In this study, we found that adding iodoacetamide to the homogenization buffer used in the preparation of mouse or rat liver plasma membranes resulted in an increase of insulin receptor autophosphorylation by 4-5-fold and receptor kinase activity by about 2-fold. Similar effects were obtained with iodoacetate and p-chloromercuriphenyl sulfonate. The effect of iodoacetamide was minimal when it was added to membranes prepared without the thiol reagent. The enhancing effect of iodoacetamide on insulin receptor autophosphorylation was the result of a more than 2-fold decrease in the K_m and a more than 3-fold increase in V_max for ATP. The presence of iodoacetamide in the preparation of plasma membranes also greatly increased the solubilization of the insulin receptor from the plasma membrane by Triton X-100. We propose that iodoacetamide acts to alkylate some unknown thiols released during tissue homogenization and that in its absence these thiols form mixed disulfides with the insulin receptor, thus adversely affecting the process of receptor activation by insulin.

The insulin receptor is a member of a family of receptors possessing ligand-dependent tyrosine kinase activity. This family includes receptors for epidermal growth factor (1), insulin-like growth factor I (2), and platelet-derived growth factor (3). It is generally accepted that insulin binding to the extracellular α subunit of its heterotetrameric (α2β2) receptor leads to the tyrosine phosphorylation of the receptor β subunit as a prerequisite for the expression of tyrosine kinase activity (reviewed in Refs. 4 and 5). Although the sites of tyrosine phosphorylation have been extensively studied (6-8), the mechanism through which the binding of insulin to the α subunit activates the phosphorylation of the β subunit remains unknown. However, it has been established that the heterotetrameric form of the receptor is required for insulin-induced receptor phosphorylation and for the expression of maximal receptor kinase activity (9, 10). Receptor phosphorylation and receptor kinase activity of solubilized and purified receptor are stimulated by the disulfide reducing agent, dithiothreitol at low concentrations (9, 11-14). Under these conditions, although the disulfides linking the two heterodimers, αδ, are reduced, the two heterodimers remain associated (13, 14). In contrast, the sulphydryl alkylating agent N-ethylmaleimide inhibits insulin receptor phosphorylation and receptor kinase activity (9, 15, 16). The inhibition by N-ethylmaleimide is more effective in the presence than in the absence of insulin, suggesting that one or more critical thiols in the receptor has become more susceptible to the alkylating agent in the presence of the ligand (16). These observations strongly implicate an important role for receptor thiols and disulfides in the mechanism of insulin activation of receptor phosphorylation and receptor kinase. So far, the effects of thiol and disulfide reducing agents have been studied by the addition of these agents in vitro to receptors isolated from the tissues. However, it is possible that during the process of receptor preparation the insulin receptor is exposed to intracellular thiols which alter the thiol state of the receptor, thus changing the response of the receptor to insulin when studied in vitro. In this communication, we report that the sulphydryl alkylating reagent iodoacetamide greatly enhanced insulin-stimulated receptor phosphorylation and kinase activity when it was added at the time of membrane preparation.

**EXPERIMENTAL PROCEDURES**

Materials—The following reagents were obtained from the respective suppliers: Sigma: leupeptin, pepstatin, phenylmethylsulfonyl fluoride, poly(Glu-Tyr) (4:1), ATP, bacitracin, bovine serum albumin, sodium orthovanadate, Triton X-100, and iodoacetamide; Fisher: benzamide, and NaF; Boehringer Mannheim: aprotinin; BDH Chemicals Canada: ethylenediamine tetraacetic acid disodium salt and sodium molybdate; Pharmacia LKB Biotechnology Inc.: Sepharose-4B; Protein A; ICN Biomedicals Canada, St. Laurent, Canada: dithiothreitol and [γ-32P]ATP (7000 Ci/mmol). Crystalline bovine insulin was a gift from Connaught Novo, Toronto, Canada.

Preparation of Liver Plasma Membranes—Liver tissues from male Wistar rats (100-150 g body weight) and 7-8-week-old female C57 mice were used for the preparation of plasma membranes by the method of Neville (17) to Step 1. The buffer used for homogenization was 50 mM Tris-HCl, pH 7.4, containing benzamidine (10 mM), bacitracin (0.08%), aprotinin (6 μg/ml), PMSF* (1 mM), pepstatin (2 μM), and leupeptin (2 μM). Iodoacetamide, when used, was added to the buffer to 5 mM except where indicated otherwise. The membranes obtained were pelleted in aliquots by centrifugation and stored at -70 °C. Protein content was determined by the method of Markwell (18), using bovine serum albumin as standard.

Receptor Phosphorylation—Liver plasma membranes were solubilized for 90 min in ice-cold buffer containing 50 mM Tris-HCl, pH 7.5, 5 mM Trition X-100, 2 μM leupeptin, 2 μM pepstatin, 10 mM benzamidine, 1 mM PMSF, bacitracin (1 mg/ml), and aprotinin (70 μg/ml). The buffer also contained 150 mM NaCl if the solubilized membrane proteins were used directly for immunoprecipitation of the insulin receptor. In experiments where receptor phosphorylation was assayed, 90-150 μg of membrane protein were solubilized in 20-50 μl of solubilization buffer. The membrane suspension was centrifuged for 5 min at 133,000 × g in an air centrifuge.

Receptor phosphorylation was measured using either one of two methods. In Method I, insulin receptors in the solubilized supernatant were precipitated with protein A-Sepharose 4B conjugated to anti-insulin receptor antibodies. The precipitated complexes were washed and assayed for receptor phosphorylation. In Method II, the solubilized membrane preparations were used directly for immunoprecipitation in a similar manner. The receptor complexes were washed and assayed for receptor phosphorylation in the presence of 0.1 mM ATP, 100 μM MgCl_2, 5 mM NaF, 5 mM DTT, and 5 μg/ml bacitracin. These conditions were used to achieve optimal receptor phosphorylation in the presence of insulin. The receptor complex was then fractionated by SDS-PAGE on 4-16% gels, and the expression of receptor kinase activity was determined by autoradiography.
were first precipitated by incubating an aliquot of the supernatant with 7.5 μl (90 μg protein) of an anti-receptor immunoglobulin (ARS-2) in a final volume of 100 μl at 4 °C overnight, followed by the addition of 7 mg of Protein A-Sepharose beads suspended in 50 μl of Tris-buffered saline (TBS). After incubation for 90 min at 4 °C, during which time the beads were kept in suspension by rotation, the beads were pelleted by centrifugation for 5 min in a microcentrifuge. The pellet was then washed three times with TBS/Triton (0.1%) containing 1 mM PMSF. The pellet was then incubated with 20 μl of 8.7 x 10^{-7} M insulin in the TBS/Triton buffer for 20 min at room temperature. Phosphorylation was initiated by the addition of 45 μl of a solution containing 8 mM MgCl₂, 67 mM Tris-HCl, pH 7.5, 29 μCi of [γ-32P]ATP. The concentration of ATP was 6 μM unless otherwise indicated. After 15 min at room temperature, the reaction was stopped by adding 0.25 ml of a solution containing 80 mM Tris-HCl, pH 6.8, 0.45 M DTT, 56 mM EDTA, 30% sucrose, and 6% SDS, followed by boiling for 5 min. The supernatant obtained after centrifugation for 5 min in a microfuge was saved for SDS-PAGE (19) in 7.5% gel and radioautography. The radioactive β subunit bands were excised for the determination of radioactivity by Cherenkov counting. In Method II, 10 μl of 50 mM Tris-HCl buffer, pH 7.5, 10 μl of 80 mM MnCl₂, in 400 mM Tris-HCl buffer, pH 7.5; and 20 μl of 2 μM insulin were added to 20 μl of the solubilized membrane supernatant containing up to 150 μg of protein. After 20 min at room temperature, 20 μl of a stock solution were added to give a final concentration of 10 mM sodium molybdate, 10 mM sodium fluoride, 2 mM sodium orthovanadate, and 67 μM ATP containing 29 μCi of [γ-32P]ATP. The phosphorylation reaction was terminated after 15 min at room temperature by the addition of 40 μl of 35 mM EDTA and 6.7 μl of 1.5 M NaCl. Insulin receptors were immunoprecipitated and analyzed by SDS-PAGE, radioautography, and determination of radioactivity as described for Method I. The amounts of solubilized membrane protein used from different membrane preparations were adjusted for equal insulin-binding capacity, which was determined by Scatchard analysis of binding of [125I]Tyr²²-insulin, as described previously (20), and by radioactivity labeling described below.

Receptor kinase activity was assayed using the synthetic peptide poly(Glu-Tyr) (4:1) as substrate (21). The solubilized membrane preparation, 10 μl containing 25–75 μg of total protein, was incubated with 30 μl of 600 nM insulin in TBS containing 1% bovine serum albumin or with 30 μl of buffer alone at 24 °C for 20 min. Receptor phosphorylation was initiated by the addition of 30 μl of a solution containing 8 mM ATP and 10 mM MnCl₂. After 30 min, 7.5 μl of ARS-2 anti-receptor immunoglobulin was added, and the reaction mixture was incubated at 4 °C overnight in a total volume of 40 μl containing 6 mM NaF, 1.2 mM sodium orthovanadate, 6 mM sodium molybdate, and 1% Triton. Protein A-Sepharose beads were used to precipitate the receptor-antibody complex as described above. The beads were washed three times with 1 ml of TBS-Triton (0.2%) containing 1 mM PMSF. The beads were then incubated at room temperature for 15 min with 10 mM poly(Glu-Tyr), 12 mM MnCl₂, and 10 μM ATP containing 15 μCi of [γ-32P]ATP in a total volume of 40 μl. The reaction was terminated by adding 8 μl of 5 mM ATP and centrifuged in a microcentrifuge for 5 min. Aliquots (15 μl) of the supernatant were spotted onto Whatman P-81 paper which was then washed twice with 10% trichloroacetic acid containing 10 mM sodium pyrophosphate, once with 5% trichloroacetic acid, once with ethanol, and once with ether and then dried. Radioactivity remaining on the filter paper was determined by liquid scintillation counting.

**Results**

**Effect of Iodoacetamide on Insulin Receptor Solubilization**

Data presented in Table I show that while the total photolabeling of insulin receptors was not significantly different between mouse liver membranes prepared without iodoacetamide and membranes prepared with iodoacetamide, the amount of photolabeled insulin receptors solubilized by 1% Triton was about 2.8-fold higher with membranes prepared in the presence of iodoacetamide and proportionally more photolabeled receptor was recovered in the Triton-insoluble pellet with membranes prepared without iodoacetamide. In the case of rat liver plasma membranes, about 2.9-fold more receptor was solubilized from membranes prepared with iodoacetamide (data not shown). This differential effect of iodoacetamide on receptor solubilization was also confirmed by direct insulin binding (data not shown).

**Effect of Iodoacetamide on Receptor Autophosphorylation**

The radioautograms presented in Fig. 1 show that when plasma membranes were prepared from rat or mouse liver in the presence of 5 mM iodoacetamide, basal and insulin-stimulated autophosphorylation of insulin receptors solubilized from these membranes was greatly enhanced when assayed by Method II. In this method, autophosphorylation of the insulin receptor with or without insulin was carried out before the separation of the receptor by immunoprecipitation. Fig. 2A demonstrates that the effect of iodoacetamide also was observed using Method I, in which the insulin receptor was immunoprecipitated before autophosphorylation in the presence or absence of insulin. Under these conditions, in the case of mouse liver plasma membranes, insulin-stimulated receptor autophosphorylation was 4–5 times higher with membranes prepared with iodoacetamide. The effect was less with rat liver plasma membranes. The relatively high degree of auto-

**Table I**

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**Fig. 1.** Radioautograms showing the effect of iodoacetamide on insulin receptor autophosphorylation. Plasma membranes were prepared from mouse (upper panel) or rat (lower panel) liver plasma membranes. 4-5 times higher with membranes prepared with iodoacetamide. The effect was less with rat liver plasma membranes. The relatively high degree of auto-

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phosphorylation obtained in the absence of insulin was due
to the insulin-mimicking action of the anti-receptor immu
noglobulin ARS-2. Other thiol reagents including
PCMPs, NEM, and DTNB were also tested for their ability
to enhance receptor autophosphorylation when added to
the homogenization buffer. Only PCMPs was found to be effective (Fig. 2B). The addition of DTNB caused a decrease in
receptor autophosphorylation. The increased receptor auto-
phosphorylation produced by the presence of iodoacetamide
during homogenization occurred entirely on tyrosine residues
as shown by phosphoamino acid analysis (Fig. 3). Iodoacetate
showed a stimulatory effect similar to that produced by iodo-
acetamide (Fig. 4A).

It has been reported that the presence of iodoacetamide or
EDTA during homogenization inhibited the degradation of
the β subunit to a 84-kDa fragment and allowed the receptor
to retain its normal activity of autophosphorylation (23). We
therefore carried out experiments to study the effects of the
presence of various protease inhibitors in the homogenization
buffer on receptor autophosphorylation and the appearance of
the 84-kDa fragment. Data presented in Fig. 4B show that in
the absence of protease inhibitors iodoacetamide alone
failed to inhibit the formation of the 84-kDa fragment and
that any three of the four protease inhibitors (bacitracin, benzamidine, leupeptin, and pepstatin) routinely used to-
gether with PMSF prevented the formation of the fragment.
The 84-kDa fragment was detected when all four protease
inhibitors were omitted from the homogenization buffer.
Furthermore, the addition of EDTA to the homogenization buffer
had little or no effect on increasing receptor autophospho-
rylation (Fig. 4A).
The effect of iodoacetamide was concentration-dependent
(Fig. 5). In the case of rat insulin receptor, more than 60% of
the maximal effect was obtained at an iodoacetamide concen-
tration of 0.5 mM. A higher concentration (1 mM) was required
to produce the same effect in the insulin receptor of the
mouse. In both cases, the effect reached a plateau when the
concentration of iodoacetamide was higher than 5 mM. The
enhancing effect of iodoacetamide was not duplicated by

Fig. 2. A. insulin-stimulated autophosphorylation of insulin receptor from mouse or rat liver plasma membranes prepared in the absence (C) or presence (IAM) of iodoacetamide. Assay of autophosphorylation in the presence of 15 μM ATP was carried out by Method I as described in the text. The extent of phosphorylation is expressed as radioactivity (CPM) incorporated into the receptor β subunit. The assay was done in triplicate, and the vertical bar indicates S.E. B. insulin-stimulated autophosphorylation of insulin receptor from mouse liver plasma membranes prepared in the absence (Control) or presence of 1.5 mM p-chloromercuribenzen sulphonate (PCMPs), 2.5 mM N-ethylmaleimide (NEM), and 2.5 mM 5,5′-dithiobis-2-nitroben-
zoic acid (DTNB). Assay of autophosphorylation was carried out as in panel A.

Fig. 3. Radioautogram showing the presence of only phospho-
tyrosine (P-Tyr) in the autophosphorylated β subunit of insulin receptor from mouse liver membranes prepared in the presence of iodoacetamide. Phosphoamino analysis by two-dimen-
sional electrophoresis of the radioactive β subunit band excised from the gel was carried out as described previously (34).

Fig. 4. A. radioautogram showing the insulin-stimulated autophosphorylation of the β subunit of insulin receptor from mouse liver plasma membranes prepared in the absence (C) or presence of iodoacetamide (IAM), 5 mM iodoacetate (IAA), and 5 mM ethylenediamine tetracetaete (EDTA). Receptor autophosphorylation was car-
ried out by Method I as described in the text. B. radioautogram showing the insulin-stimulated autophosphorylation of the β subunit of insulin receptor from mouse liver plasma membranes prepared in the absence of various protease inhibitors, but in the presence of iodoacetamide. Inhibitors that were omitted from the homogenization buffer are indicated: benzamidine (BZ), leupeptin (L), pepstatin (P), bacitracin (BC). Control membranes (C) were prepared in the presence of all inhibitors and iodoacetamide. The arrow indicates the 84-
kDa fragment of the subunit. Assays were carried out by Method I.
fluoride, molybdate, or vanadate, and these compounds did not increase further the effect of iodoacetamide (data not shown).

The enhancing effect of iodoacetamide on insulin receptor autophosphorylation was obtained only when iodoacetamide was present in the buffer used for homogenization. Thus, iodoacetamide showed only a very small effect when added to insulin receptors solubilized from mouse liver plasma membranes prepared without iodoacetamide but was inhibitory when added to receptors solubilized from membranes prepared in its presence (Table II). NEM was inhibitory when added to receptors solubilized from both types of membranes (Table II).

We considered the possibility that the increased receptor autophosphorylation in response to insulin could be the result of an increase in insulin binding. However, Scatchard analysis of insulin binding by membranes prepared in the absence or presence of iodoacetamide showed similar affinity of binding \( \text{Kd} = 0.42 \pm 0.04 \text{ nM} \) and binding capacity \( \text{nM} = 8.9 \pm 0.29 \text{ nM} \) for membranes prepared with iodoacetamide was similar. However, compared with receptors from control membranes, insulin-stimulated kinase activity in control membranes and in membranes prepared with iodoacetamide was similar. However, compared with receptors from control membranes, insulin-stimulated kinase activity was about 2-fold higher in receptors solubilized from membranes prepared in the presence of iodoacetamide. These effects were obtained with both mouse and rat liver insulin receptors.

**DISCUSSION**

In the present study, we have shown that insulin receptors from liver plasma membranes prepared in the presence of iodoacetamide exhibited an enhancement of receptor autophosphorylation (Fig. 2) and tyrosine kinase activity (Fig. 7) when compared with receptors from membranes prepared without iodoacetamide. Furthermore, the effect of iodoacetamide was obtained only when it was present in the homogenization buffer since the addition of iodoacetamide to membranes or receptors prepared in the absence of this thiol reagent was not effective (Table II). The effect of iodoacetamide...
mide was attributed to its function as a thiol alkylating reagent since a similar effect was obtained with iodoacetate (Fig. 4A) and PCMPS (Fig. 2B). However, consistent with the findings by others (16) that the autophosphorylation activity of purified insulin receptor was inhibited by NEM and DTNB, we found that both reagents were inhibitory (Fig. 2B).

A thiol-sensitive and cation-dependent proteolytic activity which degraded the phosphorylated receptor β subunit to a phosphorylated 84-kDa fragment was reported to be responsible for the uncoupling of insulin binding from receptor autophosphorylation and receptor tyrosine kinase activity (23). The addition of either iodoacetamide or EDTA to the homogenization buffer was reported to inhibit the formation of the 84-kDa fragment and to restore the coupling of insulin binding and receptor kinase activity. Therefore, we thought it possible that the effect produced by iodoacetamide in the present study was due to an inhibition of proteolytic activity. However, we found that the addition of iodoacetamide alone to the homogenization buffer, in the absence of protease inhibitors, did not prevent the formation of the 84-kDa fragment of the receptor β subunit while its enhancement on receptor autophosphorylation remained (Fig. 4B). Furthermore, the addition of EDTA to the homogenization buffer was also without effect (Fig. 4A). Thus, it is unlikely that the results we have obtained with iodoacetamide were due to the inhibition of a thiol-sensitive and cation-dependent proteolytic process that uncoupled insulin binding from receptor autophosphorylation.

An increase in receptor autophosphorylation or kinase activity could also be due to an increase in insulin binding. However, this possibility can be ruled out since the amounts of protein used in each experiment were at equivalent insulin binding capacity. Also, we found that the affinity of insulin binding was not altered in receptors from plasma membranes prepared in the presence of iodoacetamide. Therefore, we conclude that the effect of iodoacetamide on receptor autophosphorylation and kinase activity was not due to an alteration of insulin binding.

The $k_a$ for ATP obtained with membranes prepared in the presence of iodoacetamide was more than 2-fold lower than that obtained with control membranes (50 versus 143 μM). The published values of $k_a$ for ATP in the autophosphorylation of insulin receptors prepared from a variety of tissues in the absence of iodoacetamide range between 50 and 200 μM (15, 24, 25). The reasons for such a wide range of variation are not apparent but may reflect the degree of receptor inactivation during membrane preparation. We found that in addition to a decrease in $k_a$, the $V_{max}$ was increased more than 3-fold in membranes prepared in the presence of iodoacetamide. Thus, the presence of iodoacetamide in the homogenization buffer produced a profound and positive effect on the interaction between the receptor and ATP. We therefore suggest that the enhancing effect on receptor autophosphorylation and receptor kinase activity, obtained with iodoacetamide added to the homogenization buffer, was due to changes in both $k_a$ and $V_{max}$. These changes could be the result of decreased phosphatase or ATPase activity produced by the presence of iodoacetamide. However, this appears to be unlikely because the addition of iodoacetamide subsequent to the preparation of membranes was virtually ineffective (Table II). Furthermore, we have employed two different methods of assay for receptor autophosphorylation. In Method I, the assay was performed on receptor bound to anti-receptor antibody and thus was essentially free of contaminating enzyme activities. In Method II, the assay was carried out in the crude total solubilized membrane, thus in the presence of all putative contaminating enzymes. However, the effect of iodoacetamide on insulin receptor autophosphorylation measured by either method was similar. Furthermore, the addition of solubilized membranes prepared without iodoacetamide to receptors solubilized from membranes prepared with iodoacetamide did not reduce the enhancing effect of iodoacetamide (data not shown). Therefore, it is unlikely that iodoacetamide acts as an inhibitor of enzyme activities to produce the observed enhancement of receptor autophosphorylation.

The mechanism(s) by which the binding of insulin to the receptor α subunit leads to receptor autophosphorylation and the expression of tyrosine kinase activity remains unknown. One possibility is that insulin binding releases the conformational constraints by the α subunit on the β subunit (26, 27). As already discussed, receptor thios and disulfides appear to play a role in the activation of the receptor by insulin. The conformational change induced by insulin may thus involve the participation of receptor thios and disulfides in intrareceptor thiol/disulfide exchange reaction. Thiol/disulfide exchange reaction has been shown to modify reversibly the activity of a number of enzymes (see Ref. 28 for review). If thiol/disulfide exchange reaction is involved in the activation of insulin receptors, experimental conditions favoring the preservation of receptor reactive disulfides involved in the exchange reaction would be expected to have an enhancing effect. Therefore, we propose that the presence of iodoacetamide in the homogenization buffer acted to preserve the receptor thiol(s) and disulfide(s) involved in the exchange reaction. It is most likely that iodoacetamide functioned as a
thiol alkylating reagent because two other thiol alkylating reagents, iodoacetate and PCMPS, also enhanced receptor autophosphorylation when added to the homogenization buffer. Since iodoacetamide was effective only when it was present during homogenization and was without effect when added later, its reaction with thiols must have occurred during tissue homogenization and membrane preparation. We suggest that, in the absence of iodoacetamide, these thiols react with the reactive disulfide(s) of the receptor to generate mixed disulfides, with the consequence that the putative intrareceptor thiol/disulfide exchange reaction induced by insulin binding cannot take place. Alkylation of these thiols by iodoacetamide during membrane preparation prevents the formation of the mixed disulfides, thus preserving the receptor reactive thiols in intact cells or tissues may likewise affect the activity of the insulin receptor, and thus the level of reactive thiols exists as disulfides is not known, though surprisingly in the native receptor the two dimers are apparently linked by only three disulfides, and evidently there is only one NEM-titratable thiol located in the α subunit. It was suggested that this thiol was located near the β subunit of each αβ dimer (33). If receptor activation by insulin is dependent on a thiol/disulfide exchange reaction as discussed above, the reaction could involve this NEM-titratable thiol. It was suggested that this thiol was located near the ATP-binding site because its alkylation was partially inhibited by ATP or a nonhydrolyzable ATP analogue (33).

In this study, we have demonstrated that insulin receptor autophosphorylation and kinase activity were enhanced when liver tissues were homogenized in buffer containing iodoacetamide. We have presented evidence that the enhancement of receptor autophosphorylation was the result of a decrease in the Kₐᵣ and an increase in the Vₐᵣ for ATP. We propose that iodoacetamide acts by alkylating one or more unknown reactive thiols which have been released during tissue homogenization. In the absence of iodoacetamide, these thiols form mixed disulfides with the receptor and thus interfere with the activation of the receptor by insulin, a process possibly involving receptor thiol/disulfide exchange. Reactive biological thiols in intact cells or tissues may likewise affect the activity of the insulin receptor, and thus the level of reactive thiols could play a role in the regulation of tissue response to insulin.

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REFERENCES
Thiols and Insulin Receptor Kinase