receptors, perhaps as a result of ligand-induced conformational change.

EXPERIMENTAL

Materials

CHOT cells and the purified IRK protein were a gift from Dr. Leland Ellis (Texas A & M, Houston, TX, U.S.A.). CHO Δ CT69 cells were a gift from Dr. Jeremy Tavaré (Bristol University, Bristol, Avon, U.K.). Antibodies CT-1 and 83-7 were a gift from Dr. Ken Sidle (University of Cambridge, Cambridge, U.K.).

Rabbit anti-(mouse immunoglobulin) antibody was purchased from Dakopatts (Bioscientific P/L, Gympsea, New South Wales, Australia). DTT, GSH, IAM, NEM and other chemicals were from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). [γ - 3 P]ATP (3000 Ci/mmol) and Hyperfilm MP were from Amersham (North Ryde, New South Wales, Australia). Protein A-Sepharose was purchased from Pharmacia (North Ryde, New South Wales, Australia). Reagents for SDS/PAGE were from Bio-Rad (North Ryde, New South Wales, Australia) except the pre-stained high- M_r standard proteins (BRL, Glen Waverley, Victoria, Australia).

Unlabelled EGF was extracted and purified from male mouse submaxillary glands, essentially by the method of Savage and Cohen (1972).

Cell culture

CHOT cells (overexpressing transfected human insulin receptor) and CHO Δ CT69 cells (overexpressing a mutant human insulin receptor lacking the C-terminal 69 amino acids of the β -subunit) were maintained in alpha-modified essential medium (MEM) containing 10% (v/v) fetal-calf serum. A431 cells (overexpressing EGF receptor) were maintained in Dulbecco's MEM containing 25 mM glucose and 10% (v/v) fetal-calf serum. Cells were plated in appropriate dishes for each experiment and grown to 80–100% confluency.

Incubation with sulphhydryl reagents

Intact cells

Cells (CHOT or A431) in six-well plates were washed twice in buffer A (Earle's balanced salt solution containing 25 mM Hepes, pH 7.5, and 0.1% BSA) and incubated in 1 ml of this buffer with or without insulin (1 μ g/ml) or EGF (0.5 μ g/ml) as appropriate for 15 min at 22 °C. Sulphhydryl agents (GSH, DTT, NEM and IAM) were added during the incubation with insulin or EGF at concentrations as indicated in the Results section. After incubation the cells were washed twice in buffer A before lysis [with 50 mM Hepes, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 10% (w/v) glycerol, 1% (w/v) Triton X-100, 0.15 unit of aprotinin/ml, 10 μ g/ml leupeptin and 1 mM phenylmethanesulphonyl fluoride] and immunoprecipitation of receptors was carried out as described below.

Isolated receptors

CHOT, CHO Δ CT69 or A431 cells were washed and treated with insulin (1 μ g/ml) and EGF (0.5 μ g/ml) respectively, as for intact cells above. After cell lysis and immunoprecipitation of receptors, as described below, the washed pellets were resuspended in 50 μ l of buffer A, to which was added the various sulphhydryl reagents, as described in the Results section, and incubated for 15 min at 22 °C with agitation. The pellets were washed twice in PBS/0.1% Triton X-100 before autophosphorylation of receptors was carried out. For protection studies, IAM (10 mM) was added for 15 min at 22 °C, and the pellets were then washed twice in buffer A before addition of NEM (5 mM) for a further 15 min at 22 °C. Sulphhydryl reagents were added directly to the IRK protein (10 ng) in a final volume of 20 μ l (25 mM Hepes, pH 7.5/0.1% Triton X-100) and incubated for 15 min at 22 °C.

Immunoprecipitation of receptors

To 20 μ l of Protein A-Sepharose (50%, w/v) coated with 10 μ g of rabbit anti-(mouse IgG) antibody (CT-1, 83-7) was added anti-receptor antibody (20–50 μ g of either CT-1 or 83-7, insulin receptors; or R1, EGF receptors) for 10 min at 22 °C. After two washes in PBS (NaCl, 161 mM/ Na_2HPO_4 , 1.6 mM/ NaH_2PO_4 , 4 mM) cell lysate was added and the mixture agitated for 1–2 h at 4 °C. After washing in high- and low-salt buffers as described previously (Clark et al., 1988) the immunoprecipitates were autophosphorylated. Recovery of receptor protein was monitored by immunoblotting receptors from control or insulin-stimulated CHOT cells treated with or without sulphhydryl agents. Transferred proteins were probed with antibody CT-1 (Ganderton et al., 1992) directed against the last 15 amino acids

of the insulin-receptor β -subunit. None of the sulphhydryl agents altered recovery of receptor protein (results not shown).

Autophosphorylation of receptors

Immunoprecipitates were incubated in phosphorylation buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 12 mM MnCl_2 , 12 mM MgCl_2 , 10 μM Na_2VO_4 , 0.2% Triton X-100 and 20 μM ATP containing 5 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP/sample}$) for 10 min on ice; for EGF receptors the buffer contained MnCl_2 at a concentration of only 2 mM. IRK protein was incubated in 10 mM Hepes, pH 7.5, 1 mM EDTA, 2 mM MgCl_2 , 0.2 mg/ml BSA, 20 $\mu\text{g/ml}$ protamine and 100 μM ATP containing 5 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP/sample}$ for 10 min on ice. All reactions were stopped by addition of SDS/PAGE sample buffer and samples were heated at 100 °C for 3 min. Samples were analysed by SDS/PAGE [7.5% (w/v) acrylamide for EGF receptor, M_r 170 000 and insulin receptor M_r 90 000; 10% (w/v) acrylamide for IRK protein, M_r 48 000] under reducing conditions. Pre-stained standard marker proteins were also run. Phosphorylated bands were visualized by autoradiography with Hyperfilm-MP and the level of autophosphorylation quantified by excision of labelled bands and Cerenkov counting in a liquid scintillation counter. In some instances phosphorylated immunoprecipitates were resuspended in SDS/PAGE sample buffer containing 10 mM GSSG and proteins separated in 5% (w/v) non-reduced acrylamide gels. All results are expressed as percentages of the counts obtained in the absence of sulphhydryl agents, and are the means $\pm$ S.E.M. of at least three independent experiments. The lysis buffer contained no vanadate (phosphatase inhibitor), resulting in complete dephosphorylation of receptors before the *in vitro* autophosphorylation assay commenced. Insulin stimulated phosphorylation of its receptor 5.7 ± 0.5 -fold and EGF 2.9 ± 0.4 -fold.

^{125}I -labelled insulin binding assay

CHOT cells were plated in 24-well plates and grown to confluence. Cells were washed twice in buffer A and then incubated in 1 ml of this buffer containing 20 000 c.p.m. ^{125}I -labelled insulin (140 $\mu\text{Ci}/\mu\text{g}$) in the presence or absence of the various sulphhydryl agents for 15 min at 22 °C. At the end of the incubation period cells were washed three times in ice-cold buffer A and solubilized in 0.5 ml of PBS containing 0.2% Triton X-100. Bound radioactivity was measured in an autogamma spectrometer. Non-specific binding was determined by incubation of cells in 10 $\mu\text{g/ml}$ unlabelled insulin and was always less than 10% of the total binding.

RESULTS

Reduction of insulin and EGF receptors by GSH

GSH is cell impermeant (Meister and Anderson, 1983) and, when added to intact CHOT cells, had no effect on insulin binding (Table 1), receptor structure, as defined by a change in receptor mobility (Figure 1, lane B), or kinase activity, as measured by receptor autophosphorylation (Table 2). Even when the concentration of GSH was increased to 50 mM no effect on receptor kinase activity or receptor structure was apparent (results not shown).

Removal of the insulin receptor from its cellular environment produced a significant change in the response to GSH. When

Table 1 Insulin binding to intact CHOT cells in the presence of various sulphhydryl-active agents

Specific ^{125}I -insulin binding to intact CHOT cells was measured in the presence or absence of the sulphhydryl agents for 15 min at 22 °C as described in the Experimental section. Results are the means $\pm$ S.E.M. of triplicates from two separate experiments.

Agent	Specific binding (% of control)
GSH	99 $\pm$ 4
DTT	104 $\pm$ 9
NEM	85 $\pm$ 4
IAM	87 $\pm$ 3

Figure 1 Structure of the insulin receptor after treatment with GSH or DTT in intact cells or after receptor isolation

CHOT cells were treated with (lanes A–C, H–K) or without (lanes D–G) insulin (1 $\mu\text{g/ml}$) for 10 min at 37 °C. Cells were either left intact (lanes A–C) or receptors isolated by immunoprecipitation (lanes D–K) and then treated without (lanes A, D and H) or with 1 mM GSH (lanes B, E and I) or 50 mM GSH (lanes F and J) or with 1 mM DTT (lanes C, G and K) for 10 min at 22 °C. After immunoprecipitation of all receptors autophosphorylation was measured by addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as described in the Experimental section. Non-reducing sample buffer was added and proteins separated by 5% SDS/PAGE. Receptor bands were visualized by autoradiography.

Table 2 Autophosphorylation of insulin or EGF receptors treated with GSH in intact cells

CHOT cells were treated with insulin (1 $\mu\text{g/ml}$) and A431 cells with EGF (0.5 $\mu\text{g/ml}$) for 10 min at 37 °C and then with GSH (1 mM) for a further 10 min at 22 °C. Receptors were immunoprecipitated, autophosphorylated and proteins separated by SDS/PAGE as described in the Experimental section. After visualization by autoradiography, receptor bands were excised and the radioactivity in each band measured by Cerenkov counting. The results are expressed as a percentage of the autophosphorylation obtained in the absence of GSH and are the means $\pm$ S.E.M. of at least four individual experiments.

	Autophosphorylation (%)	
	Insulin receptor	EGF receptor
Basal	100 $\pm$ 11	98 $\pm$ 7
Ligand activated	128 $\pm$ 23	109 $\pm$ 3

Table 3 Autophosphorylation of isolated insulin or EGF receptors treated with GSH or DTT

CHOT or CHO Δ CT69 cells were treated with insulin (1 μ g/ml) and A431 cells with EGF (0.5 μ g/ml) for 10 min at 37 °C. Receptors were isolated as described in the Experimental section and treated with or without GSH (1 mM) or DTT (1 mM). The IRK protein was incubated with or without GSH (1 mM) or DTT (1 mM) for 10 min at 22 °C. Receptors were autophosphorylated and quantified as described in Table 2. Results are the means $\pm$ S.E.M. of at least four individual experiments. Statistical significance: * P < 0.05, ** P < 0.01.

Receptor	Autophosphorylation (%)	
	1 mM GSH	1 mM DTT
Insulin receptor		
Basal:		
Wild type	205 $\pm$ 37*	142 $\pm$ 17*
Δ CT69	174 $\pm$ 32*	—
Insulin activated:		
Wild type	72 $\pm$ 6*	162 $\pm$ 20*
Δ CT69	79 $\pm$ 9*	—
Insulin receptor kinase	50 $\pm$ 10**	108 $\pm$ 6
EGF receptor		
Basal	234 $\pm$ 39*	105 $\pm$ 9
EGF-activated	127 $\pm$ 36	107 $\pm$ 20

Figure 2 Incubation of insulin receptors with GSH either before or after stimulation with insulin

CHOT cells were lysed and insulin receptors immunoprecipitated as described in the Experimental section. Isolated receptors were incubated with buffer (lanes A, D and G) or 1 mM GSH (lanes B, E and H) or 10 mM GSH (lanes C, F and I) for 15 min at 22 °C either before (lanes G, H and I) or after (lanes D, E and F) insulin or buffer (lanes A, B and C). After autophosphorylation, proteins were separated by 5% SDS/PAGE and bands visualized by autoradiography.

isolated receptors were treated with 1 mM GSH, in the absence of insulin stimulation, the receptor kinase activity was significantly enhanced (Table 3), but without conversion of the dimeric insulin receptor to the monomer (Figure 1, lane E). The insulin-receptor Class I disulphides have been shown previously to be highly resistant to reduction by GSH (Chiacchia, 1988). We also find that one has to reach a concentration of 50 mM GSH before observable dissociation of the insulin receptor occurs (Figure 1, lanes F and J), and this does not correlate with stimulation of kinase activity by GSH. In direct contrast with the results with non-stimulated receptors, the kinase activity of insulin-stimulated receptors was significantly (30%) inhibited by 1 mM GSH (Table 3), again without visible change to receptor structure (Figure 1, lane I). These results, where the effects of GSH were observed only with isolated receptors, suggest that the site of action of GSH is cytoplasmic and was investigated further with the constitutively active IRK protein which consists solely of the cytoplasmic domain of the insulin receptor. When the IRK

protein was treated with 1 mM GSH a significant (50%) inhibition of the kinase activity was observed, analogous to the results with insulin-stimulated but not basal insulin receptors (Table 3). These results confirm that the site of action of GSH is probably within the cytoplasmic domain of the receptor. Additionally, it appears that the basal and activated states (insulin-stimulated or constitutive IRK protein) of the receptor kinase are not equivalent with respect to the actions of GSH, i.e. basal autophosphorylation is enhanced by GSH whereas insulin-stimulated and IRK autophosphorylations are inhibited. This difference was further demonstrated by pre-treating isolated insulin receptors with GSH before addition of insulin (i.e. receptors in the basal state). Under these conditions no inhibition of the insulin-activated autophosphorylations was observed at either 1 mM or 10 mM GSH (Figure 2, lanes H and I); indeed insulin activated autophosphorylation to the same level as in the absence of GSH.

Changes in receptor conformation upon activation could explain these results, and the C-terminal domain of the insulin receptor is one region that has been shown to undergo such changes (Baron et al., 1990). We investigated the effect of GSH on a mutant insulin receptor (Δ CT69) truncated at amino acid 1286 (Chin et al., 1991), which lacks cysteine residue 1308. This mutant responded to GSH in a similar fashion to wild-type receptors, with stimulation of basal and inhibition of insulin-activated autophosphorylation (Table 3).

The EGF receptor, being a single polypeptide, has no interchain disulphide bonds. The structure of the EGF receptor in SDS/PAGE is thus unaffected by reduction under all conditions (not shown) and, although the EGF receptor may dimerize upon activation there is no evidence that this occurs via disulphide-bond formation (Cochet et al., 1988). Addition of GSH or DTT to intact EGF-receptor-expressing A431 cells had no effect on receptor kinase activity (Table 2). However, once the basal EGF receptor had been isolated from the cell, similar results to isolated insulin receptors were obtained; basal EGF-receptor autophosphorylation was stimulated by 1 mM GSH (Table 3). The kinase activity of isolated EGF receptors that had been activated by EGF was not significantly stimulated by GSH (Table 3), again suggestive of a difference between the basal and EGF-activated state. None of the sulphhydryl-active agents had any effect on EGF binding (results not shown).

As observed previously (Wilden et al., 1986), and confirmed here with insulin receptors from CHOT cells, DTT stimulates the kinase activity of both basal and insulin-activated receptors (Table 3) and is associated with receptor reduction (Figure 1, lanes G and K). This effect is probably entirely due to its actions on the external domain of the insulin receptor, as DTT (1 mM) was without effect on either the IRK protein or basal or EGF-stimulated EGF receptor (Table 3).

Alkylation of receptors

Our results with GSH indicate that basal and activated (insulin or EGF) receptor kinase react qualitatively differently (stimulation compared with inhibition), whereas previous studies (Wilden and Pessin, 1987), with NEM, suggested a quantitative effect (inhibition of basal autophosphorylation, IC_{50} 73 μ M compared with 25 μ M with insulin). We decided to study NEM in our system along with a second alkylating agent, IAM. Both reagents can alkylate and block free sulphhydryl groups, but previous results show that their effects are opposing (Wilden and Pessin, 1987; Li et al., 1991).

Insulin receptors isolated from basal and insulin-activated CHOT cells or the soluble IRK protein show stimulation of

Figure 3 Protection by IAM of Insulin receptor, IRK protein and EGF receptor from NEM-induced inhibition of autophosphorylation

(a) Isolated insulin receptors that had been treated with (insulin-stimulated) or without (basal) insulin, as described in Table 2, or the IRK protein were incubated with IAM (solid bars), NEM (open bars) or IAM followed by NEM (hatched bars) as described in the Experimental section. The phosphorylated receptors were prepared and quantified as described in Table 2. Proteins were separated by reduced SDS/PAGE. Results have been normalized in each panel to the activity in the absence of any sulphhydryl agent (100%) and are the means $\pm$ S.E.M. for at least three individual experiments. (b) As described for (a) except that EGF receptors isolated from A431 cells and treated with (EGF-stimulated) or without (basal) EGF were examined.

autophosphorylation after treatment with IAM (Figure 3a). In contrast, and as reported previously (Wilden and Pessin, 1987), NEM inhibits autophosphorylation of basal or insulin-activated receptors and also the IRK protein (Figure 3a). Although IAM and NEM inhibit insulin binding this effect is minor and could not account for their effects on autophosphorylation (Table 1). The results with the IRK protein suggest that the actions of both IAM and NEM are localized within the cytoplasmic domain of the receptor, although perhaps not at the same sites. We examined the interaction between these two agents by initially treating insulin receptors with IAM, before the addition of NEM, to see if pre-alkylation with IAM would protect against the inhibitory effects of NEM. When the receptor kinase is activated IAM pretreatment cannot protect against the effects of NEM. The insulin-stimulated receptors or the IRK protein pre-treated with IAM are protected only marginally (20%) from the effects of NEM (Figure 3a). In contrast, when receptors with basal kinase activity are treated with IAM, before addition of NEM, the inhibitory effect of NEM is lost and the receptors show almost the full activation observed with IAM alone (Figure 3a). Fur-

thermore, if these receptors with basal activity are treated with NEM before incubation with IAM autophosphorylation is inhibited to the same extent as with NEM alone (results not shown). These results again suggest that the basal and activated kinase respond qualitatively differently. Similar results could be shown for EGF receptor where IAM pre-treatment of receptors with basal kinase activity protected against the inhibitory effects of NEM but only partial protection was afforded to EGF-stimulated receptors (Figure 3b).

DISCUSSION

Our results lend further support to the important role played by sulphhydryls/disulphides in insulin receptor and, for the first time, EGF-receptor function. We would suggest that most of these effects can be localized to the intracellular domain of the receptors, despite the high cysteine content of the external domain and, for insulin receptors, the presence of interchain disulphide bonds. We have demonstrated that the physiologically relevant reductant, GSH, alters both basal and activated receptor autophosphorylation via modulation of the intracellular domain of insulin- or EGF-receptors and suggest that some receptor kinases could be modulated by physiological or pathological conditions which change GSH levels. GSH produced similar effects on autophosphorylation of insulin- and EGF-receptors and inhibited autophosphorylation of IRK as for insulin-activated receptors. These effects of GSH contrast with those of DTT which appears to react with the extracellular domain of the insulin receptor to stimulate receptor autophosphorylation. DTT failed to alter the autophosphorylation of the IRK protein, which has no extracellular domain and no interchain disulphide bonding. Furthermore, DTT did not stimulate EGF-receptor autophosphorylation. The EGF receptor while sharing much sequence similarity with the insulin receptor, in both the tyrosine kinase domain and to some degree the organizational structure of the external domain (Bajaj et al., 1987), does not possess any interchain disulphide bonds. The contrasting effects of GSH and DTT may be due to the difference in oxidation potential of the two reagents (Gilbert, 1990) with 1 mM DTT being able to effect changes in receptor structure, observable on SDS/PAGE, whereas GSH has minimal effects even at 50 mM on receptor structure. In addition, GSH, being a monothiol, is able to form mixed disulphides with proteins, whereas DTT, a dithiol with a much greater oxidation potential leads to complete reduction of disulphides.

The mechanism of action of GSH upon the intracellular domain of the receptor is unclear. It is unlikely that any cytoplasmic interchain disulphide bonds exist within the reducing environment of the cell. While there is no direct evidence for cytoplasmic intrachain disulphide bonds, these could theoretically exist where two cysteine residues are held in close proximity (Creighton, 1984). Of the ten β -subunit cysteine residues, one is postulated to exist as a free thiol group (Finn et al., 1990; Chiacchia, 1991) and is probably located close to the ATP-binding site of the kinase domain (Finn et al., 1990). Under drastic reducing conditions only two additional cysteine residues could be labelled with [3 H]NEM (Finn et al., 1990; Chiacchia, 1991). Although this could represent reduction of an intrachain disulphide, presumably one of these residues is the external β -subunit cysteine involved in Class II disulphide bonds with the α -subunit. Thus potentially only one of the remaining five intracellular cysteine residues can be reduced and labelled, and indeed this cysteine residue may not be intracellular but extracellular. It is not clear whether [3 H]NEM is actually labelling all available thiol groups. As yet no confirmatory studies have

appeared with a second labelling agent such as IAM. In all of these studies it should be noted that isolation of the receptor may result in alterations to disulphide configuration in the oxidizing environment of the test tube (which may also affect our results with sulphhydryl agents) (Xu et al., 1990). Therefore in the absence of intracellular disulphide bonds how may GSH be acting, and in what manner can it be different from DTT? From the reactions that GSH can undergo we postulate that formation of mixed disulphides between GSH and one or more cysteine residues of the intracellular domain of both insulin- and EGF-receptors results in the effects we have observed.

Of interest is our observation that GSH has opposing effects on basal compared with insulin/EGF-activated autophosphorylation. Inhibition of ligand-activated autophosphorylation by GSH is not due to inhibition of ligand binding (Table 1) and is also observed with the constitutively activated IRK protein, which lacks a ligand-binding domain. Earlier studies with sulphhydryl-active agents, including NEM, have shown a greater sensitivity to inhibition in the insulin-activated compared with the basal state, suggestive of a differential response (Wilden and Pessin, 1987). Our results suggest that a conformational change in the receptors upon activation may be altering their reactivity to GSH. This is further highlighted when insulin receptors are treated with GSH first before insulin. In this situation GSH does not inhibit insulin-activated autophosphorylation, presumably because GSH reacted with the basal conformation of the receptor. There are several studies demonstrating conformational changes in the insulin receptor upon activation. First, antibodies to the autophosphorylation domain can discriminate between the phosphorylated and non-phosphorylated forms of the receptor (Herrera and Rosen, 1986; Perlman et al., 1989). This is not due merely to the presence of phosphotyrosine as one antibody could recognize both forms equally well by immunoblotting but could only immunoprecipitate phosphorylated receptor, suggesting a conformational change of the native receptor (Herrera and Rosen, 1986). This type of conformational change, however, may not be relevant to our studies as the GSH is added before the autophosphorylation reaction commences. A second set of antibodies can distinguish between the basal and insulin-bound states of the receptor (Baron et al., 1990). These antibodies have epitopes within the C-terminus of the β -subunit (residues 1306–1338), including a cysteine residue at 1308; their reactivity is decreased once insulin is bound to the receptor independently of any receptor phosphorylation. Our results with a mutant receptor which contains a deletion (residues 1287–1355) removing cysteine 1308 suggest that this residue is not involved in the GSH effects as this mutant gave similar autophosphorylation results to wild-type receptors. However, a receptor with a point mutation at cysteine 1233 showed GSH-dependent stimulation of both basal and insulin-activated autophosphorylation (L. Macaulay, M. Haritou, M. Frenkel and C. Ward, personal communication), implicating this residue in the GSH effects. Thus it appears likely that as well as inducing conformational changes within the external α -subunit, as might be expected upon insulin binding, conformational changes are also transmitted through to the cytoplasmic domain of the β -subunit. These changes could lead to the differential effects of GSH on basal versus insulin-activated receptors that we observe. One could propose similar conformational changes occurring in the EGF receptor upon ligand binding.

Our studies with NEM and IAM have led to two main findings. First, we have demonstrated that these two agents, which theoretically undergo the same type of reaction to block free thiol groups, have opposing effects on receptor

autophosphorylation. This can be explained either by the different chemical reactions that these reagents undergo [nucleophilic addition to the double bond (NEM) has stereochemical and mechanistic features different from those for S_N2 displacement (IAM)], or by the difference in their molecular size (Liu, 1977). NEM is a large bulky group which may block reactivity of nearby residues, unlike the smaller IAM molecule. Thus the inhibition of kinase activity by NEM could be due either to blocking of a reactive sulphhydryl (less likely, as IAM should then give the same result) or to steric hindrance via binding to a sulphhydryl close to other reactive groups such as the ATP-binding site (Finn et al., 1990). IAM has been reported previously to have no effect on insulin-receptor autophosphorylation (Wilden and Pessin, 1987), or, when liver receptors are prepared in IAM-containing buffers, a stimulation of autophosphorylation is observed (Li et al., 1991). In these studies IAM also stimulated autophosphorylation of control membranes. IAM (being a much smaller molecule) may bind, but not sterically hinder, nearby groups and may in fact protect receptor sulphhydryls, resulting in enhanced kinase activity. Bauskin et al. (1991) have demonstrated IAM-stimulated autophosphorylation of the Ltk tyrosine kinase, which correlated with the appearance of Ltk disulphide-linked multimers. Ltk is an endoplasmic transmembrane protein kinase with similarity to the insulin-receptor cytoplasmic domain. It is also possible that IAM is reacting with groups other than cysteine residues, as it is the least specific of the reagents. However, under the conditions of our experiments (short incubation times and neutral pH) additional reactions should be minimized.

Secondly, when we use the two alkylating agents in tandem we again demonstrate differential reactivity of basal and activated receptors. This is a more subtle effect than seen with GSH, as it is only observed when we attempt to protect insulin- and EGF-receptors from NEM inhibition by pre-treatment with IAM. If IAM binds to the same residue as NEM it should protect the receptor from subsequent NEM inhibition when added first. This is observed for receptors in their basal state, but not for insulin- or EGF-activated receptors or the activated IRK protein, where only minimal protection is afforded. This suggests that IAM may react at a separate site to NEM and only in the basal configuration of the receptor does IAM prevent NEM from also binding to the receptor. As we found similar results with EGF receptor one might propose similar mechanisms for both receptors. To date insulin-receptor labelling studies have not been performed with [^{14}C]IAM to ascertain how it compares with [^3H]NEM labelling and whether ATP and Mn can prevent such labelling.

Finally, our results suggest that the IRK protein assumes the same conformation as the activated insulin receptor and that this is different from non-stimulated receptors. In all of the studies reported here the IRK protein responds in the same way as the insulin-activated intact receptor. If insulin activation results in a conformational change in the cytoplasmic domain of the receptor then, in the presence of Mg^{2+} and protamine, the IRK protein probably has a similar conformation. Previous results show similar phosphorylation patterns (Tavaré et al., 1991) and kinetics (Cobb et al., 1989) for the IRK protein compared with intact receptor and we now show similar reactivity with several sulphhydryl-active agents. The only exception is DTT, but, as discussed earlier, it is our contention that DTT reacts with the external domain of the insulin receptor, which is not present in the IRK protein.

The interaction of sulphhydryl-reactive agents with insulin- and EGF-receptors is obviously complex. However, we have shown that the predominant site of reactivity is the intracellular domain and that activated and non-activated receptors respond

differently. Mutational analysis of the individual cysteine residues may allow dissection of the reactions involved.

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REFERENCES

- Bajaj, M., Waterfield, M. D., Schlessinger, J., Taylor, W. R. and Blundell, T. (1987) *Biochim. Biophys. Acta* **916**, 220–226
- Baron, V., Gautier, N., Komoriya, A., Hainaut, P., Scimeca, J.-C., Miljenko, M., Lavielle, S., Dolais-Kitabgi, J. and Van Obberghen, E. (1990) *Biochemistry* **29**, 4634–4641
- Bauskin, A. R., Alkalay, I. and Ben-Neriah, Y. (1991) *Cell* **6**, 685–693
- Chen, J.-J., Kosower, N. S., Petryshyn, R. and London, I. M. (1986) *J. Biol. Chem.* **261**, 902–908
- Chiacchia, K. B. (1988) *Biochemistry* **27**, 4894–4902
- Chiacchia, K. B. (1991) *Biochem. Biophys. Res. Commun.* **176**, 1178–1182
- Chin, J. E., Tavaré, J. M., Ellis, L. and Roth, R. A. (1991) *J. Biol. Chem.* **266**, 15587–15590
- Clark, S., Cheng, D. J., Hsuan, J. J., Haley, J. D. and Waterfield, M. D. (1988) *J. Cell. Physiol.* **134**, 421–428
- Cobb, M. H., Sang, B.-C., Gonzalez, R., Goldsmith, E. and Ellis, L. (1989) *J. Biol. Chem.* **264**, 18701–18706
- Cochet, C., Kashles, O., Chambaz, E. M., Borrello, I., King, C. R. and Schlessinger, J. (1988) *J. Biol. Chem.* **263**, 3290–3295
- Creighton, T. E. (1984) *Methods Enzymol.* **107**, 305–351
- Czech, M. P. (1976) *J. Biol. Chem.* **251**, 1164–1170
- Ellis, L., Levitan, A., Cobb, M. H. and Ramos, P. (1988) *J. Virol.* **62**, 1634–1639
- Finn, F. M., Ridge, K. D. and Hofmann, K. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 419–423
- Fujita-Yamaguchi, Y. and Kathuria, S. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6095–6099
- Ganderton, R. H., Stanley, K. K., Field, C. E., Coghlan, M. P., Soos, M. A. and Siddle, K. (1992) *Biochem. J.* **288**, 195–205
- Gilbert, H. F. (1990) *Adv. Enzymol.* **63**, 69–172
- Hercbergs, A., Brok-Simoni, F., Holtzman, F., Bar-Am, J., Leith, J. T. and Brenner, H. J. (1992) *Lancet* **339**, 1074–1076
- Herrera, R. and Rosen, O. M. (1986) *J. Biol. Chem.* **261**, 11980–11985
- Kanner, S. B., Kavangh, T. J., Grossmann, A., Hu, S.-L., Bolen, J. B., Rabinovitch, P. S. and Ledbetter, J. A. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 300–304
- Li, C., Moule, M. L. and Yip, C. C. (1991) *J. Biol. Chem.* **266**, 7051–7057
- Liu, T.-Y. (1977) *The Proteins* (Neurath, H. and Hill, R. L., eds.), vol. 3, 240–404, Academic Press, New York
- Ludvigsen, C. and Jarett, L. (1982) *Metabolism* **31**, 284–287
- Massague, J. and Czech, M. P. (1982) *J. Biol. Chem.* **257**, 6729–6738
- McLennan, S. V., Heffernan, S., Wright, L., Rae, C., Fisher, E., Yue, D. K. and Turtle, J. R. (1991) *Diabetes* **40**, 344–348
- Meister, A. and Anderson, M. E. (1983) *Annu. Rev. Biochem.* **52**, 711–760
- Perlman, R., Bottaro, D. P., White, M. F. and Kahn, C. R. (1989) *J. Biol. Chem.* **264**, 8946–8950
- Savage, C. R., Jr. and Cohen, S. (1972) *J. Biol. Chem.* **247**, 7609–7611
- Sweet, L. J., Morrison, B. D., Wilden, P. A. and Pessin, J. E. (1987) *J. Biol. Chem.* **262**, 16730–16738
- Tavaré, J. M., Clack, B. and Ellis, L. (1991) *J. Biol. Chem.* **266**, 1390–1395
- Wilden, P. A. and Pessin, J. E. (1987) *Biochem. J.* **245**, 325–331
- Wilden, P. A., Boyle, T. R., Swanson, M. L., Sweet, L. J. and Pessin, J. E. (1986) *Biochemistry* **25**, 4381–4388
- Xu, Q.-Y., Paxton, R. J. and Fujita-Yamaguchi, Y. (1990) *J. Biol. Chem.* **265**, 18673–18681
- Zick, Y. (1989) *CRC Crit. Rev. Biochem. Mol. Biol.* **24**, 217–269